# METABOLIC SHIFTING: NUTRITION, EXERCISE AND TIMING

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# METABOLIC SHIFTING: NUTRITION, EXERCISE AND TIMING

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EDITORIAL
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# Editorial: Metabolic Shifting: Nutrition, Exercise, and Timing

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**Editorial on the Research Topic**

**Metabolic Shifting: Nutrition, Exercise, and Timing**

From the teleological standpoint, metabolic shifting evolved due to the periodic nature of the environment, circadian cycles being one of the conditions the life on Earth confronts. Metabolic shifting occurs on a regular basis due to the demand-availability interplay of energy resources, expenditure, and depots, all of which have periodic nature due to the environment's circadian rhythmicity. In modern societies, however, there are discrepancies between the evolutionary acquired metabolic setup and our current dysrhythmic, artificial environment. Thus, the so-called "diseases of civilization" are thought to be due to the changes in the environment that are too fast to cause adequate adaptations in our genome. On the other hand, an inherent fundamental feature of metabolism at large is flexibility. Out of the many diverse manifestations of metabolic flexibility, this Research Topic addresses the triad: "*Timing, Nutrition, and Exercise*" for the apparent reason—they are areas where they depend on each other and intertwine.

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## NUTRITION

Just a few days of nutritive imbalance, e.g., on the obesogenic diet (high in both fat and sugar), resulted in weight gain and loss of metabolic control resulting in impaired hypothalamic glucose sensing and hippocampal insulin signaling. These pathologies, in turn, led to obesity, metabolic syndrome, and the hippocampal-dependent memory function (Garcia-Serrano and Duarte). Nutrients entrain peripheral tissue clocks via the molecular clocks. Carbohydrate intake-induced insulin secretion activates clock genes. Glucose tolerance is higher at the onset of the daytime than in the evening. Fuel selection in a skeletal muscle during exercise depends on the time of day. At night, high-fat meal intake caused a more noticeable increase in triacylglycerol levels than in the daytime. Some nutrients like selenium or the flavonoid nobiletin, to name just two, are capable of enhancing circadian rhythms, e.g., via the clock genes (Geng et al.), which may have significant practical applications in cases of environmental or pathological dysrhythmias (Potter and Wood).

The master clock in the suprachiasmatic nucleus is the lead light-dark pacemaker of the body. Still, the bodily structures insensitive to light nevertheless participate in metabolic alignments via their responsiveness to feeding and fasting. Shift work, feeding during the inactive phase, permanent food availability (the *ad libitum* experimental protocol), overly palatable foods—all adversely influence the peripheral molecular clocks and metabolic homeostasis (Pickel and Sung).

An essential part of timing as a whole is feeding/eating regimens restricting food availability to a certain period of the active phase of the day. For instance, intermittent fasting and time-restricted feeding are mentioned in most of the participating papers (Bae et al.; BaHammam and Almeneessier; Garcia-Serrano and Duarte; Norgren et al.; Potter and Wood; Pickel and Sung; Wang et al.).

Restricting feeding prevents diet-induced obesity without changing energy intake than the *ad libitum* protocol while improving the essential metabolic biomarkers. On the other hand, unrestrictive obesogenic diets adversely impact circadian rhythms and metabolic parameters (Bae et al.). The undernutrition approach to disease prevention and treatment (e.g., fasting) is known since ancient times. It is in use in our days as an antidote to obesogenic overnutrition and environments in the form of complete or intermittent fasting or time-restrictive eating/feeding protocols including.

From the physiological viewpoint, Ramadan fasting is a time-restrictive eating regiment, which does not seem to disturb the circadian rhythms and, as time-restrictive eating/feeding or intermittent fasting generally, has beneficial effects, e.g., on lipid, glycemic, and inflammatory biomarkers (BaHammam and Almeneessier).

## EXERCISE

Homeostatic balance is based on constant monitoring of energy intake vs. energy expenditure. Exercise is a potent entrainment factor for central as well as peripheral clocks, including such in muscle, where fuel selection depends on the time of day as are postprandial effects of nutrients. It may prevent autophagic or apoptotic dysfunctions. Aerobic exercise training improves risk factors of metabolic syndrome such as glucose intolerance, hyperlipidemia, high blood pressure, low high-density lipoprotein content, and visceral obesity. Improved aerobic fitness increases neurogenesis, enhances memory, and may prevent brain atrophy. Adequate-intensity exercise reduced prenatal depression and shortened early labor stages. Physical activity enhances cognitive functions in adolescents, and young adults attenuate or prevent such autophagic or apoptotic dysfunctions (Andreotti et al.).

## TIMING

There is the two-way communication between metabolic signals and circadian processes such as rhythmicity of feeding, gene expression, enzymatic, and metabolic pathways. The authors of the Topic address the consequences of these communications for a diverse array of interactions, including the sleep-wake cycle, body temperature, hormonal processes, dietary composition, human social, and occupational behaviors. The environmental cycles (diurnal rhythms, temperature variations, seasonal food opportunities) interact with the endogenous ones at the cellular and organismic levels. Such a complex networking mechanism is not immune to the rhythmic desynchronization resulting in a plethora of metabolic disarrays (Bae et al.).

The circadian clock drives physiological functions controlling energy homeostasis. In their turn, parameters of energy intake and expenditure influence the circadian clock, e.g., the expression of the clock genes. Food intake, fuel selection, and exercise capacity depend on the circadian clock and are time-of-day dependent (Aoyama and Shibata). Feeding rhythms and dietary compositions are sensitive to many aspects of feeding behaviors and thus are capable of regulating the transcriptional, enzymatic, metabolic, and microbiome processes (Bae et al.).

Other essential confounders impacting the metabolic markers are the light exposure and sleep-wakefulness timing and energy expenditure fluctuations. Restriction of food availability exclusively to the active phase prevents weight gain and metabolic syndrome independently from caloric restriction while eating during the inactive period (nighttime eating in humans) increases the likelihood of obesity (BaHammam and Almeneessier). The timing of food intake may contribute to metabolic shifting, for instance, to ketosis, depending on the macronutrient ratio and calorie intake (Norgren et al.). Diet composition and timing affect the circadian clock and are powerful enough to potentially help shift workers adapt to their schedules (Potter and Wood).

Summing up, this Topic offers the latest experimental and analytical works concerning characteristics of environmental cyclicity, its effects on metabolic adaptations and maladaptations.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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# At the Interface of Lifestyle, Behavior, and Circadian Rhythms: Metabolic Implications

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Nutrient metabolism is under circadian regulation. Disruption of circadian rhythms by lifestyle and behavioral choices such as work schedules, eating patterns, and social jetlag, seriously impacts metabolic homeostasis. Metabolic dysfunction due to chronic misalignment of an organism's endogenous rhythms is detrimental to health, increasing the risk of obesity, metabolic and cardiovascular disease, diabetes, and cancer. In this paper, we review literature on recent findings on the mechanisms that communicate metabolic signals to circadian clocks and *vice versa*, and how human behavioral changes imposed by societal and occupational demands affect the physiological networks integrating peripheral clocks and metabolism. Finally, we discuss factors possibly contributing to inter-individual variability in response to circadian changes in the context of metabolic (dys)function.

**Keywords: metabolic syndrome, circadian rhythms, chronodisruption, shift work, metabolism**

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## INTRODUCTION

Organisms have evolved to anticipate and adapt to the cyclic nature of the environment on earth, most notably diurnal variations in sunlight, and temperature over the earth's 24-h rotation about its axis. The circadian timing system aligns oscillations in biological processes such as food intake, alertness, and energy expenditure to the earth's solar day, producing rhythms in physiology, and behavior in organisms ranging from bacteria to mammals (1). The alternating environmental cues such as the light/dark cycle, changes in temperature, and availability of food, act as *zeitgebers (timekeepers)*, that synchronize the endogenous timing systems of every cell in an organism. A variety of physiological activities, including immune response, metabolism, sleep cycles and endocrine signaling, are under circadian regulation and modulation. Of importance is the reciprocal control between the circadian clock and metabolism, whose sensitivity to a variety of genetic and behavioral factors leads to disruption of the rhythms and consequently, metabolic dysfunction.

The endogenous time keeping system is organized as a hierarchical, interconnected network of clocks. The clocks receive input from environmental cues external to the host, processes them

to generate a rhythm, which then regulates various output pathways which, in turn, regulate key biological functions (2). The central clock lies in the suprachiasmatic nucleus (SCN) of the brain and oscillates in an autonomous manner (3). The central clock synchronizes the network of peripheral clocks, which are present in all tissues and cells (4). The synchronization of peripheral clocks is necessary for proper metabolic, physiological, and behavioral processes of the host. The central pacemaker is synchronized to the cycle of the ambient light that in mammals is exclusively detected by specialized receptor cells the eyes. Rods, cones, and melanopsin containing retinal ganglion cells process the light and transduce signals to the SCN via neuronal pathways (5, 6). However, the light/dark cycle is not the only *zeitgeber*. Circadian clocks are also under the control of energy intake and the timing thereof, ensuring that the peripheral rhythms can be altered to the food availability, effectively misaligning the rhythms in the periphery from the phase in the SCN. In return, circadian clocks regulate components of metabolism by controlling the rhythmic expression of genes encoding regulators and enzymes in various metabolic pathways. Feeding rhythms influence key clock components and more importantly clock outputs via enzymatic reactions and transcriptional regulations (7–9).

The intricate relationship between circadian rhythms and metabolism of glucose, lipid, amino acids, etc. has been studied in the context of enzymes and hormones involved in digesting the nutrients and their interaction with peripheral clocks (10, 11). The rest/active cycles and fast/feed cycles that mammals experience diurnally lead to alternating nutrient supply throughout the day. The metabolic organs of the host must manage these diurnal fluctuations in nutrients while also maintaining physiological homeostasis (12). The circadian regulation of metabolism is thought to provide the host organism with the flexibility in regulating metabolic activities in response to changing environmental conditions. Considering the intricate relationship between circadian rhythms and metabolism, it is not surprising that chronic disruption of circadian rhythms is associated with the development of metabolic syndrome, which may lead to cardiovascular diseases and diabetes (13–17). Furthermore, time-restricted feeding studies (restricted temporal access to food without calorie restriction) in mice has shown that metabolic cues influence circadian rhythmicity enabling, for instance, restoring circadian oscillations of some peripheral clock components in clock-deficient mouse livers (18).

In this paper, we review literature on the networks of metabolic signals and circadian clocks, and how human behaviors driven by societal demands and lifestyle choices affect the physiological networks integrating peripheral clocks and metabolism. Understanding of these networks can facilitate future research in overcoming metabolic syndrome due to chronodisruption by pharmacological means and behavioral modification. We will first discuss the molecular basis on the interaction between clock and metabolic components. We discuss the extended network of interactions between endogenous rhythms and the entraining environmental cues. Then we will review a few behaviors (for both human and animals) that are known to induce metabolic syndrome associated with chronodisruption. We discuss shift work, irregular meals, and social jetlag as behaviors which cause misalignment between the peripheral and central clocks. We further elaborate on sex differences, microbiota, genetic makeup, including race and ethnicity, and lifestyle as the factors that generate interindividual variability in circadian rhythms manifesting to metabolic activities, as these factors may prove useful in developing personalized care for patients of metabolic syndrome. Finally, we provide a brief overview of mathematical models that aim to understand the bi-directional regulation that occurs between peripheral clocks and metabolism, which aim to develop pharmacologic approaches targeting the circadian clock in the context of metabolic disorders.

## THE MAMMALIAN CIRCADIAN SYSTEM

In mammals, the circadian clock system is organized into a distributed, hierarchical system composed of the central pacemaker that resides in the SCN in the brain and peripheral oscillators synchronized to the central clock (19). The clocks give rise to rhythms in hormonal and metabolic signaling even in the absence of environmental cues, and the signaling establishes phase relations among the various peripheral clocks. These rhythmic signals play a major in regulating immune and metabolic functions (20, 21). The ability for mammalian organisms to diverse biological functions in a temporal manner confers and adaptive advantage as they cope with 24-h changes in the environment. The central clock in the SCN receives the photic input through the retinal ganglion cells in the eye (5, 6), and transduces the information to the peripheral clocks, within other brain regions, and outside of the brain. The necessity of proper SCN function is evident in studies with SCN lesioned animals and tissue explants (22, 23), where the phase synchrony among the cells were gradually decreased over time due to period differences in individual cells. The peripheral clocks then drive tissue specific processes. It is now well-recognized that in order to benefit the host, the local, and central rhythms must be synchronized to each other as well as to the outside environment. The oscillators have identical molecular makeup in the SCN neurons and the peripheral cells, with the peripheral oscillators coupled to the SCN via synaptic and paracrine signals (24).

The autonomous oscillation of the circadian clocks is driven transcriptional and translational feedback loops of clock genes. The CLOCK and BMAL1 form a heterocomplex and activate transcription of their own repressors, *Per* and *Cry* (25). CLOCK/BMAL1 heterodimers bind to the E box region of *Per1/2* and *Cry 1/2* genes and activate the transcription of these genes. PER and CRY proteins accumulate as a result of this transcriptional activation and form the PER/CRY heterodimer complexes, which translocates to the nucleus (26). The auto-repression of *Per* and *Cry* genes by their own products, PER and CRY, occurs through inhibition of CLOCK/BMAL1 induced transcription by the nuclear PER/CRY complex (25). The PER/CRY complex also stimulates the expression of BMAL1 (27–29). Aside from PER and CRY, the CLOCK/BMAL1 heterocomplex also transcriptionally activates

the nuclear receptors REV-ERBs and RORs (25). REV-ERBs represses the transcription of *Bmal1* while RORs activate it (25, 30).

## CIRCADIAN CLOCK AND METABOLISM CROSSTALK

In this section we discuss the molecular basis for the interaction between circadian clock and metabolism. In particular, we discuss the role or nutrient sensors that bridge the circadian clocks to energy metabolism, since metabolic syndrome linked with behavior-induced circadian disruption often involve taking in nutrients at naturally inactive times. The nutrient sensors can entrain the local clocks in the metabolic tissues away from the SCN due to the nutrient cues, causing a phase misalignment between the central and peripheral clocks and leading to a break in metabolic homeostasis. Nutrient sensors such as sirtuins (SIRTs), AMP-activated protein kinase (AMPK), and poly ADP-ribose polymerase 1 (PARP1) exhibit circadian behavior and interact with core components of the clock system, while also playing key roles in metabolic activities (31, 32).

SIRTs are $NAD^+$ activated class III histone deacetylases (HDAC), homologous to Sir2 (silence information regulator 2) in yeast (8). Among the 7 members of mammalian sirtuins, SIRT1, SIRT3, and SIRT6 interact with circadian genes. SIRT3 is involved in the circadian mitochondrial function (33), while SIRT6 controls the circadian clock at a chromatin level (34). SIRT6 is involved in the recruitment of the circadian transcriptional machinery (CLOCK and BMAL1) to E-box containing core clock gene promoters (35). According to microarray analysis of hepatic sirt1 and sirt6 deficient mice, SIRT6 regulates a set of core clock output genes different from those regulated by SIRT1 (34).

The CLOCK/BMAL1 complex also acts with SIRT1 to transcriptionally activate nicotinamide phosphoribosyltransferase (NAMTP) the rate-limiting enzyme of the $NAD^+$ salvage pathway (36). SIRT1 is a key candidate for bridging the circadian clocks and metabolism. The activity of SIRT1 occurs in the nucleus, modulating metabolism of lipids, proteins, and carbohydrates, and enhancing mitochondrial activity (37). As such, SIRT1 has been studied extensively in the context of oncogenesis, aging, and metabolism (38). SIRT1 acts as a nutrient sensor as its enzymatic activity requires binding of nicotinamide adenine dinucleotide ($NAD^+$) into its catalytic site along with the substrate. Understanding the activity of SIRT1 would require an accurate portrayal of the $NAD^+$ level in the cell, since it directly activates SIRT1. In addition to synthesizing $NAD^+$ from amino acids, cells can also recover $NAD^+$ from the $NAD^+$ salvage pathway (39), where nicotinamide (NAM) is released from $NAD^+$, NAM is converted to nicotinamide mononucleotide (NMN), and NMN is converted back to $NAD^+$, completing the cycle.

$NAD^+$, the oxidized form of the nicotinamide adenine dinucleotide, serves an important role in linking clocks to metabolism, acting as the cellular energy sensor and modulating the activity level of SIRT1. In liver, $NAD^+$ level oscillates throughout the 24-h day (40), due to contributions from a variety of factors including the feeding/fasting state of the host, oscillations in the NAMPT activity, and activation of PARP-1 (7). SIRT1 is co-activated by $NAD^+$ through direct binding, and regulates the transcriptional activity of CLOCK/BMAL1, creating a bi-directional relationship between the circadian clocks and metabolism (36). Additionally, SIRT1 mediates deacetylation and thereby degradation of PER2 protein (41). SIRT1 activity has complex relationships with the $NAD^+$ salvage pathway and the peripheral clocks. First, SIRT1 activity requires binding of $NAD^+$, thus $NAD^+$ has an activating effect on SIRT1. Second, SIRT1 activity is inhibited by NAM, the precursor of the rate-limiting step. Third, expression of nicotinamide mononucleotide adenylyltransferase (NAMPT), the rate-limiting enzyme of the $NAD^+$ salvage cycle, is under the control of SIRT1, which is a co-transcription factor for the NAMPT gene along with peripheral clock genes (PCGs). In summary, SIRT1 functions as a nutrient sensor, being under the influence of the energy state of the cells, represented by $NAD^+$. It is also under the effect of the circadian rhythmicity presented by $NAD^+$, NAM, and NAMPT. Another potential $NAD^+$ sensor is PARP-1, whose activity is also connected to circadian gene expression. When mice transition from *ad libitum* feeding to day-time feeding, the phase inversion for clock components occurs more slowly in *Parp-1* knockout animals, indicating that PARP-1 is involve in entraining the circadian rhythms in the periphery (42).

Like the $NAD^+$ levels, the AMP/ATP ratio depends on the feeding/fasting state of the host. AMP-dependent protein kinase (AMPK) responds to the AMP/ATP ratio; when the ratio increases, AMP binds to the γ subunit of AMPK and allosterically modifies the α subunit to be phosphorylated (43). Active AMPK influences the circadian clock by directly phosphorylating and facilitating the degradation of CRY protein (44). Thus, AMPK activity level oscillates with a 24-h period, antiphasic to CRY protein oscillation. AMPK also appears to promote the degradation of PER2 protein indirectly (45).

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors that function as transcription factors that play a vital role in energy metabolism (46), and also serve as a linker between circadian clocks and metabolism. Recent advances in understandings of PPARs show that all three isoforms of PPARs (α, β/δ, and γ) are expressed and function in rhythmic manner (47). PPARα and PPARγ regulate clock components *Bmal1* and Rev-erbα (48–50). PPARα is also a target of BMAL1 and CLOCK (51).

The nutrient sensors that interact with peripheral clocks bridges the clocks to metabolism of nutrients by regulating the expression of metabolic enzymes and hormones. We illustrate this with clock and metabolic components involved in gluconeogenic gene expression, shown in **Figure 1**. The fasting/feeding state of the host changes the $NAD^+$ level, eventually influencing the transcription of gluconeogenic genes such as *G6pc* and *Pck1* to control the generation of glucose (52). From the *G6pc* genes, G6PC (Glucose-6-phosphatase, catalytic subunit) is expressed, which hydrolyzes glucose-6-phosphate into glucose. The *Pck1* gene encodes for PEPCK (Phosphoenolpyruvate carboxykinase), which is the catalyzer for

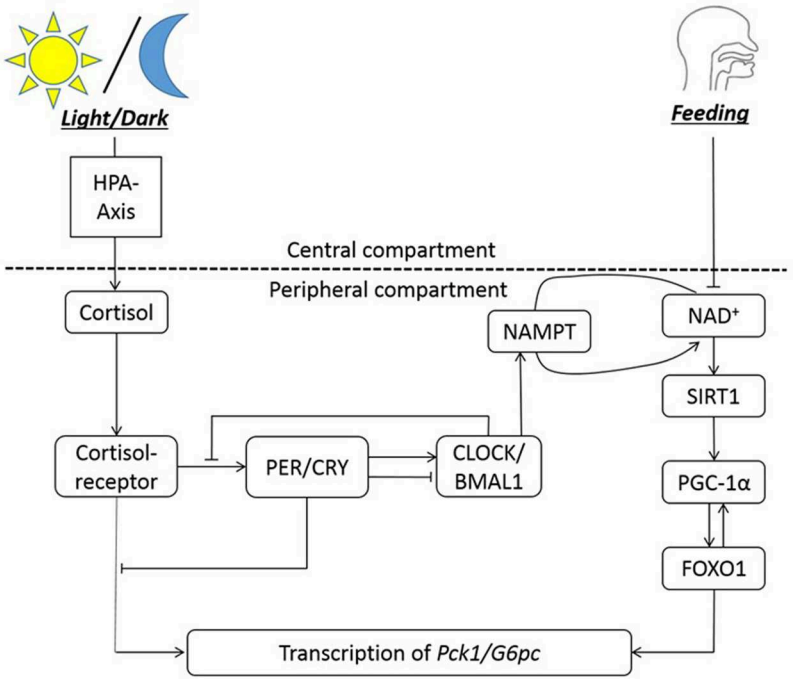


**FIGURE 1 |** The circadian regulation of hepatic gluconeogenesis illustrates an example of bi-directional regulation between clock genes and metabolism. The HPA axis is entrained to the light/dark cycle, and $NAD^+$ availability is entrained to feeding rhythms. NAMPT facilitates $NAD^+$ salvage pathway. $NAD^+$ co-activates SIRT1, which then activates PGC-1α. PGC-1α activated FOXO1 is necessary for transcription of Pck1 and G6pc genes, along with the cortisol-receptor complex. Cortisol bound to its receptor entrains the clock genes to the light/dark cycle. In summary, the transcription of gluconeogenic genes (Pck1/G6pc) is regulated by cortisol-receptor complex, clocks, and FOXO1.

conversion of oxaloacetate into phosphoenolpyruvate and carbon dioxide. These two enzymes are involved in the very beginning and end of the chemical steps for endogenous production of glucose in liver. When the host experiences long-term fasting (more than 6 h), SIRT1 upregulates the transcription of the two genes encoding for these enzymes. One of the mechanisms in which the transcription of *G6pc* and *Pck1* is initiated is through SIRT1-mediated deacetylation and activation of PPARγ-coactivator α (PGC-1α) and Forkhead box O1 (FOXO1) (53), with the aid of hepatic nuclear factor-4α (HNF-4α) (54). Among a series of rodent studies, a restricted feeding study demonstrates the alteration in G6Pase and PEPCK level and activity due to circadian disruption. The activity of the two enzymes peak slightly before the dark phase in rats fed *ad libitum*, in 12:12-h light/dark cycle. When rats are fed for restricted period during the light phase, G6Pase activity peaks right before the feeding time. T6Pase protein amount oscillate in a bimodal way, with the major peak slightly before the feeding time. For PEPCK, enzyme activity oscillates bimodally with the major peak at the transition from dark to light phase, and protein amount peaks around 2 h after the feeding start time (55).

$NAD^+$ is also consumed as a substrate of SIRT3, a mitochondrial Sirtuin protein that also functioning as a protein deacetylase (56). Circadian regulation of *Nampt* transcription by CLOCK, BMAL1, and SIRT1 governs the amount of NAD+ and regulates the deacetylation activity of SIRT3 (56), which activates several key enzymes involved in the citric acid cycle (57) and fatty acid metabolism (58, 59) in mitochondria. Therefore, circadian regulation of mitochondrial function is another pathway to control metabolism by circadian clock (60).

Recent findings suggest that mTOR signaling pathway has interactions with the circadian clock. mTOR signaling centers on mTORC1 and mTORC2, which are multiprotein complexes that involves mTOR, a serine/threonine protein kinase (61, 62). Activated mTOR signaling regulates some of the fundamental biological process including lipid and glucose metabolism (61, 62). One of the ways in which mTOR controls the circadian clocks is through the regulation of photic entrainment of the SCN via mTORC1 (63). mTORC1 is involved in the synchronization of SCN neurons (64). In the periphery, mTOR integrates intracellular signals that involve energy status and nutrients, and can therefore serve to link the cellular metabolic state and circadian clocks, as revealed by RNAi screening of human cells (65).

Insulin secretion is another relevant example in describing the involvement of a clock-interacting metabolic hormone, and one of the earlier evidence was observed in 3-dimentional culture of hepatocytes where insulin appeared to play a role in synchronizing the hepatic clocks (66). For many components involved in different stages of insulin action, including insulin receptor on erythrocytes (67), circadian profiles are observed. The promoter of PGC-1, an activator of gluconeogenic transcription along with FOXO1 and cortisol-receptor complex, includes insulin response sequences (IRSs) (68). Bmal1 is

post-transcriptionally regulated by signals from insulin (69), while also controlling proper release of insulin. Insulin promotes postprandial Akt-mediated Ser42-phosphorylation of Bmal1 to induce its dissociation from DNA, eventually leading to nuclear exclusion. Ultimately insulin activity on Bmal1 results in the suppression of Bmal1 transcriptional activity (69). Insulin is also a target of GSK3β, which is involved in robust clock regulation by phosphorylating BMAL1 (70). More recent findings suggest that insulin and IGF-1 receptor signaling induces the synthesis of PERIOD proteins, therefore signaling the cellular clocks of feeding time of the host (71).

Focusing on the secretion of insulin itself, rhythmic release of insulin from pancreatic islets has been observed in various studies. Perfused rat pancreatic islets exhibited circadian rhythmicity in insulin release with a period close to 24 h (72). In the same study, adding melatonin advanced the insulin secretion phase by 9 h while enhancing the amplitude of oscillation, demonstrating that insulin release is under the regulation circadian clock components. In healthy humans, glucose clamping experiments showed that glucose-stimulated insulin secretion rate varies depending on the time of the day, with a nadir between midnight and 6 a.m. and a peak between noon and 6 p.m. (73). The observations from this study suggest that increase in insulin secretion during the day and decrease in secretion during the night may contribute to higher glucose tolerance and insulin response in the morning than at night. A question remains as to if the duration of prior fast for different meals throughout the day is a contributing factor in changing insulin response, as fasting duration prior to breakfast is the longest among the three meals. This was ruled out as a reason for differential insulin secretion in a study that fed subjects identical meals at 6- or 12-h intervals (74).

At a molecular level, functional clock genes are required for proper pancreatic islet function and insulin secretion. Mutations of *Clock* and *Bmal1* in mice resulted in hypoinsulemia and diabetes (75). The mutant mice had defects in size and proliferation of pancreatic islets which worsened with age, suggesting that chronic circadian disruption may affect human insulin secretion at the level of β-cells. Genes that were involved in growth, survival, and synaptic vesicle assembly were altered at the transcriptome level, leading to β-cell dysfunction. Knockout of *Clock* in human pancreatic islet cells significantly decreases both acute and chronic glucose-stimulated insulin secretion (76). Furthermore, the rhythm of insulin secretion became asynchronous with genetic clock disruption. The same study revealed that clock genes regulate the level of insulin by controlling insulin secretion rather than production. Among ∼300 altered genes, regulators of insulin secretion (*gnaq, atp1a1, atp5g2, kcnj11*) as well as transcripts required for granule maturation and release (*vamp1, stx6, slc30a8*) were affected. To test whether an independent pancreatic clock regulates the secretion of insulin, both global and β-cell specific *Bmal1* deletion were tested in a study (77), and both resulted in insufficient glucose-stimulated insulin secretion, leading to β-dysfunction and diabetes. *Bmal1*-knockout mice lose the rhythms in insulin and are locked into the trough stage of insulin secretion (78), and experience increased risk of obesity under high-fat diet. Although the exact mechanism of clock control in insulin release is still under investigation, CLOCK/BMAL1 heterocomplex is thought modulate rhythmic metabolic activities in pancreas together with cell-type specific enhancer PDX1 (79) in a distinct manner. Another study has found that the presence of pineal gland is required for proper synchronization of metabolic rhythms including glucose-stimulated insulin secretion (80). Indeed, when mRNA expression levels were compared between cultured human islets from deceased donors, *Per2*, *Per3*, and *Cry2* were under-expressed in type 2 diabetes mellitus (T2DM) patients compared to healthy donors (81), alluding that metabolic function correlates with robust clock rhythms. The donors in this study included both men and women of varying ages for both healthy and T2DM groups.

As the above examples illustrate, robust, and synchronized circadian rhythms in the periphery are required for metabolic health of mammals. Disruption of the rhythms induced by mutation of clock genes or restriction of food is detrimental for metabolic function. Restricting food access in a manner antiphase to the animals' natural sleep/wake cycle effectively resets the phase of the peripheral clock oscillations, uncoupling from the central pacemaker in the SCN (82). Thus, food availability acts as the primary *zeitgeber* for the peripheral clocks in metabolic organs such as kidney and liver, while leaving the SCN to be coupled to the light/dark cycle. The glucocorticoid signaling from the SCN acts as an inhibitor in feeding-induced decoupling, resulting in slower phase resetting for feeding time changing from active to rest period. On the other hand, a recent study exploring methods for mitigating jet-lag illustrates the advantage of synchronized light and feeding cues. In mice, changing the feeding cycle in synchrony with the light/dark cycle results in faster re-entrainment of circadian rhythms, due to the extra-SCN oscillators phase-resetting to the feeding cues immediately and synchronizing with the SCN clocks right away (83). The rate at which peripheral clocks re-entrain to the feeding rhythms and decouple from the SCN are different for each organ, with liver resetting the more rapidly than kidney, heart, or pancreas (84). However, all organs completely re-entrain in phase to the feeding rhythms after 1 week of restricted feeding. However, the resulting rhythms desynchronized from the SCN are less robust, having oscillations with smaller amplitude. The disrupted synchrony between central and peripheral clocks is quite common due to lifestyle choices and occupational demands of the modern society. The next section will examine specific human behaviors that cause metabolic implications in relation to disruption in circadian rhythms.

## METABOLIC SYNDROME AND BEHAVIORAL CIRCADIAN DISRUPTION

The metabolic syndrome is a cluster of metabolic abnormalities, including obesity, dyslipidemia, hyperglycemia, and hypertension increasing the risk for heart disease, stroke, and diabetes (85). The spectrum of disorders representing metabolic syndrome continues to increase in the industrialized world. With an incidence rate estimated to be between 25 and

40% (ages 25–64) the syndrome is becoming a major public health issue. Although genetic and environmental factors, such as increased food intake and lack of physical activity, have been known to contribute, it is becoming increasingly more clear that behaviorally-driven disruptions in circadian rhythms –a light at night or work during the rest period—substantially contribute to the disease (86). The central clock in the SCN plays a major role in maintaining metabolic homeostasis by regulating the systemic metabolic rhythmicity (87). SCN control of blood glucose level illustrates the importance of the central clock's role in modulating the metabolic activity to the environmental cues. During the active phase, glucose is taken up by liver and muscle cells via insulin dependent transporters GLUT4 and GLUT2, which are partly regulated by the clock (88). During the inactive phase, depleted glucose is replenished by glucose excretion from the liver by GLUT2 (89). As summarized in **Figure 2**, circadian disruption resulting from altered behaviors that create a mismatch between the host's endogenous rhythms and environmental cues, often manifests itself in the form of modifications to the oscillatory characteristics of the circadian rhythms in peripheral tissues and organs, including: reduction in the amplitude of oscillations, loss of synchrony, and peripheral clock phase misalignment relative to the SCN. Given the established circadian regulation of metabolic process (90–92), it is only natural to expect that behavioral and environmental factors leading to circadian dysregulation would adversely impact metabolism.

## TEMPORAL EATING PATTERNS AND TIME-RESTRICTED FEEDING

Time restricted feeding studies with animals have tried to deepen the understanding about the effects of circadian misaligned meals on hosts' metabolism (93, 94) while few studies have considered implications in humans (95). Consistent with the observation in shift workers, mice fed with high-fat diet during the light (inactive) phase gained significantly more weight than mice on high-fat diet during the dark (active) phase (96). The increased risk of weight gain in day-time fed mice is accompanied by misalignment in peripheral clocks with the SCN (96). In contrast, restricting feeding to the night time (active phase for mice) leads to robust oscillation of peripheral rhythms and prevent obesity (52), although there is no difference in between caloric intake for restricted feeding group and *ad libitum* group. Interestingly, mice on high-fat diet under *ad libitum* schedule become obese, but mice consuming the same diet under restricted feeding during the dark phase consume reduced amount of food and are protected from obesity (97). Mice on restricted feeding with high fat chow had food available to them for 4 h in the middle of the active period, and their overall daily caloric intake was similar to the group of mice that had low fat food available *ad libitum*. However, these mice still showed decrease in body weight, reduced cholesterol levels, decreased in TNF-α levels, and improved insulin sensitivity, highlighting the health benefits of robust peripheral clock oscillations. Recent evidence suggests

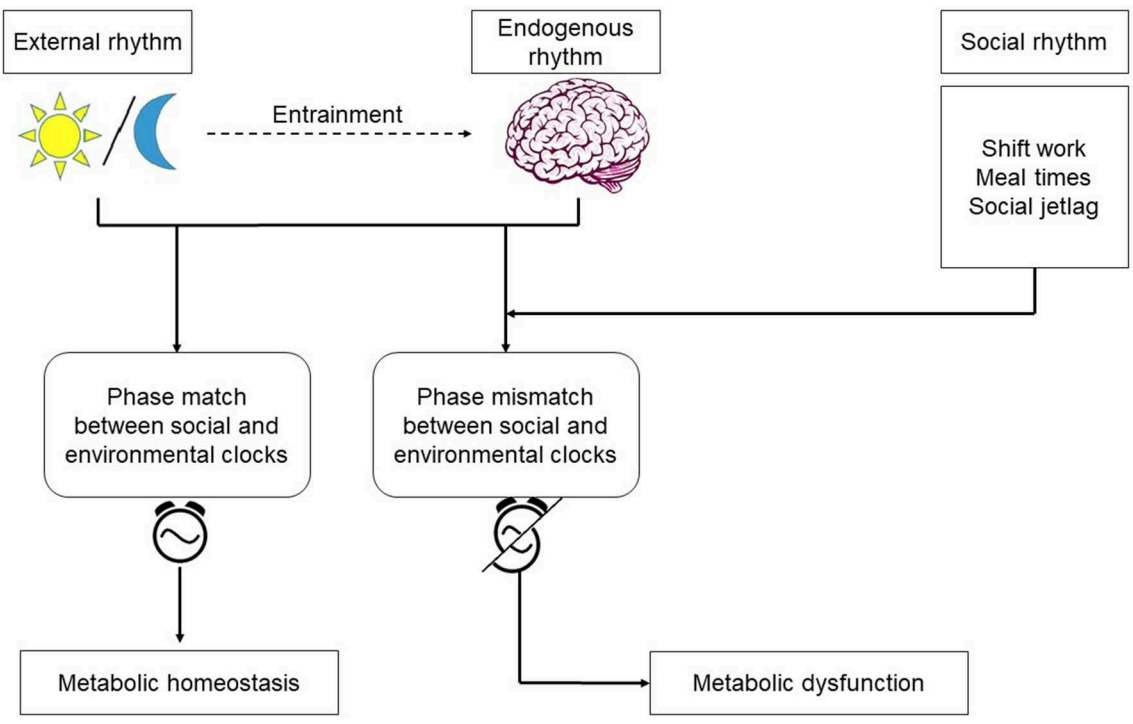


**FIGURE 2 |** Endogenous rhythms and environmental cues: the central pacemaker in the brain is entrained by the light/dark cycle. Shift work, irregular meals, and social jetlag are behaviors which cause misalignment between the environmental and social clocks. Phase mismatch between the clocks increases the risk for metabolic dysfunction.

that the benefits of clock oscillations are mainly due to sustaining daily rhythms and balance between nutrients and cellular stress responses. As restricted feeding prevents metabolic syndrome in the absence of circadian clocks, rhythmicity of clock outputs may give more health benefits than the clock itself from the metabolic standpoint (98). The relationship between diet composition and circadian rhythms appears to be bidirectional since high-fat diets have also been shown to adversely impact circadian rhythms (99).

The increased metabolic risk associated with shift work may be related to the altered eating patterns due to work schedules, at least in part (100). Shift work can lead to irregular meal patterns due to both biological and social mismatch. Night workers experience a decrease in appetite because the hypothalamus that controls the eating behavior is central to synchronizing the clocks of the body to the environmental light/dark cycle. Furthermore, shift work forces the workers to eat at times different from their families and friends, creating a difference in both the timing of food intake and nutritional composition of the meals (100).

Irregular feeding patterns drive disturbances in homeostatic control through endocrine signaling (101) as evidenced by altered level of satiety hormones such as ghrelin and leptin upon sleep deprivation (102). Reduced leptin level is observed with mismatched phase relations between behavioral pattern and circadian rhythms, independent of the lack of sleep (103).

The majority of studies tend to confirm that the total caloric intake between day workers and shift workers is comparable, despite differences in the distribution of the meals during a 24-h day between the two groups (104, 105). The meals tend to become more irregular during shift work, taking snacks rich in carbohydrate and fat, as the meal times are driven more by work schedule rather than hunger (106). Furthermore, the quality of the meals does not match the day meals, possibly due to ease of preparation or the limited availability of food at workplace (100). However, such practical constraints may not be the only reason for difference in meal composition as a study conducted with nurses working night shifts found that workers opted for cold snacks when hot prepared meals were available (106). Irregularity of meals resulting from shift work could contribute to increased risk of metabolic syndrome in some part, as evidenced by population-based cross-sectional studies indicating that middle-aged men and women were more prone to metabolic syndrome if they were irregular as opposed to regular eaters (107). Furthermore, night eating syndrome (NES), where more than 20% of the daily caloric intake occurs after the evening meal, is positively associated with BMI and binge eating. It also occurs more in males compared to females (108). Interestingly, increasing meal frequency is positively correlated with lower risk of obesity, whereas skipping breakfast increases the risk of obesity (109). People who concentrated their caloric consumption earlier in the day compared to later in the day were more likely to lose weight (110).

## SHIFT WORK

Epidemiological studies seem to point to a strong link between shift work and developing metabolic syndrome (111) likely through a complex interplay between circadian rhythms and sleep disruption (112). This is a growing concern as shift work is an unavoidable part of the modern society. According to 2004 data from Bureau of Labor Statistics, ∼15 million Americans work full time on night shifts, evening shifts, or otherwise irregular shifts arranged by their employers (113). An extensive number of studies suggests that chronic disruption of circadian rhythms due to shift work, especially rotating shift work, results in negative health effects, including metabolic disorders, cardiovascular disease, and cancer (114). However, a clear mechanism that links circadian disruption to the development of metabolic syndrome is yet to be established. Longitudinal studies have demonstrated that alternating shift work was associated with increase in BMI over a 14 year period (115) and have established shift work to be a risk factor for weight gain (116). By and large, individuals who engaged in night shift work for longer period of time were at a greater risk for obesity compared to workers who started as day workers and switched to night shift, suggesting that chronic exposure to a life style mismatching the internal clock to environmental cues leads to higher risk for weight gain. An observational study conducted on nurses' cohort also found that exposure to night work can lead to more frequent weight gain (117).

Population studies indicated that obesity and increased triglycerides were more prevalent in shift workers for women and some age groups in men, even after adjusting for age, and socioeconomic factors (16). Hypertriglyceridemia is frequently observed in patients suffering from obesity, diabetes, and stroke, often accompanied by low level of high-density lipoprotein (HDL) cholesterol (118, 119). However, it is difficult to determine if the increased risk of metabolic syndrome is due to the circadian disruption or the other factors involved in shift work. Indeed, comparing shift and day workers performing similar duties, more job strain, and higher total and at-work physical activity were observed for shift workers compared to the day workers (120). After accounting for potential covariates such as physical activity and job strain, the study determined that shift work is still highly associated with metabolic syndrome. Furthermore, shift workers exhibit different meal distribution patterns from day workers, as expected, and further analysis indicated that while caloric intake during breakfast and lunch is usually favorable for good health, high energy intake at lunch is deleterious to shift workers (120). The effect of meal distribution will be discussed in more detail in a later section.

In addition to factors contributing to metabolic syndrome such as obesity and high triglycerides, shift work is also correlated to abnormal glucose levels, likely indicating that alternating shift work increases the risk of impaired glucose metabolism (121). In both young and middle-aged women, a positive association was found between T2DM and accumulation of years in rotating night-shift work, although the relationship was not strong if body weight is taken into account (122). Short-duration and poor quality of sleep increases the risk of T2DM, marked by an increase in hemoglobin A1c (123).

Efforts aiming at understanding the implications of eating and sleeping at different phases of the circadian demonstrated that such abnormalities resulted in not only major phase shifts of the cortisol rhythms, but also decreased leptin and increased insulin levels, increased mean arterial pressure but, in some cases, resulted in glucose response typical of prediabetic state (103).

Therefore, circadian misalignment resulting from shift work is accompanied by adverse cardiometabolic implications.

Overall, chronic exposure, rather than acute exposure, to circadian disruption seems to increase the risk for metabolic syndrome. In a prospective study conducted among Belgian men, the risk for metabolic syndrome gradually increased with accumulated years of rotating shift work (124). In addition to metabolic biomarkers such as body mass index, waist-hip ratio, and fasting insulin level, leukocyte count was also correlated with shift work (125). Since leukocyte count is a biological marker for systemic inflammation, its association with metabolic syndrome and cardiovascular events should be further explored. Given the well-established links between low-grade, chronic inflammation, and obesity (126), it is very interesting to identify the positive correlation between shift work and systemic inflammation biomarkers (127).

## CHRONOTYPE AND SOCIAL JETLAG

There is a large variability in the way humans organize their sleep and wakefulness during the 24-h day. The manifestation of a person's underlying circadian rhythms is known as their chronotype (128). The chronotype is usually assessed by questionnaires including questions about sleep habits and shows a normal distribution ranging from very early chronotype to very late chronotype (128–131). Falling asleep and waking up at different times is not the only varying factor in human sleeping behavior. People also sleep for different durations, which adds ambiguity to chronotype determination. Sleep time and sleep duration appears to be two independent traits, as these separate when factor analysis is performed (129). However, there is an association between chronotype and duration of sleep on work and free days. People with late chronotypes exhibit the largest difference in sleeping time between work days and free days. This group of people wake up earlier than their biological wake up time during the work days due to work schedule, resulting in social jet-lag. Social jetlag is defined as the difference between the social clock and biological clock (128), usually due to work and social constraints (132). Since the circadian clock determines when people can fall asleep, people with later chronotypes often accrue sleep debt during the work week to be compensated on free days, which may cause conflicting schedules with family members and friends, leading to social/psychological stress that may worsen sleep disorder. Mutations in *per2, cry2,* and *csnk1d* have been shown to cause advanced sleep phase disorder, while a mutation in the *cry1* gene results in delayed sleep phase disorder. All of these are negative feedback protein-encoding genes. Genome-wide association studies of chronotype have both confirmed associations between specific genetic mutations and chronotype (133).

In addition to genetic (134, 135) and environmental factors (130), chronotype is also dependent on age. Chronotype is usually early in childhood, then is delayed as people mature, reaching the latest stage at around age 20 (129). From then on, early chronotype gradually returns as humans age. Women matures in chronotype a little bit earlier than men until age 30. However, the difference in chronotype between the sexes disappears around 30, and men become more morning-oriented than women starting at 45 years of age (136). Since this is the average age for menopause, the change in chronotype over a life span may be at least partly due to endocrine signaling (137). Given that different chronotypes (early vs. late) characterize diverse phase relations between endogenous rhythms and *zeitgebers* it was recently established that metabolic disorders are highly correlated with chronotype. In fact, late (evening) chronotypes are associated with diabetes and metabolic syndrome (138).

Social jetlag positively correlates with increased risk of adverse endocrine, behavioral, and cardiovascular risk profiles in apparently healthy subjects, putting them at a risk of developing metabolic diseases (139, 140). It has been estimated that the difference between the social clock and biological clock is $>1$ h in 70% of the individuals and $>2$ h in 1/3 of the population. The metabolic and mental health implications of mismatching clocks should be carefully considered in discussions of Daylight Savings Time as well as work and school start times (141). Social jetlag, along with short duration of sleep, appears to be a predictor of BMI, especially in over-weight individuals (141).

Often social jetlag is further accompanied with higher risks of diabetes and inflammation (142). Among non-shift workers, social jetlag was associated with numerous clinically assessed measurement indicative of metabolic diseases and obesity. Within the obese group, higher social jetlag levels were observed for metabolically unhealthy individuals, defined with biomarkers such as high waist circumference, high blood pressure, low HDL cholesterol, high glycated hemoglobin (risk factor for diabetes), and high triglycerides. In the metabolically unhealthy obese group, social jetlag was also correlated with elevated glycated hemoglobin and high-sensitivity assays of C-reactive protein (hsCRP), an indicator of inflammation.

Social jetlag is also associated with additional indicators of cardiometabolic risks, including lower HDL cholesterol level, higher triglycerides, higher fasting plasma insulin, insulin resistance, and adiposity, even after taking multiple covariates into account (143–145). Evening chronotypes associate with low level of HDL cholesterol, while social jetlag positively correlates with lower HDL cholesterol, higher triglycerides, higher fasting plasma insulin, insulin resistance, and adiposity (143). In this study, cofounding factors such as subjective sleep quality, depression, and health behaviors were adjusted for. The results of these studies demonstrate that living against the internal clock is contributing to the epidemic of obesity in industrialized societies, and improving the synchrony between the social and biological clocks may be one of the approaches for fighting obesity (141).

## DIET AND MICROBIOME

The gut microbiota, the mixture of microorganisms inhabiting living the intestine, has been demonstrated to play a critical role in insulin resistance, obesity, and metabolic syndrome (146). Furthermore, several studies have proposed that aside from the well-studied circadian *zeitgebers* such as the light/dark cycle and meal timing, gut microbiome may influence the rhythmic

expression of the host's internal body clock (147), while at the same time composition oscillations of the gut microbiome during the day further strengthen the bidirectional interaction between the gut microbiome circadian rhythms and the host's circadian rhythms (148, 149). Therefore, exerting an influence directly in the gut and through modifying the circadian rhythms.

The regulation of circadian rhythms and gut microbiota appears to be reciprocal. In germ-free mice fed either low or high-fat diets, impaired central and hepatic clock gene expression is observed even when the light/dark signals persist (150). This observation leads us to believe that the existence of microbiota is necessary for proper functioning of circadian clocks in the host. The host's internal clocks receive diurnal variation in microbial signals in 2 folds. The microbiota itself exhibits rhythmic community composition, metabolite secretion, localization, and adherence to the intestinal epithelium (150, 151). Therefore, the gut epithelium experiences differential bacterial species and their metabolites depending on the time of the day. In addition, toll-like receptors (TLRs) in intestinal epithelial cells that detect microbial metabolites are rhythmically expressed (152), adding another layer to the circadian rhythms of microbial signals that peripheral organs are exposed to.

The circadian microbiota signal, particularly short chain fatty acids such as butyrate, is known to reset the hepatic circadian gene expression in addition to the intestinal clock gene expression (150, 151). Transcriptomic analysis of the liver in germ free (GF) mice and specific pathogen-free (SPF) mice shed some light onto the microbiome-liver axis (153). Axenic mice exhibit reduced amplitude and phase delay in core clock genes and altered liver transcriptome. The gut microbiome and its metabolites seem to play an essential role in activating some of the key nuclear receptors in the liver. The expression level of the nuclear receptors such as PXR, CAR, LXR, and PPARα, related to detoxification or lipid metabolism, were altered only slightly. However, the target genes of these receptors showed very little expression or dramatic dampening of the rhythms. The gut microbiome most likely influences the liver via portal vein in which the bacterial metabolites travel to the liver. The influence of gut microbiome on hepatic clock is visualized in **Figure 3**.

The variation in enteral microbial structure and function is dependent on dietary composition and meal timing. High fat diet changes the community composition of gut microbiota, reducing the microbial diversity, and blunts the daily changes of luminal gut microbiota abundance (150). The diet-dependent diurnal patterns of metabolite production by the microbes directly modulate hepatic clock gene expression. For conventionally raised mice, high fat diet causes circadian disruption and exposes the animals to risk of obesity. The host's internal clock is perturbed because its liver is exposed to the changes in microbe-dependent metabolites. However, germ-free mice stay lean regardless of the composition of diet. The hypothesis is that diurnal cues from the gut microbiota is missing in these animals, resulting in dampened circadian gene expression even under the presence of light/dark signals. The disturbed rhythms then lead to a heightened metabolic state, resulting in lean body mass regardless of the dietary composition. Since the composition of the diet alters the diversity of gut microbiome while also influencing the proper function of internal rhythms in the peripheral organs, person-to-person variability in metabolism could result from the type of food one chooses to consume as well as the meal times and regularity. The gut microbiome is also under the regulation of sex hormones, which will be discussed in the next section.

In humans circadian oscillations are observed in the relative abundance of 60% of the total gut bacteria, representing ∼15% of various bacterial taxa (150, 151, 154). The circadian rhythmicity of the gut microbiota composition may be due to the signals from the host's peripheral circadian rhythms. The evidence for this is that disrupting the subject's circadian rhythms by the means of jetlag or clock gene mutation abolishes the oscillations in composition of the enteral microbiota (155, 156). The circadian rhythmicity of the microbe itself may also play a role, as at least one species of gut microbiota (*Enterobacter aerogenes*) exhibit endogenous circadian rhythms in swarming and motility while being entrained to melatonin secretion (157).

## INTERPERSONAL VARIABILITY IN METABOLISM: SEX, RACE, AND ETHNICITY

In addition to the degree of misalignment in circadian rhythms due to work shift and life style choices, there are additional factors that may cause person-to-person variability in metabolism. The regulation and progression of metabolic disease differs between males and females due to the effects of gonadal hormones such as estrogens and androgens on the brain and peripheral organs (158, 159). The asymmetry in metabolic homeostasis between the sexes are due to evolutionary paradigm for females to resist the loss of energy state and males to increase muscle mass and exert energy from storage promptly (160).

The gonadal hormones are responsible for sex differences in circadian timing, which then influences metabolic homeostasis. The SCN, where the master clock for mammals reside, exhibit morphological differences between male and female mammals, in volume, number of synapses, and length of rostrocaudal axis (161). The electrical activity in the SCN is also sex-dimorphic. In the SCN core region, the action potential threshold and greater amplitude for males is higher during the dark phase (162), suggesting that females may adjust more easily to new patterns in environmental cues due to low amplitude. In the SCN, androgen and estrogen receptors are present, with males having higher level of androgen receptor expression in human (163) and females having higher level of estrogen receptor alpha expression (164). Male mice that received gonadectomy show longer circadian rhythm period, and treatment with testosterone or dihydrotestosterone can reverse these responses (161), showing the necessity of androgen for entrainment of internal rhythms to environmental cues. Sex difference also exists in HPA functioning through differential HPA response to stress and rhythms or hormone secretion (161). Proestrus female rodents show more increase of basal plasma ACTH and CRH mRNA expression under stress (165). Under basal and stress conditions, female rats show higher level of glucocorticoid

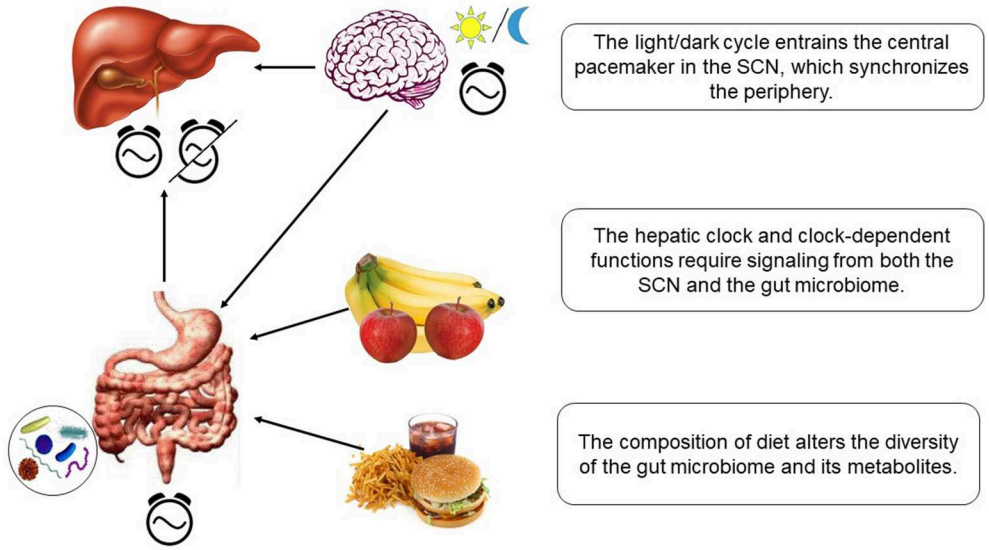


**FIGURE 3 |** Hepatic clock and metabolism is regulated by diet-dependent gut microbiome and light-dependent SCN.

secretion, along with a higher peak in its circadian rhythms (166). In females, corticosterone levels increase and decrease with the estrus cycle, as estrogen increases ACTH release by increasing the duration of secretion (167). In contrast, androgen has an inhibitory effect on the magnitude of ACTH release (166). In addition to the morphological differences and SCN signaling activities, gonadal hormones also appear to affect sleep behavior, and homeostasis. Women have a tendency to go to sleep earlier than man throughout childhood and adulthood; however, the difference disappears at menopause (129). Women also report poorer sleep quality and are more likely to develop insomnia compared to men, despite reporting longer hours of sleep (168). Sleep debt accumulates more quickly in women than men, and has a greater negative impact on women's health (169). As the peripheral clocks entrained by the HPA axis and cortisol interact closely with the metabolic activities of the host, the sex differences in the regulation of these would manifest to variability in metabolism as well. In addition to the gonadal hormones, the effects of sex chromosomes on sex differences in metabolism was recently explored (170). Gonadectomy has differential effects on energy and metabolism of gonadal males and females. Gonad-intact females consume food earlier in the day than males. Gonadectomy phase-advances the feeding time, especially in XX mice. However, gonadectomized XY mice still show earlier acrophase of feeding compared to gonad-intact females (170).

The sex hormones appear to have an influence on the microbiome of the host, as evidenced by male and female mice exhibiting differences in microbiome composition (171). Gonadectomy-associated changes in gut microbiota and bile acid composition can be prevented by administration of testosterone, further confirming that sex hormones, and related clock genes are involved in controlling the enteral microbiome (171). The difference in gut microbiome of rats is at the single strain level, meaning that the sex differences could be masked by individual genetic variation and dietary changes (171). In humans, the sex differences in gut microbiota are observed at the bacterial phylum, genus, and species levels (172), showing more distinction between the microbiota of two sexes compared to rodents. The differences are also associated with obesity or BMI (172). For example, the abundance of *Bacteroides* genus decreases with BMI in men, but remains unchanged with increasing BMI in women, resulting in lower abundance in men than women at high BMI (172). The discrepancy between gut microbiota of men and women appears to have an influence in the gender differences in the susceptibility to cardiometabolic diseases. For example, females are more susceptible to type 1 diabetes (T1D) in both humans and rodents. However, the difference does not exist for germ free mouse model of T1D (173). The gut microbiome of the T1D mouse model is similar in composition for both sexes up to puberty, but in adulthood, males have greater abundance of some genera, including *Roseburia*, *Coprococcus*, and *Bilophilia* (173, 174). Furthermore, castration of male mice leads to a gut microbiota composition similar to that of the females and increases the T1D incidence (173, 175).

Sex, race, and ethnicity play intertwined roles in the development of metabolic syndrome and related comorbidities (176, 177). Recent epidemiological studies reveal that although adolescent African-American males are much less likely to be diagnosed with metabolic syndrome that non-Hispanic whites, even though they are more likely to be obese. On the other hand, non-Hispanic African-American women are more likely than non-Hispanic white women to develop metabolic syndrome. Recent evidence also suggests that obesity in white, post-menopausal women is not associate with increased cardiovascular risk, unless accompanied by metabolic syndrome,

whereas overweight, or obese, African-American women had elevated cardiovascular risk, even if they did not have metabolic syndrome (178).

Given the intimate connection between metabolic syndrome and circadian rhythms, it is interesting to explore what is broadly known regarding the relationships between race/ethnicity and circadian rhythms to rationalize some of the aforementioned observations. Recent studies have compared the endogenous circadian rhythms between African-Americans and non-Hispanic European Americans (179) to determine that the former had slightly longer period than the latter, likely the result of adopting more favorable periods prior to migrating out of areas near the equator. Even more interestingly, males of European American ancestry had longer period than females. A hypothesis for this observation is that out of the equator area, longer circadian period is advantageous in tracking dawn, which is important for males as hunters but not so much for women as gatherers (179). The evolutionarily driven adaptation of the endogenous (free running) rhythms could possibly provide clues as to the differential response of various ancestry characteristics on circadian misalignment. This was test in a study assessing the impact of chronodisruption in African-Americans and non-Hispanic European-Americans (180). Interestingly the longer period of European-Americans was associated with longer phase delays following chrono disruption. The key message being that the endogenous rhythms, as evolutionary developed, determine the ability of subject of different decent to adapt to circadian misalignment. These finding could potentially shed light on the race dependencies of chrono-disruption.

However, deciphering individualized effects of circadian misalignment is a complex issue. Two insightful early studies attempted to characterize the intrinsic qualities of individuals not being affected by shift work (181, 182). Interestingly, these early studies seem to indicate that individual tolerant to shift work appear to (i) exhibit more robust circadian amplitude; and (ii) appear to adjust slowly to changing light-dark schedules. It is therefore evident that genotypic differences, establishing the dynamic of endogenous rhythms, underlie the ability of the host to cope with *zeitgeber* changes. The issue of the emergence of such trade-offs in the context of circadian misalignment was further discussed in (183).

## THE CIRCADIAN CLOCK AS A TARGET IN METABOLIC DISORDERS

The wealth of information connecting circadian disruption and metabolic syndrome has prompted an interest in the development of pharmacological clock-targeting compounds aiming at attenuating disease symptoms by resetting dysregulated circadian rhythms (184) whereas a wide range of small molecules are currently explored as likely pharmacologic leads (185, 186). Preliminary studies have established that diets rich in certain nutritional supplements, such as selenium (187), or certain flavonoids (188) have been shown to enhance circadian rhythms with positive health implications (189). Pharmacologic studies using *nobiletin,* a flavonoid, natural polyphenol, isolated from citrus peel, demonstrated that strengthening circadian amplitude as a plausible pharmacological intervention for metabolic disorders (188). Furthermore, nobiletin was shown to ameliorate lipid metabolism in hepatocytes, mediated via modulations of the clock gene *Bmal1* (190). Similarly, nobiletin was shown to restore circadian rhythms in embryonic fibroblasts (191).

## MATHEMATICAL MODELING OF CIRCADIAN METABOLISM

Since the understanding of the intricate network consisting of circadian clocks and metabolic activities is still under development, mathematical modeling of the system is useful in testing hypotheses on the relationship between circadian disruption and metabolic dysfunction. In addition to aiding in understanding of interaction between metabolism and circadian rhythms, modeling can also help define optimized feeding regimen for various photic environment for healthy and diseased hosts. Over the past decade the circadian system has been modeled using different approaches such as differential equations (192) automata, and Petri Nets (193). The earliest circadian model was based on a simple feedback loop comprising of an activator and inhibitor (194). Later, the coupling of ultradian oscillators to generate a circadian rhythmicity was also demonstrated in a mathematical model (195). More recent models target the proteins that form the core clock functions in the SCN and in the periphery, such as PER, CRY, BMAL1, and CLOCK (192, 196). The models describing the core clock machinery were later utilized to study the entrainment mechanism or to describe downstream biological functions that are closely regulated to circadian clocks. For example, a mathematical model that described the entrainment of PCGs by cortisol (197) was expanded to describe the entrainment of clock genes by the light/dark cycle (198). The model was used to explore the effects of seasonal variations in light duration on the HPA axis response (199, 200). The effects of sex differences and interindividual variability on the activity of HPA axis and cortisol secretion were also modeled (201), which would have implications for the clock genes entrained to cortisol and the biological functions tied to the clock rhythms.

More recently, there have been efforts to integrate the newly discovered transcriptional/translational regulation of core circadian clock by the feeding induced signals. The integration of feeding signals transmitted through SIRT1, PARP1, and HSF1 to the clocks with a Petri Net model was studied in (202) in order to study the effects of different feeding regimens to demonstrate that circadian system can be entrained to the differential feeding rhythms and that the feeding signals were transmitted to the PCGs. The model shows that 2 or 3 meal/day feeding regimens are beneficial for robust circadian rhythms while less or more meals/day have negative effects. The integrated role of feeding/fasting cues to the hepatic circadian clock was studied by incorporating SIRT1 and AMPK as the metabolic sensors (203). The content of the diet (normal, fasting, and high-fat) were simulated as the activation pattern of AMPK, and the

model predicted the observed decrease in clock gene expression for high-fat diet.

Several studies have explored the effects of circadian disruption on clock gene expression and metabolic syndrome. Along those lines, a model that takes in the feeding/fasting rhythms through $NAD^+$ mediated SIRT1 activation and light/dark cycle through the HPA axis modeled with Goodwin oscillator predicted asymmetrical entrainment of clock genes to the two environmental cues was recently discussed in (204). The model was further developed to study the effect of *zeitgeber* phase mismatch on expression of gluconeogenic genes such as *G6pc* and *Pck1* (205). This model reproduced the finding that genetic circadian disruption through *Clock* gene knockout results in altered level of gluconeogenic gene expression and predicted that certain phase relations between the two *zeitgebers* can recover the wild-type expression level in knockout cells. The effect of light-feeding mismatch was also modeled for pancreatic β cells (206). Consistent with the previous findings, restricting food access to rest phase results in phase shift of clock gene expression as well as metabolic abnormalities such as hypoinsulinemia and hyperglycemia. Recent studies further help elucidated the complex regulatory impact of SIRT1 on the regulation of clock function through actions on PER2, and PGC1α and CLOCK/BMAL1 (207).

## CONCLUDING REMARKS

Organisms have evolutionarily adapted their circadian machinery to allow flexibility in processing the changing environment such as the light signal, temperature, and food availability throughout the 24-h day. However, the biological clock is greatly impacted by the modern society and technologies that imposes a lifestyle which often misaligns the endogenous rhythms from the earth's rotation. Disrupted rhythms manifest in the form of phase mismatch and muted amplitude in expression of clock genes with serious health implications including metabolic syndrome. Genetic modifications in animal models have shown that proper functioning of clock genes at a molecular level is necessary for homeostatic metabolic activity. For instance, homozygous mutation of *Clock* in mice result in suppressed metabolic rate rhythm and overall decrease in metabolic expenditure, increased risk of obesity, hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulemia (208). Although mechanisms discovered in clock knockout animal models cannot directly translate to human circadian disruption, it can start to provide insight into and generate hypotheses around human physiology relating to circadian driven metabolic dysfunction. Recent findings on signal transduction of feed/fast cycles that influence the clock proteins are beginning to shed light on the mechanism of metabolic syndrome resulting from circadian disruption, but much work remains to gain comprehensive enough understanding to begin prevention and treatment of the metabolic conditions.

Shift work, variable eating patterns, and social jetlag strongly suggest that chronic exposure to misaligned rhythms is more detrimental to health compared to acute exposure. Although mathematical modeling of circadian metabolism is beginning to provide insight into effects of *zeitgeber* misalignment and implications of restricted feeding, they have not yet been able to address the question of chronic phase mismatch intensifying the degree of metabolic dysfunction. Understanding the network of clocks and metabolic components is challenging because in addition to the underlying complexity of the system, there are also many sources for interindividual variability. Inconsistencies could arise from behaviors, life style choices, genetic makeup, and socioeconomic factors, accumulating convolution in studying long-term effects. However, current understanding suggests that behavioral changes (i.e., restricted feeding) can reverse chronodisruption (205, 209) due to genetic or behavioral causes, and broadening the understanding of mechanism behind circadian metabolism would be beneficial for chronotherapy and discussion in more biologically pertinent social clock.

## AUTHOR CONTRIBUTIONS

S-AB: wrote the manuscript. MF, VR, and HZ: edited the manuscript. IA: conceived the idea and edited the manuscript.

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REVIEW
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# Time-of-Day-Dependent Physiological Responses to Meal and Exercise

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The mammalian circadian clock drives the temporal coordination in cellular homeostasis and it leads the day-night fluctuation of physiological functions, such as sleep/wake cycle, hormonal secretion, and body temperature. The mammalian circadian clock system in the body is classified hierarchically into two classes, the central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus and the peripheral clocks in peripheral tissues such as the intestine and liver, as well as other brain areas outside the SCN. The circadian rhythm of various tissue-specific functions is mainly controlled by each peripheral clock and partially by the central clock as well. The digestive, absorptive, and metabolic capacities of nutrients also show the day-night variations in several peripheral tissues such as small intestine and liver. It is therefore indicated that the bioavailability or metabolic capacity of nutrients depends on the time of day. In fact, the postprandial response of blood triacylglycerol to a specific diet and glucose tolerance exhibit clear time-of-day effects. Meal frequency and distribution within a day are highly related to metabolic functions, and optimal time-restricted feeding has the potential to prevent several metabolic dysfunctions. In this review, we summarize the time-of-day-dependent postprandial response of macronutrients to each meal and the involvement of circadian clock system in the time-of-day effect. Furthermore, the chronic beneficial and adverse effects of meal time and eating pattern on metabolism and its related diseases are discussed. Finally, we discuss the timing-dependent effects of exercise on the day-night variation of exercise performance and therapeutic potential of time-controlled-exercise for promoting general health.

**Keywords: circadian rhythm, chrono nutrition, chrono exercise, time-restricted feeding, meal pattern**

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## INTRODUCTION

Several human physiological functions such as sleep/wake cycle, blood pressure, hormone secretion, body temperature, and physical activity exhibit around 24 h cycles called circadian rhythm. The anticipated diurnal change of a physiological function is also observed prior to the diurnal changes in environmental conditions such as light/dark cycle and temperature changes due to the rotation of the earth. This anticipative adaptation is driven by a circadian clock system existing in several tissues. The mammalian circadian clock system has an established hierarchy to distinguish between a central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral clocks in peripheral tissues including liver, lung, kidney, skeletal muscle and adipose

tissue, as well as brain areas outside the SCN (1). The photic signal transmitted from the retina to SCN entrains the central clock, or master pace maker, that provides temporal cues to circadian clocks in the whole body. The temporal information of the central clock is transmitted to the peripheral clocks via neural and endocrine pathways, such as the sympathetic nervous system and glucocorticoid signaling (2, 3). The peripheral clocks are entrained by not only a light-induced signaling from the SCN but also other stimuli such as feeding, exercise, and stress in a SCN-independent manner (4–7). Nutrients entrain peripheral clocks (e.g., liver) via the activation of transcriptional and translational regulation of molecular clocks (see below) [for review, see (7, 8)]. For example, the ingestion of carbohydrate increases the insulin secretion, following the activation of the transcription and translation of clock genes and proteins (especially *Period2*), via the activation of insulin signaling (9, 10). Likewise, exercise entrains the circadian clocks in the peripheral tissues such as muscle, liver and lung via the sympathetic nervous system and glucocorticoid signaling (11, 12). These effects of nutrient and exercise on circadian clock are observed not only in rodents, but also in humans (13, 14).

The molecular mechanisms of circadian clock systems in mammals have been investigated since the discovery of *Clock* gene (*Circadian locomotor output cycles kaput*) in 1997 (15). Several core clock genes have been identified in mammals, including *Bmal1* (*Brain and muscle ARNT-like 1*), *Clock*, *Per1* (*Period1*), *Per2*, *Cry1* (*Cryptochrome1*), and *Cry2*. These genes interact with each other via transcriptional and translational negative feedback loops to exhibit a 24 h cycle (**Figure 1**). The heterodimer of CLOCK and BMAL1 works as transcriptional factors and has a basic helix-loop-helix PAS domain. The binding of this heterodimer to an E-box binding element in the promoter regions of *Pers* and *Crys* activates the transcription of these genes (16). The translated PER1/2 proteins are phosphorylated by CKIε/δ (Casein kinase Iε/δ) in the cytoplasm (17). The phosphorylated PER1/2 proteins are unstable and are degraded by the ubiquitination-proteasome pathway (18, 19). Similar degradation is seen in the CRY1/2 proteins due to ubiquitination systems via FBXL3 (F-box and leucine rich repeat protein 3) (20). The CRY1/2 and PER1/2 proteins in the cytoplasm promotes the formation of PERs/CRYs/CKIε/δ complex. This complex then transfers to the nucleus and suppresses the transcription induced by the heterodimer of CLOCK and BMAL1. The transcription of *Clock* and *Bmal1* is negatively and positively controlled by REV-ERBs (nuclear receptor subfamily 1, group D) and RORs (RAR-related orphan receptor), respectively via binding to a ROR-responsive element (21, 22). Similarly, *Pers* and *Crys*, the *Rev-erbs* and *Rors* genes are also the target of the BMAL1 and CLOCK complex (21, 22). The BMAL1/CLOCK complex temporally controls the transcription of other genes, which are called clock-controlled genes (CCGs), such as *Dbp* (*D-site of albumin promoter binding protein*) and *Pparα* (*Peroxisome proliferator activated receptor* α) via binding to respective responsive element sequences (23–25). This negative feedback loop of clock genes exists in nearly all tissues in mammals.

Circadian transcriptomics revealed that the expression of rhythmic genes occurs in a tissue-specific manner (26, 27). Through the analysis of mice with tissue-specific clock gene mutations, the importance of peripheral clocks in tissue-specific functions is being revealed (28, 29). Especially, nutrient metabolism exhibits a clear day-night variation in tissues with high metabolic activity, such as liver, muscle and adipose tissue, where its diurnal change is directly regulated by an intrinsic clock (30–34). From these results, it is thought that one of the major roles of the peripheral clock is to prepare for the transition from the rest phase to the active phase, and responding to high energy demand (26, 30, 31, 35). Considering that the metabolic process of each nutrient is diurnally controlled, it is expected that the postprandial response of metabolic functions depends on the feeding time. Also, fuel selection in a skeletal muscle during exercise depends not only on the nutritional state, but also on the time of day (36, 37). Additionally, some of exercise-regulated factors such as AMPK (AMP-activated protein kinase) are temporally activated by circadian clocks (38). In this review, we discuss the time-dependent physiological response to nutrients and a role of circadian clock in these time-dependent effects. Finally, we also summarize the time-dependent effects of exercise on physiological functions and athletic performance.

## TIME-OF-DAY-DEPENDENT POSTPRANDIAL RESPONSE OF MACRONUTRIENTS

Generally, we take meals three times a day. Although there are many reports focusing on the postprandial metabolic response to a single meal, it is rare that the research focuses on the comparison between metabolic responses to breakfast, lunch, and dinner. In this section, we review the time-of-day effects on the postprandial metabolic responses of macronutrients and the influence of circadian rhythm to these time-of-day effects.

### Lipid Metabolism

It is observed that the postprandial triacylglycerol (TG) response is dependent on the eating time. Sopowski et al. investigated the blood TG response to identical high-fat meal consumed during the daytime (13:30) and the night time (01:30) in healthy men and women. They reported higher and longer postprandial elevation of TG at the night time than that at daytime (39). The meal-time-specific postprandial response of blood TG is also different between breakfast and lunch, and the increasing of TG levels after lunch is ∼2-fold less than that after breakfast in men (40). A weak postprandial response to lunch is also exhibited when breakfast had been skipped, suggesting that the endogenous circadian rhythm is involved in the differential effects observed after breakfast vs. lunch and dinner. In addition, addition of stable-isotope-labeled palmitic acid to the test meal was used to distinguish between the meal-derived TG and endogenous TG. The postprandial labeled-palmitic-acid level was not changed between breakfast and lunch, suggesting that the lower response of blood TG level after lunch involves fatty acids derived from endogenous sources but not the meal itself (40). Insulin suppresses the release of free fatty acids from adipose tissue (41). Considering that the change of insulin level also depends on

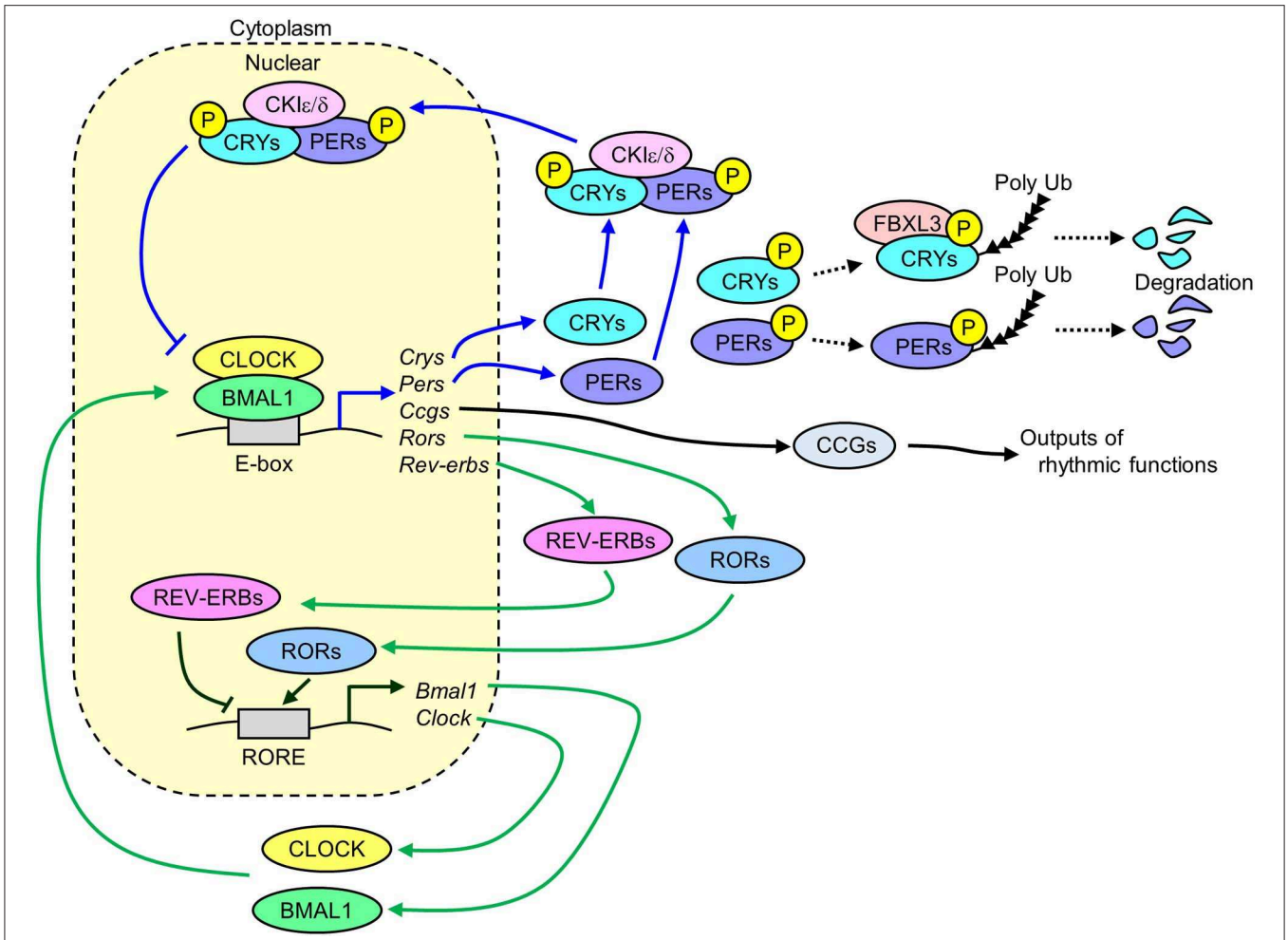


**FIGURE 1 |** Transcriptional and translational negative feedback loop of core molecular clocks in mammals. The heterodimer of CLOCK and BMAL1 activates the transcription of *Crys*, *Pers*, *Rors*, *Rev-erbs,* and *Ccgs*. The translated and phosphorylated PERIODs and CRYs form a complex along with CKIε/δ and then this complex is translocated into the nucleus to inhibit its own transcription induced by the heterodimer of CLOCK and BMAL1 (blue lines). A part of phosphorylated CRYs and PERIODs is degraded via ubiquitin-proteasome pathways. The translated REV-ERBs and RORs inhibits and activates the transcription of *Bmal1* and *Clock* genes via binding to RORE, respectively (green lines). The rhythmic expression of *Ccgs* results in oscillation of several physiological functions (Crys, Cryptochrome1/2; Pers, Period1/2; Rors, Retinoid-related orphan receptors; Rev-erbs, reverse-Erb receptors; Ccgs, Clock-controlled genes; CKIε/δ, Casein kinase 1ε/δ; RORE, retinoic acid receptor response element).

the meal time, it is suggested that the lower elevation of TG after lunch could be dependent on insulin levels. The day-night variation of postprandial TG levels is reported in studies carried out on animal models, and the higher response of postprandial TG at the rest phase compared with the active phase is attributed to lower uptake of fatty acids into skeletal muscles and brown adipose tissues (42). This study reported that the postprandial day-night variations are not observed in SCN lesioned rats. Furthermore, lipid utilization is also directly regulated by the intrinsic muscle clock (43). Thus, it suggests that the circadian-clock-driven day-night variation of lipid uptake and utilization is related to the difference of postprandial TG response among meals. Recently, it was observed that the preventive effects of fish oil on hepatic steatosis and hyperlipidemia depend on feeding time in mice (44). In this study, Oishi et al. developed and used the two-meals-per-day feeding model. The blood levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are higher in the mice fed with a fish oil during the time of activity onset compared with that during the onset of inactive phase, suggesting that feeding-time dependent therapeutic effects of fish oil rely on the temporal capacity of intestinal absorption of DHA and EPA.

## Glucose Metabolism

Like the effects observed on blood TG levels, the postprandial glucose levels exhibit a time-of-day-dependent response to meals. Glucose tolerance is higher in the morning than in the evening, in humans (37). It is known that the difference of glucose tolerance between the morning and evening is due to the temporal regulation of glucose utilization and pancreatic β cell function (see below). In fact, the dysregulation of glucose metabolism is observed in the whole-body or liver-, muscle-,

or pancreatic βcell-specific clock gene mutant mice (30, 32, 33, 45, 46). In pancreatic β cells, a circadian clock controls the rhythmic transcription of insulin-secretion-related genes, and the decrease of a nutrient-induced insulin secretion is observed in the pancreatic β-cell-specific *Bmal1* knock out mice (45). Glucose uptake and utilization in peripheral tissues such as liver and skeletal muscle are differentially regulated (30, 32). For example, the murine muscle clock temporally regulates glucose uptake into skeletal muscle via the recruitment of GLUT4 (glucose transporter 4) to the plasma membrane and increased expression of glycolytic genes. Considering that this temporally regulated surge is observed prior to the active phase, it is thought that the muscle clock has a role in preparation for high energy demand at the beginning of active phase. In addition to animal studies, Morris et al. reported that the human intrinsic circadian system affects day-night variation of glucose tolerance and insulin-secretion by the use of circadian alignment and misalignment protocols (47). The magnitude of its effect is larger than effect of behavioral rhythm such as sleep/wake cycle and fasting/feeding cycle in humans. In addition, circadian misalignment between endogenous and behavioral rhythms also exacerbates glucose tolerance in shift workers (48), thus highlighting the importance of alignment between both rhythms for prevention of diabetes in shift-workers. Thus, the diurnal variation of glucose tolerance is regulated by both endogenous and behavioral rhythms, and the molecular clock in peripheral tissues drives endogenous rhythms such as glucose uptake and insulin secretion.

## Amino Acid Metabolism

There have been studies focused on the feeding-time-dependent postprandial response of amino acids and peptides and the diurnal absorptive capacity of these nutrients. In the small intestine of rodents, the absorption of some amino acids and peptides was activated in the early active phase rather than in the early rest phase (49). $H^+$-coupled peptide transporter (PEPT1) is localized at the apical membrane of intestinal epithelial cells and has major role for di- or tri-peptide transportation in the small intestine. Pan et al. reported that the absorption of glycyl-sarcosine, which is one of the substrates of PEPT1, depends on the administration time in rodents (49). The blood glycyl-sarcosine level is higher after its administration in the early active phase than that in the early rest phase (49). In addition, the PEPT1 mRNA and protein levels in the rat duodenum and jejunum exhibit day-night variation and are elevated before active phase (49, 50). The pattern of day-night variations of PEPT1 level is associated with the diurnal pattern of glycyl-sarcosine uptake in the duodenum (49), suggesting that the diurnal variation of PEPT1 levels is involved in the time-dependent absorption of peptides in small intestines. Pan et al. also reported that another PEPT1's substrate, the antibiotic ceftibuten, is absorbed in a time-dependent manner in rodents (51). The time-dependent absorption and the diurnal rhythm of PEPT1 level are not observed under the fasting condition, suggesting that feeding cycle is important for the time-dependent effect. In fact, time-restricted-feeding led to a shift in the phase of diurnal PEPT1 level in the duodenum of rats (52). Albumin D site-binding protein (DBP) is one of the clock-controlled genes and its transcription is activated by the heterodimer of BMAL1 and CLOCK and suppressed by PERs and CRYs (53). In addition, DBP activates the transcription of several genes including *Pers* via DBP binding site and the expression of target genes shows the diurnal rhythmic pattern (53). *Pept1* has a DBP binding site in its promotor region. A luciferase assay designed using the promotor region of *Pept1* showed that DBP activates PEPT1 promotor activity (54). Okamura et al. reported that bile-acid-regulated PPARα activity leads to the diurnal expression of *Ppet1* in the intestinal cells of mice (55). The feeding-fasting cycle induced the day-night variation of cholic acid in the intestinal epithelial cells. Cholic acid decreases the *Pept1* levels before active phase, which corresponds to the peak time of *Pept1* expression, but not before rest phase, which is its trough time. The cholic-acid-dependent regulation of *Pept1* expression is suppressed by knockdown of PPARα. In addition, time-dependent absorption of carnosine, which is one of the substrates of PEPT1, is also not observed in PPARα-null mice. These reports suggest that absorption of peptides in small intestine exhibits diurnal variation via the DBP- and PPARα-mediated circadian control of PEPT1 expression. In recent years, time-dependent intestinal absorption of amino acids has been reported (56, 57). Jando et al. reported that isoleucine absorption is higher in the active phase than in the rest phase, although the protein levels of intestinal amino acid transporter B0AT1 in the rat intestine are not changed between the two time points (57). This study suggested that circadian expression and/or post-transcriptional modulation of other amino acids transporters is involved in the time-dependent intestinal isoleucine absorption. The branched-chain amino acids, such as leucine, valine, and isoleucine, are absorbed via LAT4 (SLC43A2), a basolateral neutral amino acid transporter (58). LAT4 phosphorylation at Ser274 is higher at the beginning of the rest phase than at the beginning of the active phase in mice (56). LAT4 shows high activity under dephosphorylated condition, suggesting that post-translational modulation such as phosphorylation could be involved in the time-dependent amino acid absorption. In studies involving human subjects, comparing the postprandial response between morning and evening using metabolomics, revealed that 16 amino acids such as arginine and leucine were detected at higher levels in blood in the morning than in the evening (59). These data suggest that the postprandial amino acids response in humans depends on the feeding time.

# EFFECTS OF TIME-RESTRICTED-FEEDING

The feeding activity of a rodent is rhythmic and occurs mainly during the active phase, especially in the early active phase. The perturbations of feeding rhythm relate to the metabolic dysfunctions, leading to the onset of obesity, diabetes and lipidosis (60–63). Feeding a high-fat diet dampens the diurnal feeding/fasting cycle, resulting in more food intake during the inactive phase (64). The restriction of feeding time prevents the high-fat diet induced metabolic disorders, such as excessive body weight gain, glucose intolerance, hepatic steatosis, and inflammation (65, 66). Considering that the time-restricted feeding (TRF) also protects against the high-fat-diet-induced dampening of clock genes such as *Per2*, *Bmal1*, *Rev-erbα,* and *Cry1* in the liver (65), it is contemplated that the TRF prevents several metabolic dysfunctions via a rescue of rhythmic

peripheral clock gene expression. However, the preventive effects of the TRF are also observed without changes of locomotor activity or calorie intake, in mice lacking a circadian clock, such as whole-body *Cry1/2* double knockout, liver-specific *Bmal1* or *Rev-erbα/β* knockout mice (67). Transcriptomic analysis of different mouse lines reveals that the transcripts to observed to be oscillating in the wild type mice under the TRF are mostly unaffected in the clock gene deficient mice under the TRF, thus suggesting that one of the main effects of TRF in clock gene deficient mice is maintaining basal level of gene expression rather than the temporal control of expression. In addition to gene expression profile, Chaix et al. discuss the possibility that the TRF may regulate a temporal post-translational modification because the TRF drives the oscillation of post-translational modification with greater amplitudes rather than those of transcripts and metabolites (68). Contrary to these studies, some reports showed that TRF had no effect on body weight loss in rodents (69–71). For example, TRF (12 h feeding window) during the light or dark phase did not change the body weight as compared to *ad libitum* feeding in rats (70, 71). Although the reason for these conflicting results is unclear, it is possible that the effects of TRF on body weight loss may depend on the periods of feeding window and diet composition in animal studies. In fact, the beneficial effects of TRF on body weight loss were especially observed in the case of high-fat diet feeding or under the shorter feeding windows (<8 h) (72). The preventive effect of TRF due to a reduction of meal frequency or a shortened feeding window is also observed in human studies (36, 73, 74). Sutton et al. reported the strict early-time-restricted feeding (6 h feeding window, with dinner time before 1500 h) for 5 weeks improves the insulin sensitivity and β cell function, blood pressure, and oxidative stress in prediabetic men (75).

As mentioned before, food intake is one of the major non-photic entraining impulses in peripheral clocks in the peripheral tissue such as liver and adipose tissue, while it does not entrain a central clock in the SCN (7). Shift of calorie intake time to the sleep-phase induces desynchronization between peripheral and central clocks (76). In other words, the disturbance between fasting/feeding cycle and sleep/wake cycle leads to the disconnection between the central and peripheral clocks, resulting in induction of several metabolic dysfunctions (60, 61, 77–80) (**Figure 2**). For example, the larger food consumption at night or the delayed onset of feeding time due to breakfast skipping, is related to body weight gain and insulin sensitivity in humans (77, 78, 81–85). The adverse effects of rest-phase-feeding are observed in experimental animal models and it has been shown that the rest-phase-feeding-induced weight gain is induced without any change of locomotor activity and food intake (60). Additionally, some researchers have developed two- or three-meals-per-day-feeding models in rodents (meals in the early, middle, late active phase are defined as breakfast, lunch and dinner, respectively) to imitate general human meal pattern (86–88). The breakfast skipping rats had larger weight gain when compared with the dinner skipping rats (88). Larger weight gain is also observed in the delayed breakfast model without a change of total food intake (87). Also, Wu et al. showed that the beneficial effects of calorie restriction depends on which meal you restrict the calories from Wu et al. (88). Greater weight loss, circumference reduction, insulin sensitivity index, and triglyceride levels are observed in obese women who restricted calories from dinner compared to those who restricted it from breakfast (89). It suggests that consuming high calorie meal during the night induces the dysfunctions of lipid and glucose metabolisms even if the feeding window is shortened, like in time-restricted feeding.

In recent years, it is known that the feeding in the rest phase affects not only metabolic diseases but also other functions. Muscle mass is decreased by the rest phase feeding via the inactivation of IGF-1 signaling (90). The murine muscle growth and protein synthesis are down-regulated byTRF in the rest phase compared with the TRF in the active phase (91). In the murine skin, the rest-phase-TRF shifts the phase and reduces the amplitude of clock genes, leading to the dysregulation of the diurnal sensitivity of UVB-induced DNA damage and a key DNA-repair-related gene (92). On the other hand, the diurnal regulation of DNA synthesis is not affected by TRF. Thus, the rest-phase-TRF leads to the mismatch of temporal regulations between DNA synthesis and repair, resulting in the increased sensitivity to UVB-induced DNA damage. In summary, TRF during the optimal time, to avoid the sleep phase, could be effective for the maintenance of several biological functions, while TRF during the sleep phase might attenuate muscle and skin functions as compared to TRF during the active phase.

# TIME-OF-DAY-DEPENDENT PHYSIOLOGICAL RESPONSES TO EXERCISE

## Diurnal Variation of Physical Performance

Athletic performance such as muscle strength and endurance exhibits day-night variations (93–95). Generally, the human athletic performance is low in the morning and its peak time is late afternoon (93–95). Its diurnal change is closely related with the change of body temperature (96, 97). A hot environment blunts the day-night variation of muscle performance, such as muscle force, power, and contractility, thus it is thought that the body temperature partially contributes to the diurnal variation of physical performance (98). The circadian clock drives the oscillation of various physiological functions including body temperature (99). In addition to the body temperature, the diurnal pattern of human physical performance is also changed by chronotype, and its amplitude is greater in the evening type persons with a lower performance in the morning (100). The chronotype-specific day-night pattern is also observed in the swimming performance (101). Endurance exercise capacity is changed in mice with some clock gene deletions, such as *Rev-erbα* and *Cry1/2* (102, 103), suggesting the regulation of exercise performance by circadian molecular clock. In fact, Ezagouri et al. report that the diurnal variation of exercise capacity in mice relies on the clock proteins PER1/2 and they discover 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), which is AMPK activator, as a key factor to induce the time-specific effects

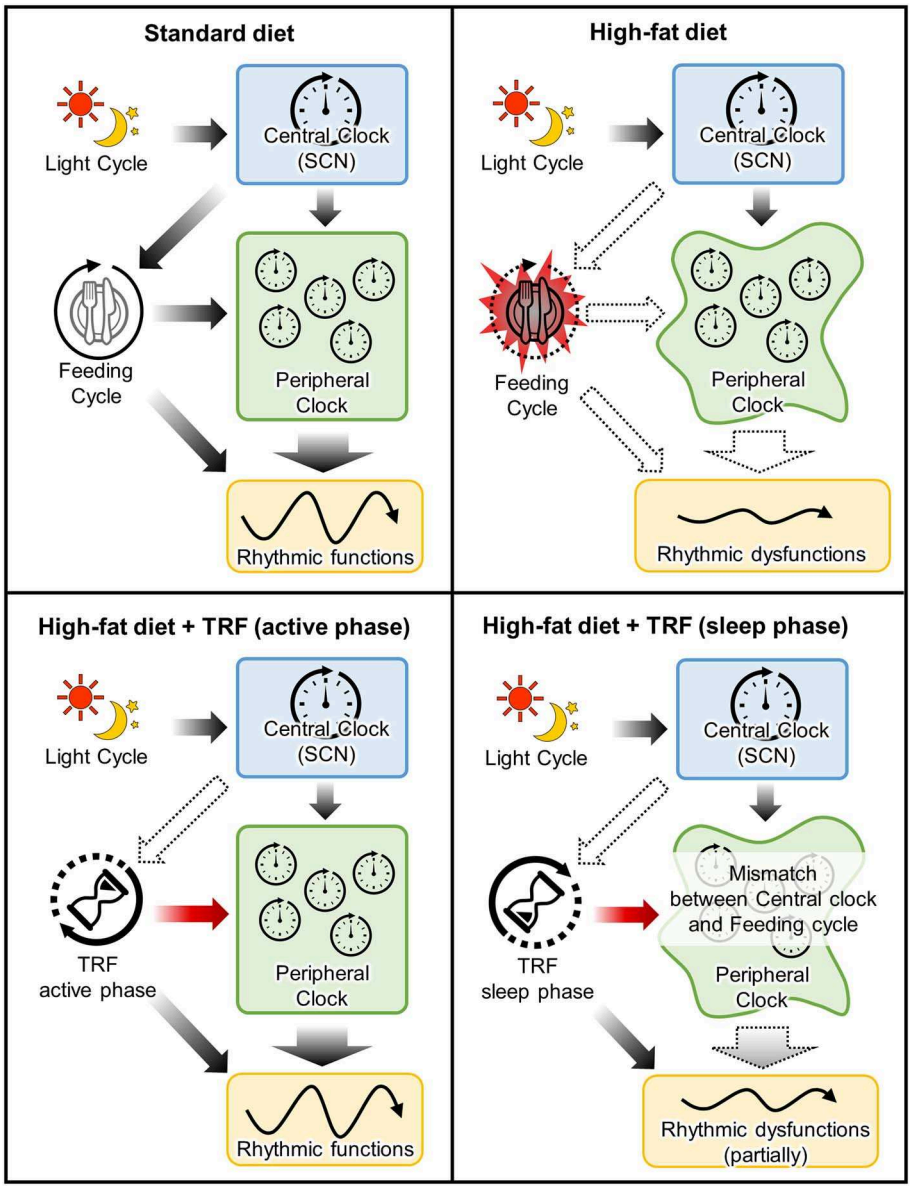


**FIGURE 2 |** Mechanistic insight into the effects of time-restricted-feeding during active or sleep phase. Upper left panel: In standard-diet-fed mice, timing of the entrainment cues between feeding cycle and central clock is matched for peripheral clocks. Upper right panel: High-fat-diet not only induced metabolic dysfunction due to a high calorie but also arrhythmic feeding cycle. This dysregulation of feeding cycle attenuates the feeding-induced-entrainment of peripheral clocks. Lower left panel: TRF during active phase rescues the attenuation of peripheral clock functions due to perturbation of feeding cycle. Lower right panel: Although TRF during sleep phase also entrains peripheral clocks, timing of the entrainment cues between feeding cycle and central clock is mismatched. It is suggested that this mismatch partially attenuates the beneficial effects of TRF.

of exercise on exercise capacity using both transcriptomic and metabolomic analyses (104).

The training time within a day affects the day-night variation of human physical performance (93, 105). As mentioned before, human muscle power exhibits a diurnal variation, where it is lower in the morning than in the evening (93, 94). In a human study, this diurnal variation of the muscle power is blunted by 12 weeks of resistant training in the morning via an enhancement of muscle power (106). The reduced daily fluctuation of muscle power due to the exercise training is specific to morning training, while it is not observed in the evening training (93, 107). On the other hand, the exercise training in the evening induces the elevation of muscle performance in the afternoon, thus the magnitude of diurnal muscle performance change is increased in humans (107, 108). In summary, a high amplitude of muscle performance within a day is observed by training in the evening, while training in the morning decreases amplitude of daily muscle performance through the enhancement of performance in the morning (**Figure 3**). Similarly, in elite college basketball players, the performance in afternoon is lower during the

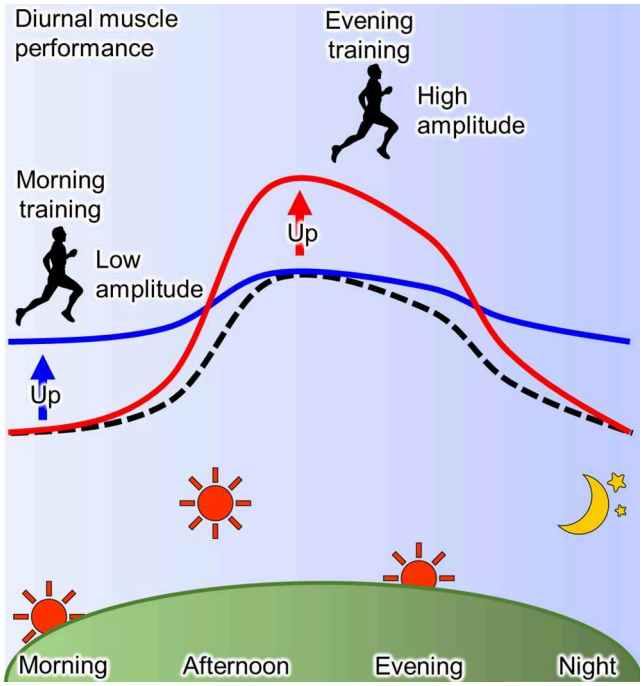


**FIGURE 3 |** Scheme for effect of training-time on diurnal physical performance. The muscle performance exhibits diurnal variation with the peak at afternoon and the trough at morning and night (dotted line). This diurnal characteristic of muscle performance is changed by training and its effect depends on the time of day (93). Morning training increases the performance in morning resulting in the low amplitude (blue line), while evening training increases it in the evening resulting in the high amplitude (red line).

morning training periods when compared with the afternoon training period, although the performance in the morning was not evaluated (109). These observations suggest that similar response to time-specific training is seen not only in common people, but also among elite athletes. In addition, it is possible that these changes of diurnal pattern are linked to competition ability. The times recorded for 200 m swimming trail in the morning were faster in the subjects who habitually train in the morning compared with those who train in the evening (101). From a practical point of view, Chtourou et al. recommend that the time-controlled training should be adjusted to same time of competition for exerting the best performance in the competition (93, 106). However, some reports show no effects of training time on the day-night variation of muscle performance (110, 111). It is a possibility that the different training conditions, such as duration and intensity, and the passive warm-up effect of the environment may have influenced the results.

## Blood Pressure and Blood Circulation

Blood pressure also exhibits a clear circadian rhythm with a lower blood pressure during the rest phase, increasing around the time of waking up, and the highest at active phase in human and rodents (112, 113). The time-dependent hypotensive effect of exercise was observed a long time ago in humans (114). Helen et al. reported the time of day effects on the post-exercise response of blood pressure in normotensive men (115). Cycling exercise at 60% $VO_{2max}$ in the early morning (0400 h) induces the transient elevation of blood pressure while the exercise in the afternoon, evening, and night, do not change or transiently reduce blood pressure compared with each pre-exercise condition (115). Although it suggests that the morning exercise is not better for the reduction of blood pressure, Helen et al. do not evaluate the blood pressure under the sedentary conditions at each time point (115). Thus, the possibility remains that the reducing effect of morning exercise on blood pressure may be masked by the circadian rising of blood pressure in the morning, called "morning surge." De Brito et al. evaluated the net change of post-exercise blood pressure, with the use of adjustment by the day-night variation of blood pressure, under the control sedentary condition in normotensive subjects (116). In the adjusted conditions, the post-exercise reduction of blood pressure is observed in both morning and evening, and its reduction is greater in morning than in the evening (116). In addition to blood pressure, the exercise-induced reduction of cardiac output and the weak response of exercise-induced increased heart rate are observed after morning exercise, while the sympathovagal balance and lower limb blood flow responses are increased after evening exercise (116). It suggests that the greater effect of morning exercise is due to cardiac and autonomic functions. Recently, it was reported that the responses of peripheral blood flow and vascular conductance after exercise are not changed between the morning and evening exercise in young subjects, suggesting that the time-of-day effects of exercise on blood pressure and vasodilation are likely reflecting central rather than peripheral regulation (117). Similarly, the higher response of blood pressure to cold exposure in hypertensive adults is reduced by the exercise in the morning but not in the evening (118), suggesting that the morning exercise promotes the reactivity of vasodilation.

The chronic anti-hypertensive effects of exercise training in the morning or evening on blood pressure were observed in anti-hypertensive-drug-treated men (119). Evening exercise training for 10 weeks (3 times a week) reduces systolic blood pressure and diastolic blood pressure during sleep, while their effects are not observed in morning trained hypertensive men. In contrast to the beneficial acute effects of morning exercise (116), the chronic effects of morning exercise are not observed. It is possible that the effect of anti-hypertensive drug masks the hypotensive effect of exercise in the morning, because all subjects took medication in the morning (119). Moreover, the net change of blood pressure is not evaluated under the adjusted condition described before, thus it is possible that some effects of morning exercise mask by the morning surge.

The timing-dependent hypotensive effect of exercise depends on the circadian characteristic of blood pressure. Park et al. investigated the hypotensive effect of exercise in the dipping or non-dipping hypertensive subjects (120). The dipping hypertensive subjects show a clear circadian rhythm of blood pressure, while the non-dipping subjects did not show it due to a less dropping in night-time blood pressure. The morning exercise reduces blood pressure with similar efficacy in the dipping and non-dipping hypertensive subjects. On the other hand, greater

reduction of night-time blood pressure due to evening exercise was observed in non-dipping hypertensive subjects than in cases of dipping hypertensive subjects. Thus, it suggests that the timing of exercise is more important for controlling dipping hypertension rather than for non-dipping hypertension. Based on this research, it is expected that the evening exercise has beneficial effects in non-dipping hypertensive men. However, in a recent human study, exercise at the evening and night-time (from 1900 to 2200 h) delays the phase of melatonin metabolites (14), thus it is possible that evening exercise progress the circadian disturbance of blood pressure via a phase-delay of circadian rhythm. Further studies are required to evaluate the effect of exercise both in terms of hypotensive effect and circadian rhythm is required for the control of circadian blood pressure in hypertensive subjects.

### Muscle Size

The exercise training increases and/or maintains muscle size via controlling the balance of muscular protein turnover (121, 122). The combination of endurance and resistance training for 24 weeks induces muscle hypertrophy, and along with training in the evening leads to larger magnitude in muscle cross sectional area compared to the same training in the morning in men (111). Sedliak et al. reported similar effects of the time-of-day-specific resistance training on muscle mass and power in men but the results were statistically insignificant (123). These results suggest that the evening is the optimal timing to promote training-induced muscle hypertrophy. However, its mechanism remains unclear. In a recent animal study, it was reported that the preventive effects of stimulus like a rehabilitation on muscle atrophy depended on its timing (124). Intermittent weight-bearing for 4 h prevents the hindlimb-unloading-induced muscle atrophy and the up-regulation of *Atrogin1* expression, which is one of the muscle catabolic genes (124, 125). These preventive effects are greater in mice, which perform weight-bearing in the early active phase compared with weight-bearing in the late period of active phase (equivalent to evening in human because mice are nocturnal) (124). In addition, these preventive effects of weight-bearing at the early active phase are not observed in the *Clock* mutant mice, suggesting that the effects of rehabilitation time is mediated via the circadian clock protein CLOCK (124). From these reports, it is possible that the beneficial timing of exercise is different by your aim. Thus, exercise in the evening is better for the induction of muscle hypertrophy, while in the morning is better for the prevention of muscle loss. However, because there are few studies in this field called chrono-exercise, further studies are expected to generate strong and conclusive evidence.

### Lipid Metabolism

Endurance exercise controls the energy metabolism and oxygen ($O_2$) consumption (126–128). Some studies show time-dependent or -independent response to acute endurance exercise (see below). In women, higher $O_2$ consumption is observed during submaximal treadmill exercise in the afternoon and evening compared with morning (129). In the normal weight and obese men, fat oxidation during the incremental running exercise test is higher in the evening than in the morning, suggesting that evening exercise is better for fat burning (130). The beneficial effects of evening exercise on lipid metabolism in men are also observed in the change of post-exercise hormone levels (131). Treadmill running in the evening increases the free fatty acid levels, subsequent to the elevation of blood adrenaline and interleukin-6 levels, compared with morning running (131). In this report, exercise was performed under the postprandial condition in each time. On the other hand, in the other study, the exercise-induced fat oxidation for 24 h in men is only observed in cases where the exercise performed in the early morning before breakfast but not in the cases where exercise was performed after breakfast, in the afternoon, and evening. As one of its mechanism, it is suggested that it is easy to shift the fuel source from carbohydrate to fat because prior to breakfast, because it is the longer fasting condition within a day, resulting in depletion of energy derived from a carbohydrate source like glycogen (132). Similar responses are observed in women (133). Thus, acute response of fat oxidation to exercise is greater in the evening while the longer effects of exercise on fat oxidation are observed before breakfast.

## SUMMARY AND PERSPECTIVES

In this review, we confirm that the postprandial response of macronutrients is different based on the feeding time, when an identical meal is ingested at each time point. Circadian clock system and behavioral pattern are involved in the time-dependent physiological responses to each meal. Additionally, the metabolic function of macronutrients could be exacerbated by a misalignment between endogenous circadian clock and life cycle, as observed in shift workers (48, 134). On the other hand, there are a few reports to elucidate a chronic physiological effect of the distribution of macronutrients to each meal. Although there are many reports about the training time-dependent regulation of the day-night variation of athletic performance, the mechanisms are not fully understood and the research in these fields is only just beginning. Further evidence is expected to lead to a clearer understanding of the molecular mechanisms leading to the interaction between circadian clock and time-of-day effects. Finally, exercise also has the potential for a timekeeper of circadian clock, especially exercise at night-time induces phase-delay in humans (14). Thus, further evidence is needed to discuss the effects of exercise timing in the context of therapeutic effects and its circadian rhythms.

## AUTHOR CONTRIBUTIONS

SA was involved in conceptualizing and writing the manuscript. SS was involved in conceptualizing and editing the manuscript.

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REVIEW
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# Recent Evidence on the Impact of Ramadan Diurnal Intermittent Fasting, Mealtime, and Circadian Rhythm on Cardiometabolic Risk: A Review

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In this article, we reviewed recent data that examined the relationship of circadian rhythm, mealtime, and intermittent fasting with the risk of cardiometabolic dysfunction. We also examined the effect of their interactions on cardiometabolic risks. Furthermore, since major differences exists between Ramadan diurnal intermittent fasting compared to other forms of experimental intermittent fasting, in this article, we further restricted the discussion to Ramadan diurnal intermittent fasting. PubMed and Google Scholar databases were searched using "intermittent fasting," "time-restricted feeding," "fasting," "mealtime," "circadian rhythm," and "cardiometabolic risk," focusing on human studies published after 2013. Recent evidence indicates that meal timing may influence circadian rhythm, as a result, it may also directly or indirectly impact cardiometabolic risk. In humans, several studies suggested that late mealtime is related to an increased risk of poor cardiometabolic health. Nevertheless, large clinical interventional studies are required to assess causality between late mealtime and cardiometabolic morbidity. Currently, evidence indicates that Ramadan diurnal intermittent fasting has several beneficial effects that may reduce the risk of cardiometabolic disorders, such as weight reduction, improvement in lipid profile and glycemic control, reduction in proinflammatory markers, and oxidative stress. Nevertheless, several changes in daily lifestyle routine, happening during the Ramadan month, may affect the all measured markers of cardiometabolic diseases. Summarily, no definitive conclusion about the impact of Ramadan intermittent fasting on oxidative stress can be formulated. Therefore, large, well-designed studies, which control for various confounding factors are required to assess the influence of Ramadan diurnal intermittent fasting on markers of cardiometabolic risk and disorders.

**Keywords: intermittent fasting, sleep, circadian rhythms, cardiometabolic disease, Ramadan, oxidative stress, proinflammatory markers**

## INTRODUCTION

Cardiometabolic disorders, a major health problem, is among the main causes of increased morbidity and mortality (1). Cardiometabolic risk denotes risk factors that increase the chances of developing vascular events or developing diabetes and obesity. This conception comprises diseases, environmental, behavioral and genetic factors, such as hypertension, dyslipidemia, poor eating habits, and smoking, in addition to recently described risk factors, such as central obesity, systemic inflammation, and genetics (2, 3).

Important under-recognized factors that have recently been linked to cardiometabolic risk include; intermittent fasting (IF), time-restricted feeding (TRF), mealtime, and circadian rhythm (4). Disturbances of circadian rhythm disrupt body functions and may increase the risk of cardiometabolic dysfunction (5). On the other hand, changes in the mealtime, such as increasing caloric intake at night, have recently been linked to cardiometabolic health in humans (6, 7). In addition, recent data indicate that IF may have positive effects on cardiometabolic function and may reduce the risk of cardiometabolic diseases. In this article, we critically reviewed studies that examined the effect of IF, mealtime, and circadian rhythm; in addition to their interactions on cardiometabolic risks.

## SEARCH METHODS

PubMed and Google Scholar databases were searched using the following words "intermittent fasting," "time-restricted feeding," "fasting," "mealtime," "circadian rhythm," and "cardiometabolic risk." We focused on human studies; however, important and relevant animal studies were reported in this review. In this review, we mainly discussed studies published after 2013; though, relevant papers published before that date were included.

## CIRCADIAN CLOCK AND CARDIOMETABOLIC RISK

All body organs, tissues and cells are under the control of a biologic clock and follow a circadian pattern. Based on the anatomical location, biologic clocks can be separated into peripheral and central clocks.

The central biologic clock is in the hypothalamus and is known as the suprachiasmatic nucleus (SCN). However, each cell in the body has peripheral clocks (8). Biologic clocks maintain the normal tissue functioning by regulating the expression of tissue-specific genes (8).

**Abbreviations:** 3D-MRI, three-dimensional magnetic resonance imaging; BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; HBA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; IF, intermittent fasting; IL, interleukin; MDA, malondialdehyde; NAD, nicotine adenine dinucleotide; Nrf2, nuclear respiratory factor-2; OR, odd ratio; PP, pulse pressure; SCN, suprachiasmatic nucleus; SIRT, sirtuins; SOD2, superoxide dismutase-2 mitochondrial; TFAM, Mitochondrial transcription factor A; TNF-α, tumor necrosis factor-alpha; TRF, time-restricted feeding; WMD, weighted mean difference.

The biologic clock of the body needs to be entrained daily to achieve external synchrony between the body functions and the external natural time, and internal synchrony between the central and peripheral body clocks (8). The SCN is entrained mainly by light, while peripheral clocks are influenced by neurohormonal factors and mealtime (7, 9).

Studies have shown that desynchrony of the circadian system predisposes to several cardiometabolic dysfunctions, such as glucose tolerance impairment, reduced sensitivity to insulin, elevated proinflammatory cytokines, increased arterial blood pressure, and reduced energy expenditure, which results in obesity (5). The effect of circadian misalignment on cardiometabolic risk is supported by epidemiologic data linking shift work with a higher risk of developing cardiometabolic disorders, such as obesity, diabetes, and cardiovascular diseases (10–12).

Recently, several investigators explored the relationship of acute disruption of circadian rhythm and mealtime with cardiometabolic risk in healthy volunteers. In a well-designed study that aimed to assess the acute impact of misalignment between mealtime, sleep/wake pattern, and internal circadian rhythm on some markers of cardiometabolic health, 10 adults were subjected to a 10-day forced desynchrony protocol where the participants ate iso-caloric meals and slept during a recurrent 28-h day cycle (13). This protocol required feeding and sleeping for about 12 h by the participants, out of phase of their usual sleep and mealtimes. The resulting misalignment caused a reduction in leptin (−17%), an increase in blood glucose (+6%) in spite of increased insulin (+22%), an absolute reversal of the circadian pattern of cortisol, increased mean arterial blood pressure (+3%), and a decrease in sleep efficiency (−20%).

A subsequent experimental study under controlled conditions examined the effects of circadian disruption (28-h day forced desynchrony protocol) accompanied by extended sleep curtailment (5.6 h/24 h), on blood glucose levels, in 21 participants, for 3 weeks (14). The investigators reported a reduction in resting metabolic rate and a rise in plasma glucose concentrations following a meal; these changes were secondary to inadequate pancreatic insulin secretion.

However, does circadian rhythm disruption affect the cardiometabolic system if sleep duration is maintained? Leproult et al. tried to answer this question by studying 26 healthy volunteers using a parallel group design. The experimental protocol comprised 3 days with a sleep duration of 10-h, followed by 8 days of sleep curtailment to 5 h with pre-set bedtimes at night (circadian alignment), or with a delay in bedtimes by 8.5 h on 4 of the 8 days (circadian misalignment) (15). Insulin sensitivity was reduced in both groups (the circadian aligned and misaligned groups) without a compensatory rise in insulin secretion (15). Moreover, C-reactive protein (CRP), which is a sensitive marker of inflammation that predicts increased risk of coronary heart disease, increased with circadian misalignment, independent of sleep loss.

The above laboratory-controlled studies suggest that acute misalignment of the circadian rhythm increases the risk of developing cardiometabolic disorders. Nevertheless, studies in the general public are required to supplement

these short-term laboratory-based results of forced circadian desynchrony. Moreover, long-term studies are needed to explore the possibility of adaptation to the changes of chronic circadian misalignment (16).

## MEALTIME AND CARDIOMETABOLIC RISK

Increasing evidence indicates that mealtime has a significant role in metabolic regulation and that mealtime closely interacts with the circadian clock (17). "Chrononutrition," which means meal timings, is a newly proposed discipline that addresses the interaction between mealtime, circadian clock, and metabolism.

Current evidence suggests that mealtime affects circadian rhythm, metabolism, and body weight (18). Eating during the wrong time of the day results in a misalignment between the peripheral circadian clocks and the central biologic clock in the SCN. The resulting desynchronization increases the risk of developing cardiometabolic diseases (7, 17). Nocturnal species eat most of their daily food requirement at night. For example, mice consume most of their daily intake of food (70–80%) during the dark phase (active phase) (19). Therefore, when mealtime is confined to the light phase (inactive phase) of the day, uncoupling between peripheral and central clocks occurs, and mice gain more weight compared to their counterparts fed during the dark phase in as little as 1 week (20). Additionally, another study in mice demonstrated that restricting food availability to the active phase (8–9 h) had a protective effect against weight gain and metabolic syndrome, secondary to atherogenic food consumption (21). Although, the protective effect of restricting food to the active phase is not only secondary to caloric restriction. Hatori et al. exposed mice to either *ad libitum* or restricted their feeding time to 8 h per day (active phase) of high-fat-diet (22). Interestingly, mice with restricted feeding time consumed comparable calories to their counterparts with *ad libitum* food access, yet food restriction to 8 h had a protective effect against weight gain, increased insulin levels, hepatic steatosis, and systemic inflammation (22).

Similarly, in humans, studies have shown very similar outcomes, where eating at the wrong time (nighttime "inactive phase") is associated with a higher risk of developing cardiometabolic dysfunction (7). In a Swedish study of 3,610 men and women, eating late at night was linked to an increased obesity odd ratio (OR) of 1.62 (95% confidence interval [CI], 1.10–2.39) compared to those who did not eat late at night (23). A recent systematic review and meta-analysis of 10 observational and experimental studies that assessed the effect of meal timing on obesity and metabolic alterations in humans reported negative impact of late meal timing on weight and metabolism (24). Additionally, both observational and experimental studies reported an association of late mealtime with hyperglycemia and diabetes mellitus (24).

In shift workers, studies have revealed an association between night eating and metabolic disturbances. In a study of 11 female nurses over 3 days of nightshift duties, standard meals of 440 kcal were served at 07:30, 23:30, and 03:30; and the glycemic response was then assessed over 4 h (25). Although baseline glucose levels were similar before each meal, postprandial levels were highest after meal intake at 23:30. Moreover, the highest insulin level was reported after meal intake at 23:30, and the lowest after meal intake was at 03:30. In another earlier randomized study, with a cross-over design, eight non-obese males were subjected to day or night work for a single shift with fixed caloric intake and fixed proportions of proteins, fats, and carbohydrates (26). Meals were served according to the day and night protocols at 01:00/13:00 and 07:00/19:00, and the snacks at 04:00/6:00. Blood samples for measurements were collected after an overnight fast, and at 8 h following the first meal in each protocol. The study demonstrated increased postprandial triacylglycerol and glucose levels in the night protocol (26).

To investigate the effect of restricted food intake to daytime or nighttime on cardiometabolic risk, non-obese ($n = 27$) participants were asked to abstain from food between 19:00 and 06:00 for 2 weeks or to continue their usual eating routine (27). The study showed that total caloric intake when eating was restricted to daytime was associated with a 0.4-kg weight loss compared with a 0.6-kg weight gain during the control period. A recent study tested the effect of early TRF as a form of TRF, which entails eating early in the day to align with human circadian rhythms in metabolism in eight men with prediabetes (28). In a randomized crossover design, the participants were assigned to early TRF (6-h period for eating, with dinner before 15:00) or a control group (12-h period for eating); and the participants were followed-up for 5 weeks, following which, they did a crossover to the other schedule. The study demonstrated that early TRF (6-h feeding period, with dinner before 3 p.m.) led to reduced blood pressure, oxidative stress, and appetite, and increased insulin sensitivity and β cell responsiveness compared to the controls (12-h feeding period) for 5 weeks (28). Another two recent studies assessed the effect of early TRF (08:00–14:00) vs. a control protocol (08:00–20:00) in a randomized crossover design on 11 overweight adults (28, 29). Early TRF significantly reduced the mean 24-h glucose levels and glycemic excursions (29). Moreover, the early time-feeding protocol led to decreased appetite without affecting energy expenditure (30).

### Breakfast and Cardiometabolic Risk

A study compared the effects of late dinner served at 23:00 with dinner served at 18:00 on 12 female university students (31). Blood glucose level was significantly higher after breakfast under the late dinnertime protocol. On the other hand, Jakubowicz et al. assessed the effect of a high-energy breakfast in non-obese women with polycystic ovary syndrome over a period of 90 days (32). The participants were randomized into two isocaloric (1,800 kcal/day) diets divided over three meals with different caloric distribution: (1) a breakfast diet group, which served 980 kcal at breakfast, 640 kcal at lunch, and 190 kcal at dinner; or (2) a dinner diet group that provided 190 kcal at breakfast, 640 at kcal lunch, and 980 kcal at dinner. The study revealed that the group with a high caloric intake at breakfast and lower calories at dinner had an improvement in insulin sensitivity parameters and a reduction in cytochrome P450c17α activity (32).

However, the beneficial effect of a breakfast meal on weight and cardiometabolic health is not unanimously reported in all

published work. A recent meta-analysis included 12 randomized controlled trials from rich countries, and aimed to assess the impact of regular breakfast ingestion on weight and energy consumption in adults (33). The paper reported a small difference in weight favoring participants who were not assigned to breakfast (mean difference 0.44 kg, 95% CI: 0.07–0.82; mean follow-up 7 weeks; range 2–16 weeks). However, there was inconsistency across trial results, and participants in the breakfast group had a higher total daily energy consumption (∼260 extra calories/day) compared to those assigned not to take breakfast (mean difference 259.79 kcal/day). Additionally, the quality of the analyzed studies was generally low; therefore, the authors concluded that the results of their study should be interpreted with caution (33). Moreover, Gonzalez et al. conducted four trials in a randomized cross-over design on 12 healthy men where they performed overnight fasting followed next day by one of the following protocols: (1) rest without breakfast; (2) exercise without breakfast; (3) breakfast followed by rest; or (4) breakfast followed by exercise (34). The study demonstrated that after 1 day of monitoring, energy intake from breakfast and energy expenditure from exercise were not compensated for at lunch. Accordingly, energy balance was most positive following breakfast and rest and least positive following breakfast omission and exercise. Nevertheless, the findings of this study should be interpreted with caution as it does not predict the longer-term outcomes of energy and fat balance due to its single-meal design (34).

Additionally, data from studies that assessed the effects of Ramadan diurnal IF (see below) on weight and cardiometabolic risk, where individuals take breakfast 30 min before dawn and dinner after sunset, demonstrated that this diurnal IF protocol reduces weight and cardiometabolic risk (4, 35).

This inconsistency indicates that large randomized controlled trials of high quality are needed to assess the effect of breakfast meal on weight and cardiometabolic risk. A recent consensus statement from the American Heart Association concluded that epidemiological data suggest a probable harmful effect of late mealtime on cardiometabolic risk. However, clinical interventional investigations that could address causality have been limited and not too focused to allow definite conclusions to be drawn, in order to formulate recommendations (7). Additionally, the consensus statement indicated that mealtime and frequency are not the only culprits; the duration between meals and the amount of caloric intake consumed in each meal are as important (7).

## INTERMITTENT FASTING AND CARDIOMETABOLIC RISK

IF means abstinence of food and drink in specified time periods. IF can be practiced in different forms, such as abstinence from food every other day (36), significant reduction in caloric consumption every other day (37), moderate caloric restriction for 2 consecutive days/week (38), restricting of food to specific times of a 24-r period, which is called TRF (21), abstinence from food for 1 or 2 days per week and then *ad libitum* food consumption for the rest of the week, as well as the diurnal IF performed by Muslims during the month of Ramadan where fasting performers abstain from food as well as drinks from dawn to sunset for the whole month (29–30 days) (39).

Researchers have developed a great interest in the health effects of IF over the past decade. Nevertheless, there is still a scarcity of long-term studies and studies that control for confounders that may affect the measured health indices, while well-designed studies included small numbers of participants.

Current evidence suggests that experimental IF is associated with weight loss, although there are no data on the long-term effects of this practice on weight. Moreover, recent data demonstrated that experimental IF lowers blood pressure (4). Data from animal studies suggest that experimental IF may have cardioprotective effects (4). Nevertheless, most of the studies that assessed the effect of experimental IF on cardiometabolic risk have major limitations. The sample sizes of most previous studies were relatively small. Additionally, most of the studies that assessed the effects of experimental IF on cardiometabolic risk did not control for major confounders that may have an impact on the measured markers, such as sleep duration and timing, energy expenditure, and light exposure (40). Furthermore, some of the proposed experimental fasting protocols, such as the alternate-day fasting protocol are not practical due to accompanying severe hunger (41). As there are major differences between Ramadan diurnal IF and other forms of experimental IF, in this article, we restricted the discussion to Ramadan diurnal IF (42).

## Ramadan Diurnal Intermittent Fasting

Diurnal IF during Ramadan month is the fourth pillar of Islam and has been practiced by all Muslims for over 1,400 years. Ramadan is the ninth month of Islamic (*Hijri*) year, which follows the lunar system. The lunar year is 11 days shorter than the Gregorian year; therefore, Ramadan occurs in a different season every 9 years. The season during which Ramadan occurs affects the duration of each fast, because fasting hours are longer in summer than in winter. This, in turn, may affect sleep patterns, due to factors, such as shorter nights and earlier dawns. Besides, the climate may affect sleep. During the hot summer, many people resort to napping in the middle of the day, which may influence night sleep. Moreover, the geographical location may affect the duration of fasting. As one moves further from the equator, daytime becomes longer in summer and shorter in winter (43). These factors may influence sleep and circadian rhythm. Therefore, when studying sleep patterns during Ramadan, it is essential to document the time of year, the location of the study, and the times of dawn and sunset.

The abstinence of food and drink during the daytime results in sudden shift in meal timing to the period between sunset and dawn.

Theoretically, restricting feeding to a limited time of the 24-h day is expected to result in reduced energy intake, and this is the essence of diurnal IF during Ramadan (42). Ideally, in Ramadan, fast performers are expected to eat two main meals, breakfast at sunset and a pre-dawn (suhur) meal ∼30 min before dawn, and to get a good night sleep. This practice is expected to result in weight

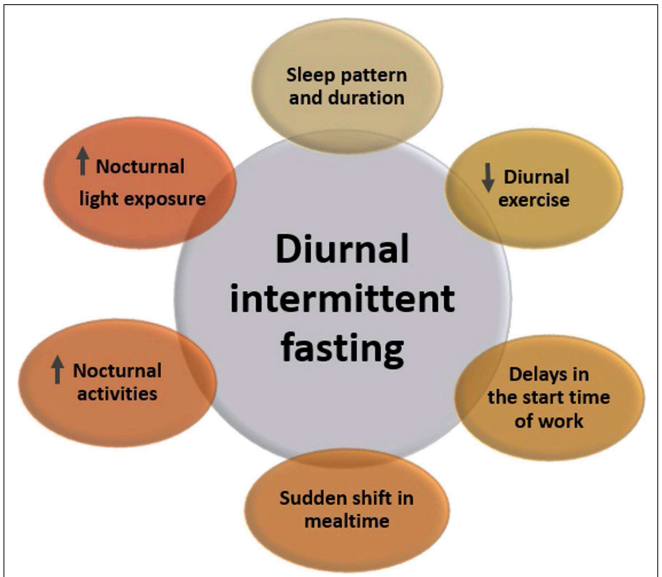


**FIGURE 1 |** Lifestyle changes that accompany the month of Ramadan and may influence the cardiometabolic risk.

loss and improved physiological parameters that are related to increased weight. However, in real life, this does not always occur as many lifestyle and cultural factors interact with the IF practice during Ramadan (**Figure 1**) (4, 42). In other words, Ramadan fasting can be considered as a TRF schedule without intentional restrictions on energy consumption, where two large meals are administered; one at sunset and the other in the early morning before dawn, and adequate sleep at night (44).

Therefore, the sleep and feeding behavior of Ramadan is different from a night shift schedule, which has been shown to be associated with cardiometabolic disorders (11, 12). Religiously, this practice aims to persuade fast-performers to get up early for the pre-dawn meal, which is followed by the dawn prayer (4).

Another important point that should be addressed when discussing the impact of Ramadan intermittent fasting on cardiometabolic risk is physical activity during Ramadan. A recent meta-analysis of studies that did not attempt to influence physical activity revealed that measures of physical activity did not indicate any significant change between pre-Ramadan and during Ramadan of either maximum effort physical activity or daily physical activity (35).

Ramadan fasting has unique characteristics, which include the long duration of the practice for 1 month. This long duration may cause some adaptations to the new behavior. Therefore, physiological changes in the first week of the month may not be similar to changes at the end of the month. Additionally, the geographic location away from the equator affects the duration of light and dark cycles and hence the fasting hours.

## Ramadan Diurnal Intermittent Fasting and Cardiometabolic Changes

### Effects on Circadian Rhythm

During Ramadan fasting, there is a sudden shift in mealtime from mainly daytime to the dark hours between sunset and dawn. Therefore, it is logical to ask whether this sudden shift in mealtime will affect the circadian pattern of the body, as this may have impact on the cardiometabolic system.

Reviewing all studies that aimed to assess the circadian rhythm changes during Ramadan reveals two types of studies: (1) studies that were conducted in a non-constrained environment and did not control for lifestyle changes, caloric intake, and nocturnal meal timings, and these studies reported rapid and significant delays in bedtime and rising time (45–47); and (2) recent studies that accounted for sleep/wake schedule, sleep duration, light exposure, caloric consumption, meal timings and energy expenditure, which demonstrated no changes in circadian rhythm during fasting (48–50). In the second group, meals were served early at night, the participants slept early according to their usual routine and then woke up for the predawn meal in the early morning.

In an earlier study, our group examined the changes in circadian pattern of the proximal skin temperature (an indicator of core body temperature) before Ramadan and during the first 2 weeks of Ramadan in an unconstrained environment using a validated portable device in six young adults who had an evening chronotype (i.e., the participants slept during daytime and wake up to eat at night before Ramadan) (47). Despite no changes in the meal timings during Ramadan, the acrophase of the proximal skin temperature was delayed; indicating a shift-delay in the circadian clock (47). Another study in Saudi Arabia in the free-living environment reported flattening of the cortisol rhythm, which is known to be linked to chronic stress or endogenous hypercortisolism and associated with insulin resistance, which supports that lifestyle changes that accompany Ramadan month are associated with disruption of the circadian rhythm (51).

These findings indicate that the shift delay in the biologic clock reported in some studies during Ramadan is not only due to mealtime shift, but other factors. Lifestyle changes may influence the biologic clock during Ramadan. Therefore, based on the available evidence, restricting mealtime to the early evening and predawn times, and getting a good night sleep during Ramadan does not affect the biologic clock.

### Effects on Weight and Lipid Profile

A meta-analysis that assessed the weight changes in fast performers during and after Ramadan diurnal IF including 25 studies (mainly from West Asia) in a free-living unconstrained environment reported significant weight loss at the end of the month (−1.24 kg; CI: −1.60, −0.88 kg). However, weight was regained 2 weeks after Ramadan (52). Another meta-analysis that included 30 cohort studies of healthy young men and women in an unconstrained environment assessed the impact of Ramadan diurnal IF on weight, lipid profile, and blood glucose level (53). The analysis revealed a reduction in low-density lipoprotein (−1.67; CI: −2.48 to −0.86) and fasting blood glucose levels (−1.10; CI: −1.62 to −0.58) in both genders (53). The meta-analysis of 21 studies that assessed the effects of Ramadan fasting on body weight, included 830 participants of both genders (men = 531 women = 299) (53). There was a slight but significant reduction in weight in men (−0.24 [−0.36, −0.12], $p = 0.001$), but no significant changes were documented in women (−0.04 [−0.20, 0.12], $p = 0.620$) (53).

A more recent meta-analysis assessed the effect of Ramadan IF on weight and body composition (35). The analysis included 70 studies (∼3,000 participants) and demonstrated a significant positive correlation between pre-Ramadan body mass index (BMI) and weight lost during Ramadan. Fasting resulted in a significant reduction in weight (−1.34 [95% CI: −1.61 to −1.07] kg, $p < 0.001$), despite not giving the participant any advice on lifestyle or dietary modifications (35). Additionally, there was a significant decrease in the percentage of fat in overweight and obese participants (−1.46 [95% confidence interval: −2.57 to −0.35] %, $p = 0.010$) (35). This reduction was not seen in normal-weight subjects (35). Nevertheless, weight and body composition returned to about the pre-Ramadan levels after 2–5 weeks of follow-up.

Another recent meta-analysis included 33 studies and assessed the effect of Ramadan diurnal IF on lipid and lipoprotein in ∼2,000 healthy participants, with no lifestyle modifications (54). The weighted mean changes at the end of Ramadan demonstrated a significant reduction in low density cholesterol levels in men (weighted mean difference [WMD] = 2.65 mg/dl; 95% CI = 5.16, −0.14) but not in women (WMD = 9.50 mg/dl; 95% CI=21.93, 2.92) (54).

One more study assessed the effect of Ramadan IF in a free unconstrained environment on 10 obese men (7 of them had metabolic syndrome) (55). The study reported that by the end of the month of Ramadan, BMI had decreased significantly and was associated with a significant reduction in homeostasis model assessment of insulin resistance (HOMA-IR) and fasting levels of blood glucose (55).

A recent systematic review and meta-analysis assessed the impact of Ramadan IF on metabolic syndrome components in healthy, non-athletes and included 85 studies (∼4,500 participants) that were performed in 23 countries (56). The study reported small but significant reductions in waist circumference, fasting blood glucose level, systolic blood pressure, and triglyceride level, and a small increase in HDL cholesterol (56).

Nonetheless, it is imperative to mention here that all the above reviewed studies occurred in the free-living unconstrained environment and did not account for potential confounders that may have affected the measured parameters, such as caloric intake and food composition (54), sleep duration (55), physical activity (57), and energy expenditure (57), which have been shown to change in Ramadan (39).

## Effects on Diabetes

A study that assessed changes in fat mass and metabolic markers in 29 patients with type 2 diabetes for 15–21 days during Ramadan reported a significant reduction in glycated hemoglobin (HbA1c) (8.6 ± 2.4% at baseline vs. 8.0 ± 2.3% toward the end-Ramadan, $p = 0.02$) (58). Moreover, there was a significant decrease in body fat mass, but no changes were detected in body weight (58). This study suggests that Ramadan diurnal IF may improve glycemic control in patients with diabetes. Nevertheless, it is important to indicate here that patients with diabetes must consult their physicians before fasting.

A new study assessed changes in 228 patients with type 2 diabetes who were on >3 medications for diabetes before and after Ramadan (59). The study reported a significant decrease in HBA1c [7.8% (62 mmol/mol) vs. 7.6% (60 mmol/mol); $p = 0.004$] after Ramadan (59).

## Effects on Blood Pressure and Cardiovascular System

Al-Shafei examined the effects of Ramadan IF on arterial pulse pressure (PP) in 40 patients with hypertension, without receiving any instructions on behavioral or dietary modifications (60). The results revealed a significant decrease in PP (17.2%), with no changes observed in the control group (60).

A recent study assessed blood pressure changes during Ramadan diurnal IF on 60 subjects with prehypertension or hypertension but who did not use antihypertensive agents (61). The study revealed that during Ramadan, there was a significant reduction in blood pressure, including the 24-h ambulatory blood pressure monitoring (61).

A recent systematic review and meta-analysis of studies that assessed the incidence of cardiovascular events during Ramadan that included 15 studies reported no significant increase in the incidence rate of acute cardiac conditions, including congestive heart failure, myocardial infarction, and stroke during Ramadan (62).

## Effects on Inflammatory Biomarkers

There is a strong association between inflammatory biomarkers and increased risk of metabolic and cardiovascular disorders (63–65). In the past few years, researchers interest was increased toward studying the effects of Ramadan diurnal IF on inflammatory biomarkers.

A recent systematic review and meta-analysis included 12 studies (from 8 countries) and assessed changes in inflammatory biomarkers in healthy subjects pre and post-Ramadan (66). The pooled data revealed that diurnal IF during Ramadan was associated with a very small decrease in interleukin-1 (IL-1) (Hedge's $g = 0.016$); CRP (Hedge's $g = 0.119$); and a small reduction in tumor necrosis factor-alpha (TNF-α) (Hedge's $g = 0.371$); and IL-6 (Hedge's $g = 0.407$) (66).

Shariatpanahi et al. conducted a study on 65 patients with metabolic syndrome (who were admitted to a hospital), to evaluate the effect of diurnal IF during Ramadan on CRP and fibrinogen levels in the unconstrained environment. Participants were served two meals, at sunset and 30 min before dawn (fasting period 17 h) (67). At the end of the month, there was a significant reduction in fibrinogen, CRP, BMI, and waist circumference compared to the baseline (67). The findings of this study suggest that limiting the mealtime during Ramadan to sunset time and early morning (pre-dawn) and obtaining good nocturnal sleep may help in maintaining good alignment between the central and peripheral biologic clocks and have beneficial physiological effects. Similar reductions in IL-1β, IL-6, and TNF-α have been reported in young, healthy volunteers (68).

An additional study assessed inflammatory biomarkers in 50 healthy adults (21 men and 29 women) and reported a significant decrease in the circulating levels of interleukin

(IL) IL-1β, IL-6, and TNF-α occurred toward the end of the third week of Ramadan (68). Another recent study assessed specific cardiometabolic risk factors twice (9 a.m. and 9 p.m.) before Ramadan and the end of the second week of Ramadan in 23 volunteers and reported improvements in serum high sensitivity CRP, gamma glutamyl transferase, and IL-1 (69).

In a recent study, Faris et al. assessed the influence of IF during Ramadan on visceral adiposity (using three-dimensional magnetic resonance imaging [3D-MRI]) and inflammatory biomarkers in 61 obese subjects (70). At the end of Ramadan, visceral fat tissue area, weight, and systolic blood pressure were significantly decreased compared to the baseline (70). Visceral adiposity has been shown to have a significant association with cardiometabolic risk, even in non-obese individuals (71, 72). Moreover, Faris et al. reported that serum levels of adiponectin, IL-6, TNF-a, and insulin-like growth factor-1 were significantly decreased. However, there was a significant increase in the serum levels of visfatin, leptin, apelin, and IL-10, at the end of Ramadan (71, 72). Previous studies have reported contradictory results regarding the effect of diurnal IF on leptin levels. While an earlier study reported an increase in serum leptin by 133% (73); another study reported no changes in leptin levels during Ramadan IF (74). However, a major limitation of all previous studies is that only a single blood sample for leptin was taken. It is known that leptin levels follow a circadian rhythm and are affected by meals (75); therefore, the levels of measured serum leptin will depend on the timing of the sample collection in relation to the timing of the last meal. Therefore, our group assessed serum leptin levels at 22:00, 02:00, 04:00, 06:00, and 11:00 at baseline; while performing diurnal IF and reported a significant decrease in leptin levels at 22:00 and 02:00 compared with the baseline concentrations (at 22:00: 194.2 ± 177.2 vs. 146.7 ± 174.5; at 02:00: 203.8 ± 189.5 vs. 168.1 ± 178.1; $p < 0.05$) (76).

Based on the above, most studies have demonstrated reductions in inflammatory biomarkers during diurnal IF. However, the above papers have major shortcomings that need to be kept in mind. A single morning sample for the measured biomarkers does not take the circadian variation of the levels of the measured biomarkers into consideration (77). Moreover, the above studies were conducted in the free-living environment, which means that there was no control to determine the influence of potential confounders related to lifestyle changes during Ramadan month that may affect the measured biomarkers, such as energy consumption (78), sleep duration (79), and physical activity (80).

To avoid the above limitations, our group conducted an experiment on 12 young, healthy males to assess the inflammatory biomarkers before and during Ramadan diurnal IF while controlling for the above-mentioned confounders (81). We measured the levels of cytokines (IL-1β, IL-6, and IL-8) at 22:00, 02:00, 04:00, 06:00, and 11:00 and demonstrated a significant reduction in cytokines levels across the 24 h period during diurnal IF compared to the baseline, mainly with IL-1β and IL-6.

## Effects on Oxidative Stress

A few studies assessed the effects of Ramadan diurnal IF on oxidative stress, which revealed contradictory findings. Some investigators demonstrated a significant decrease in lipid peroxidation (60, 82); while others reported no changes (83–85).

The pooled data of malondialdehyde (MDA), in a recent systematic review and meta-analysis that included four studies, which assessed the effects of Ramadan diurnal IF on oxidative stress, reported a very small decrease in MDA Hedge's g ($N = 117$, $K = 4$) of 0.219 ($I^2 = 0.0\%$). However, the sensitivity analysis for MDA demonstrated that the results were influenced by one study (86), and Hedge's g increased to 0.4 when the investigators removed that study.

In a previous study on 50 healthy volunteers, Faris et al. demonstrated that during Ramadan diurnal IF, markers of oxidative stress were significantly decreased in those who experienced weight loss (85). Therefore, it is difficult to figure out if the reduction in oxidative stress was due to weight loss or IF. In a recently published study, Madkour et al. examined the effects of Ramadan diurnal IF on the expression of cellular metabolism genes (Sirtuin [SIRT] 1 and SIRT3) and antioxidant genes (Mitochondrial transcription factor A [TFAM], superoxide dismutase-2 mitochondrial [SOD2], and nuclear respiratory factor-2 [Nrf2]) in 56 healthy overweight and obese subjects and 6 normal BMI controls (87). The sirtuins are a family of nicotine adenine dinucleotide $(NAD)^+$-dependent deacetylases, which work as cellular sensors to discover energy availability and modify metabolic processes, and have been reported to control several genes (88). Expression of these genes functions as endogenous reactive oxygen species scavengers, and hence protect the cells from oxidative stress damage (88). Madkour et al. reported that at the end of Ramadan, the expression of the antioxidant genes (TFAM, SOD2, and Nrf2) was significantly increased in obese subjects compared to the control group (87). On the other hand, there was a significant decrease in the SIRT3 gene (metabolism-controlling gene) toward the end of Ramadan, with a non-significant reduction in SIRT1 gene (87). The investigators concluded that Ramadan diurnal IF probably provide a protective antioxidant effect in obese patients. The investigators also proposed that the reduction in SIRT1 and SIRT3 could be explained by the fact that the sirtuins levels usually increase secondary to inflammation; the reduction in the inflammatory activity during Ramadan diurnal IF may result in a decrease in sirtuins levels (87).

As with the inflammatory biomarkers, measured biomarkers of oxidative stress are affected by the above discussed lifestyle changes that accompany Ramadan (89–96), and the relationship with the timing of the blood samples to mealtime (90). Previously discussed studies collected only a single early morning blood sample. Oxidant levels could have been affected by the sample collection time as well as by the interaction between sample collection time and mealtime (91, 93). The blood sample collection time of oxidant or anti-oxidant activity may affect the measured levels. Blood samples collected in the early morning after an overnight fast (at baseline, before Ramadan) may be different from blood samples collected after a pre-dawn (Suhur)

meal during Ramadan (97). During fasting, there is a larger utilization fat instead of glucose as an energy source (85), which may cause higher fat oxidation.

To avoid the above limitations, our group examined the impact of Ramadan diurnal IF during and outside of Ramadan on the circadian changes in MDA while accounting for the above discussed confounders (97). MDA levels were measured at 22:00, 02:00, 04:00, 06:00, and 11:00, while controlling for meal composition and caloric ingestion, light exposure, physical activity, sleep duration, and body weight monitoring (no change in weight) during the study period in a sample of healthy young males (97). The resulted demonstrated that Ramadan diurnal IF does not alter serum MDA levels in healthy subjects.

Based on the above, no definitive conclusion about the impact of Ramadan IF on oxidative stress can be formulated. Large scale studies are needed to assess oxidative stress during diurnal IF while controlling for discussed confounders.

## SUMMARY

Recent data suggest that IF, circadian rhythm, and meal timings may influence cardiometabolic physiology and risk of cardiometabolic disorders. TRF and mealtime are known to affect circadian rhythm and synchronization, between the central and peripheral biologic clocks.

Desynchronization of the circadian system has been linked to several cardiometabolic disorders, including impaired glucose tolerance, reduced sensitivity to insulin, increased markers of systemic inflammation, increased blood pressure, decreased energy expenditure, and increased weight.

The discipline "chrononutrition" has been recently proposed to address the interaction between mealtime, circadian clock, and metabolism. Animal studies indicate that eating during the wrong time of the day (inactive phase) causes a misalignment between the peripheral circadian clocks and the central clock in the SCN, which increases the risk of developing cardiometabolic diseases. Likewise, several human studies indicated an association between late meal timing and a greater risk of poor cardiometabolic health. Nevertheless, clinical interventional studies that could address causality between late mealtime and cardiometabolic morbidity have been limited and not too focused to allow definitive conclusions to be drawn.

Currently available evidence indicates that Ramadan diurnal IF does not affect the circadian rhythm when meal timings are confined to the early evening and predawn periods, with an adequate night sleep. Moreover, Ramadan diurnal IF has beneficial effects on weight, lipid profile, glycemic control, inflammatory biomarkers, and probably oxidative stress. Nonetheless, Ramadan diurnal IF is accompanied by several lifestyle changes that may affect all the markers of cardiometabolic diseases. Therefore, larger studies that account for various confounding variables are needed to examine the influence of Ramadan diurnal IF on markers of cardiometabolic disorders.

## AUTHOR CONTRIBUTIONS

AB and AA have contributed equally to review of the literature, analysis, and writing of the manuscript.

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REVIEW
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# Brain Metabolism Alterations in Type 2 Diabetes: What Did We Learn From Diet-Induced Diabetes Models?

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Type 2 diabetes (T2D) is a metabolic disease with impact on brain function through mechanisms that include glucose toxicity, vascular damage and blood–brain barrier (BBB) impairments, mitochondrial dysfunction, oxidative stress, brain insulin resistance, synaptic failure, neuroinflammation, and gliosis. Rodent models have been developed for investigating T2D, and have contributed to our understanding of mechanisms involved in T2D-induced brain dysfunction. Namely, mice or rats exposed to diabetogenic diets that are rich in fat and/or sugar have been widely used since they develop memory impairment, especially in tasks that depend on hippocampal processing. Here we summarize main findings on brain energy metabolism alterations underlying dysfunction of neuronal and glial cells promoted by diet-induced metabolic syndrome that progresses to a T2D phenotype.

**Keywords: diet-induced obesity, brain metabolism, insulin resistance, glucose, sucrose, high-fat**

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## INTRODUCTION

Diabetes mellitus is among the top 10 causes of death in the world. Insulin-resistant diabetes (or T2D) often progresses from obesity, a pandemic that is favored by a sedentary lifestyle and the widespread consumption of food products rich in saturated fat and refined carbohydrates (Swinburn et al., 2011). Many factors of the metabolic syndrome impact brain function, such as chronic hyperglycemia, microvascular complications, insulin resistance, dyslipidemia, and hypertension (Duarte, 2015; Gaspar et al., 2016). There is also a growing body of epidemiological evidence suggesting that insulin resistance is associated with increased risk of developing age-related cognitive decline, mild cognitive impairment (MCI), vascular dementia, and Alzheimer's disease (AD) (Frisardi et al., 2010; Spauwen et al., 2013; de la Monte, 2017). Brain insulin signaling deficits have been proposed to impact the brain through mechanisms that include the modulation of energy metabolism, synaptic plasticity, learning and memory, as well as interacting with Aβ and tau, the building blocks of amyloid plaques and neurofibrillary tangles (Craft et al., 1998; Steen et al., 2005; Zhao and Townsend, 2009). In addition, a plethora of studies in rodent models of diabetes suggest that both glucose neurotoxicity and deficient insulin signaling impair brain structure and function leading to behavioral and cognitive alterations (e.g., Duarte et al., 2012a, 2019; Calvo-Ochoa et al., 2014; Girault et al., 2019; Lizarbe et al., 2019b).

**Abbreviations:** Aβ, amyloid β; AD, Alzheimer's disease; BBB, blood–brain barrier; $CMR_{glc}$, cerebral metabolic rate of glucose; FDG, [$^{18}$F]-fluorodeoxyglucose; GK, Goto-Kakizaki; HFD, high-fat diet; IGF1, insulin-like growth factor 1; IR, insulin receptor; MCI, mild cognitive impairment; MRS, magnetic resonance spectroscopy; NAA, *N*-acetylaspartate; PET, positron emission tomography; T2D, type 2 diabetes.

Hyper-caloric feeding is able to trigger insulin resistance, and diet is also a modulator of brain function and neurodegeneration. In cognitively normal individuals without obesity or diabetes, nutritional patterns were found to associate with $^{11}$C-Pittsburgh compound-B (marker of β-amyloid plaques) and [$^{18}$F]-fluorodeoxyglucose (FDG; marker of glucose metabolism) accumulation (Berti et al., 2015). Berti et al. verified that an AD-protective diet includes high intake of fresh fruit and vegetables, whole grains, fish and low-fat dairies, and low consumption of sugar, high-fat food products, and processed meat. Sugar intake was also found positively associated with cerebral amyloid burden measured by [$^{18}$F]-florbetapir positron emission tomography (PET), and negatively correlated with cognitive performance in cognitively normal subjects (Taylor et al., 2017).

Dietary imbalances may trigger metabolic disorders and obesity, which in the long-term may progress to insulin resistance and T2D. Clinical studies set obesity and associated metabolic derangements as important risk factors for dementia (Pedditzi et al., 2016; Singh-Manoux et al., 2018; Bandosz et al., 2020). Li et al. (2017) observed that cortical Aβ deposition by [$^{18}$F]-florbetapir PET was decreased in T2D patients while Aβ levels increased in the cerebral spinal fluid. Insulin resistance is associated with AD markers, such as accumulation of $^{11}$C-Pittsburgh compound-B and FDG in PET scans (Willette et al., 2015a,b), or Tau-protein levels in the cerebral spinal fluid (Laws et al., 2017). Takenoshita et al. (2019) investigated AD markers, namely, amyloid and Tau protein deposition by PET, in AD associated to T2D, and concluded that there are patient subgroups with neuronal damage independent of AD pathology. It is apparent that a diabetes-related dementia can be considered a different entity from AD itself.

Although several mechanisms underpin brain dysfunction that leads to poor cognitive performance in T2D (Gaspar et al., 2016), confusion and controversy landed in the field due to the variety of phenotypes generated in experimental animal models of T2D. Nevertheless, our knowledge on brain dysfunction mechanisms upon exposure to diabetogenic diets is increasing, and may help preventing cognitive deterioration associated to poor life styles.

## MEMORY DYSFUNCTION INDUCED BY DIABETOGENIC DIETS

Many studies in T2D animal models have employed diets rich in sugar and/or fat in order to induce T2D, namely, high-fat diet (HFD), high-sucrose diet, high-fructose diet, or the combination of some of them. Glucose intolerance develops promptly in rodents exposed to HFD, followed by a progressive increase of fasting insulin levels and metabolic derangements such as hepatic lipid accumulation (Soares et al., 2018). We have also recently reported that increasing the dietary amount of lard-based fat from 10 to 45 or 60% leads to slightly different diabetic phenotypes: compared to controls that were exposed to the low-fat diet, increased fed glycemia and plasma corticosterone were observed in mice fed a 60%- but not 45%-fat diet (Lizarbe et al., 2019b).

Similar degree of insulin resistance and of stress biomarkers in liver and pancreas have been observed in rats exposed to HFD, high-fructose diet, or the combination of both, compared to control diet (Balakumar et al., 2016). In mice, HFD feeding was found to cause elevated basal insulin levels, which was not observed in mice fed a combined high-fat and high-sucrose diet, despite similar energy intake and degree of glucose intolerance (Omar et al., 2012). The authors attributed this difference to the distinct effect on insulin secretion and insulin sensitivity.

In addition to the employment of different animal species or strains, such differences in dietary fat and sugar amounts are likely to explain that a variety of metabolic profiles are developed by experimental animal models in different studies.

Major hypothalamic injury has been proposed to occur within a few days of HFD feeding, this preceding weight gain (Thaler et al., 2012). Thus, early hypothalamic alterations are important determinants for the loss of whole-body metabolic control upon exposure to obesogenic diets. Indeed, regulation of energy balance relies on glucose sensing by neuronal networks that control food intake, hepatic glucose production, and pancreatic counter-regulatory hormone secretion. The hypothalamus is a primary site for integration of peripheral and central neuronal signals and hormonal inputs (Jordan et al., 2010). Impaired hypothalamic glucose sensing is key in developing T2D in obese humans and animal models (Colombani et al., 2009; Thaler et al., 2012; Gaspar et al., 2018).

Despite differences in metabolic phenotypes, all these diabetogenic diet interventions generate metabolic syndrome phenotypes that impact brain function, particularly the performance in hippocampal-dependent memory tasks (Soares et al., 2013; Beilharz et al., 2014; Hsu et al., 2015; Lemos et al., 2016; de Souza et al., 2019; Lizarbe et al., 2019b). For example, rats exposed to one week of high-fat and fructose diet displayed impaired hippocampal insulin signaling, and smaller hippocampal size with synaptic degeneration, reduced neuronal processes, and astrogliosis (Calvo-Ochoa et al., 2014). Rats under a similar diet for 5 days displayed impaired performance in place but not object recognition tasks (Beilharz et al., 2014), which are dependent on hippocampus and perirhinal cortex function, respectively. HFD alone also impairs hippocampal-dependent memory (McNay et al., 2010; Pistell et al., 2010; Lizarbe et al., 2019b). Rodents that were allowed to drink a 35% sucrose solution for 2–3 months while fed a low fat diet also develop hippocampal-dependent spatial memory impairment (Soares et al., 2013; Lemos et al., 2016).

While memory assessments have been mostly focused on spatial memory that depends on hippocampal functioning, other functional domains remain to the thoroughly investigated.

## HYPERGLYCEMIA AND BRAIN GLUCOSE TOXICITY

It is well established that glucose neurotoxicity upon uncontrolled hyperglycemia contributes to cellular dysfunction through (*i*)

increased polyol pathway flux, (*ii*) increased advanced glycation end-product formation, (*iii*) activation of protein kinase C (PKC) isoforms, and (*iv*) increased hexosamine pathway flux (Brownlee, 2001). Since the brain has about fivefold less glucose than plasma (Gruetter et al., 1998; Choi et al., 2001; Duarte et al., 2009b), endothelial cells in cerebral vessels are more susceptible to damage by hyperglycemia than cells in the brain parenchyma. Deterioration of the cerebral vasculature can lead to impaired BBB permeability in diabetes, as well as in aging and neurodegenerative disorders (Ueno et al., 2016). However, there is controversial evidence regarding cerebral microcirculation pathology and BBB dysfunction in rodent models of diabetes or *in vitro* models of chronic hyperglycemia (Andaloussi et al., 2018; Rom et al., 2019, and the references therein).

Measurements of brain-to-plasma glucose concentrations *in vivo* have not confirmed a substantial degree of BBB leakage in streptozotocin-induced diabetic rats maintained under hyperglycemia (>20 mmol/L) for 1 month (Duarte et al., 2009a; Wang et al., 2012), or in insulin resistant GK rats that show sustained fed glycemia of 9–16 mmol/L (Duarte et al., 2019; Girault et al., 2019). Accordingly, Andaloussi et al. (2018) have not observed BBB permeability alterations or morphological changes in brain vasculature of $Ins2^{AKITA}$ mice that display sustained hyperglycemia above 20 mmol/L. Nevertheless, gene expression profiles in brain microvessels isolated from models of diabetes point toward deregulated expression of genes related to angiogenesis, inflammation, vasoconstriction and vasodilation, and platelet activation pathways (Rom et al., 2019). Proteomic analyses suggest impaired metabolic activity in microvessels from the cerebral cortex of HFD-exposed mice compared to controls (Ouyang et al., 2014), even though HDF exposure results in limited increases of blood glucose levels (Soares et al., 2018; Lizarbe et al., 2019b). Such alterations are likely to impact brain perfusion and to limit nutrient delivery for fueling neuronal energetics (Glaser et al., 2012; Bangen et al., 2018). In mice, exposure to HFD impairs vascular reactivity (relaxation and contractile responses) and cerebral blood flow of the middle cerebral artery and of intraparenchymal micro vessels in prefrontal cortex and hippocampus, without changes of baseline perfusion (Pétrault et al., 2019). Accordingly, HFD feeding also exacerbates memory impairment induced by carotid occlusion without changes in basal cerebral blood flow (Zuloaga et al., 2016).

In sum, BBB breakdown mechanisms in diabetogenic diets are unlikely to be directly linked to hyperglycemia, but may include alterations of endothelial functions.

## BRAIN INSULIN RESISTANCE

Various metabolic hormones (ghrelin, insulin, leptin, glucagon-like peptide 1), which are key in central regulation of appetite through activation of receptors expressed in brain regions such as the hypothalamus, also play a role in learning and memory (Suarez et al., 2019). Insulin has been considered of particular importance for dementia and early changes of glucose metabolism (Duarte, 2015; Gaspar et al., 2016; Lee et al., 2018). However, it has been debated whether brain insulin resistance and metabolic changes are cause or consequence of neurodegeneration (Stanley et al., 2016; Mullins et al., 2017).

Insulin resistance (when cells do not respond to insulin) occurs in T2D, is associated to increased dementia risk, partly due to poor insulin signaling in neurons (Duarte, 2015). Brains from subjects with dementia and AD downregulated insulin receptors (IRs) and pointed toward a major role of neuronal insulin signaling in AD (Duarte, 2015; Barone et al., 2016; Sharma et al., 2019). Glucose utilization by the brain declines with age and is notably impaired in subjects with early AD, which may be related to insulin action in key areas for memory/cognition (Lee et al., 2018). Interestingly, insulin resistance may be differentially associated with either glucose hypo- or hyper-metabolism across different brain areas (Willette et al., 2015b). In fact, Willette et al. found that peripheral insulin resistance is correlated with reduced glucose metabolism in the brain of AD patients, while a positive correlation was observed in the brain of individuals with MCI that then progress to develop AD. Work on animal models of AD, T2D, or insulin resistance also points toward an association between insulin signaling and AD-like pathology (Duarte, 2015; Triani et al., 2018; Sharma et al., 2019). Diverse clinical trials testing the efficacy of insulin to treat AD and MCI are being conducted (Craft et al., 2012; Chapman et al., 2018; Lee et al., 2018).

Insulin is of particular importance in some specific brain areas: the hypothalamus that centrally regulates body energy homeostasis, the fusiform gyrus that plays a role in object recognition tasks, prefrontal areas that process sensory information, and the hippocampus that is key for memory formation (Heni et al., 2015). Binding of insulin to the IR activates the IR substrates IRS1 and IRS2, which in turn activate signaling cascades for brain function regulation, including metabolic processes in the different brain cells (Mullins et al., 2017). Importantly, insulin regulates the expression of genes necessary for memory consolidation, namely, via the mitogen-activated protein kinase (MAPK) pathway (Kelly et al., 2003; Dou et al., 2005). Insulin also participates in controlling the main cellular metabolic sensor AMP-activated protein kinase (AMPK) (Hardie, 2004; Marinangeli et al., 2016), which might provide a means to afford neuroprotection through metabolic control (Marinangeli et al., 2016). Thus, impaired insulin signaling might contribute to poor fueling of brain activity.

Brain glucose uptake is not dependent on insulin, but might be under control of insulin in specific subcellular compartments (**Figure 1**). For example, activity at synapses was shown to trigger the mobilization of GLUT4 (the insulin-sensitive glucose carrier) from intracellular sources into axonal plasma membranes, a process that is mediated by the metabolic sensor AMPK, and is necessary for increasing glucose flux into neurons during periods of high metabolic demand, such as during learning (Pearson-Leary and McNay, 2016; Ashrafi et al., 2017). Interestingly, it has been shown that toxic Aβ oligomers impair insulin signaling and decrease plasma membrane translocation of the insulin-sensitive GLUT4 in the hippocampus (Pearson-Leary and McNay, 2012),

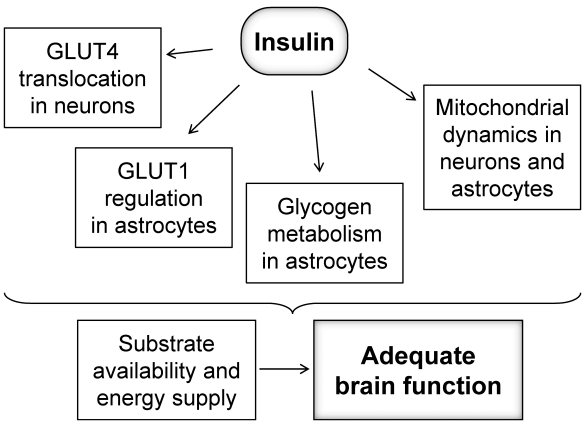


**FIGURE 1 |** Possible mechanisms by which insulin might regulate fueling of neurons to sustain adequate brain function.

which might result in poor support of energetic demands within active synapses.

While the focus of insulin signaling has been mostly on neurons, astrocytes also have receptors for insulin and IGF1 that may be key for maintaining GLUT1 at the plasma membrane (Fernandez et al., 2017), and thus regulating glucose utilization. In addition, glycogen metabolism, which is crucial for fueling glutamatergic neurotransmission and memory (Alberini et al., 2018), has also been proposed to be under insulin and IGF-1 regulation in cultured astrocytes (Muhič et al., 2015). Although insulin-dependent glycogen metabolism regulation *in vivo* remains to be elucidated, brain glycogen is mobilized rapidly for supporting glutamatergic neurotransmission *in vivo* (Gibbs et al., 2007), or metabolism during reduced fuel supply (Swanson et al., 1989; Choi et al., 2003; Duarte et al., 2017).

Mitochondria are the power-house of the cell, and mitochondrial dysfunction has been shown to be involved in neurodegenerative processes (Belenguer et al., 2019). Insulin might also control oxidative metabolism in mitochondria of neurons and astrocytes by regulating mitochondrial dynamics, biogenesis or autophagy, oxidative stress and apoptosis (Santos et al., 2014; Westermeier et al., 2015; Ruegsegger et al., 2019). The network of mitochondria is regulated by a fine balance of fission, involving the GTPase dynamin-like protein 1 (DRP1), and fusion processes involving Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) protein (Belenguer et al., 2019). Mitochondrial dynamics dysregulation plays a role in hypothalamic dysfunction upon HFD exposure (Dietrich et al., 2013), and a diabetes-induced increase of DRP1 phosphorylation was observed in the cerebral cortex (Santos et al., 2014). Recently, Ruegsegger et al. (2019) observed increased DRP1 as well as its phosphorylation without changes in Mfn1/2 and OPA1, as well as the expected mitochondrial fragmentation in the HFD-exposed hippocampus. Smaller mitochondria have been associated to reduced oxidative phosphorylation and ATP production rates (Schmitt et al., 2018; Belenguer et al., 2019). Therefore, the loss of mitochondrial metabolism regulation by insulin might result in impaired fueling of neuronal and astrocytic functions.

## SYNAPTIC DYSFUNCTION

Damage of synapses is the most important step for brain dysfunction (Morrison and Baxter, 2012), and the degree of synaptic changes correlates with the severity of cognitive decline (Sheng et al., 2012). Synaptic dysfunction and neurotoxicity in age-associated dementia and AD are mainly caused by amyloid plaques, but also neuroinflammation with reactive microglia (Moore et al., 2019). Mitochondria are also involved in synaptic degeneration due to compromised ATP synthesis (energy failure), as well as impaired $Ca^{2+}$ handling, increased production of reactive oxygen species (ROS), impaired production of metabolites that are neurotransmitter precursors, and dysregulation of mitochondrial dynamics and mitochondria-dependent cell signaling transduction (Tait and Green, 2012; Guo et al., 2017; Belenguer et al., 2019).

Cognitive dysfunction connected to diabetes is particularly associated with significant changes in the integrity of the hippocampus, a brain region considered to mediate memory formation in animals, and electrophysiological analyses indicate that diabetes impairs synaptic plasticity in hippocampal slices (Biessels et al., 2002; Trudeau et al., 2004; Duarte et al., 2019). Based on this, the vast majority of translational studies in animal models of diabetes were dedicated to the study of hippocampal structure and function. Impaired hippocampal-dependent spatial learning and memory have been demonstrated in different animal models of diabetes (e.g., Flood et al., 1990; Duarte et al., 2012a, 2019; Lizarbe et al., 2019b).

Diabetic conditions, including short- and long-term exposure to diets rich in fat and/or sugar, lead to synaptic deterioration that results in defective neurotransmission and synaptic plasticity in the hippocampus (Nitta et al., 2002; Duarte et al., 2009a, 2012a, 2019; Calvo-Ochoa et al., 2014; Girault et al., 2019; Lizarbe et al., 2019b). Interestingly, intranasal insulin treatment in insulin-deficient mice was shown to ameliorate synaptic degeneration and deficits in learning and memory, without preventing hyperglycemia (Francis et al., 2008). This indicates that impairment of central insulin signaling is indeed an important factor for diabetes-induced brain injury.

## INFLAMMATION AND GLIOSIS

The neurodegenerative process in the hippocampus of diabetes models is accompanied by neuroinflammation and astrogliosis (e.g., Saravia et al., 2002; Baydas et al., 2003; Duarte et al., 2009a, 2012a, 2019; Calvo-Ochoa et al., 2014). Inflammation and activation of microglia have been observed in animal models fed diabetogenic diets and have been linked to memory impairment. However, the activation of microglia as consequence of diabetogenic diet exposure has not been consistently observed.

Seven days of feeding a diet rich in fat and fructose induced hippocampal dendritic damage, accompanied by an

increase of reactive astrocytes associated with microglial changes (Calvo-Ochoa et al., 2014). Long-term HFD consumption (4 months) increased expression of pro-inflammatory cytokines in hippocampus of rats, namely, IL-6, IL-1β, and TNFα (Dutheil et al., 2016). In contrast, astrogliosis (elevated levels of GFAP) and microgliosis (elevated levels of Iba1) were not observed in the hippocampus of mice exposed to HFD for 6 months (Lizarbe et al., 2019b). Denver et al. (2018) showed astrogliosis and microgliosis in cortex and dentate gyrus of mice fed a HFD for 18 days, but not after 1 month, even though the expression of inflammatory genes such as IKKβ, ERK2, mTOR, NF-kB1, and TLR4 persisted upregulated for 5 months on HFD (Denver et al., 2018). It should be noted, however, that levels of GFAP or Iba1 alone might not report on changes in cellular morphology, and such simplistic assessments might contribute to reported controversies (Gzielo et al., 2017).

Proliferation of microglia might also depend on the age of HFD exposure. Aged animals appear to be more susceptible to develop HFD-induced neuroinflammation (Spencer et al., 2019). Hsu et al. (2015) also observed age-dependent inflammation effects of exposure to sugar-rich diets. Namely, diabetogenic diets rich in sucrose or fructose for 1 month, which that do not result in obesity, triggered memory impairment with some degree of neuroinflammation in the hippocampus of adolescent rats, but not in adults (Hsu et al., 2015).

In sum, neuroinflammation profiles not only change with the duration of HFD exposure, but also depend on the age of onset.

## BRAIN ENERGY METABOLISM IN DIET-INDUCED T2D

Brain function requires continuous supply of glucose and oxygen and a tight regulation of metabolic interactions between neurons and astrocytes (Sonnay et al., 2017). Loss of this metabolic regulation that fuels neuronal activity has been proposed to be the culprit of memory dysfunction (Alberini et al., 2018), followed by an important neurodegenerative process (de la Monte, 2017).

The predominant glucose carrier isoforms involved in cerebral glucose utilization are GLUT1 and GLUT3. GLUT1 is expressed in all brain cells including the endothelial cells and with very low neuronal expression, while GLUT3 is almost restricted to neurons (Simpson et al., 2007). Levels of the main BBB carrier GLUT1 were found reduced in the hippocampus of insulin resistant GK rats (Soares et al., 2019). In contrast, Vannucci et al. (1997) reported no changes in the density of GLUT1 or GLUT3, in the brain of db/db mice, relative to wild-type mice. Nevertheless, both studies found T2D-induced reduced cerebral glucose utilization (Vannucci et al., 1997; Soares et al., 2019). Lower levels of both GLUT1 and GLUT3 were found in the brain of mice under a diet rich in fat and sugar for 3 months (Kothari et al., 2017). Mice fed an HFD for 3 months also showed reduced density of the neuronal GLUT3, and of the insulin-dependent GLUT4 that is key for synaptic fueling (see above), when compared to controls (Liu et al., 2015). Altogether, this suggests that brain cells, and especially neurons, have reduced access to glucose in the insulin resistant brain.

PET scans using FDG are commonly used to evaluate brain glucose uptake in both humans and animal models, as well as the $CMR_{glc}$ utilization. Although insulin is the main regulator of peripheral glucose metabolism, it is considered to not control glucose uptake and utilization in the healthy brain (e.g., Hasselbalch et al., 1999). In contrast, insulin was shown to stimulate brain glucose metabolism in subjects with impaired glucose tolerance (Hirvonen et al., 2011) and there have been reports of inverse relations between insulin resistance and $CMR_{glc}$ (Baker et al., 2011; Willette et al., 2015b). Using FDG-PET, Liu et al. (2017) observed lower glucose uptake in the brain of mice fed an HFD for about 2 months, relative to controls.

Mitochondria are key in neurodegeneration processes, namely, due to oxidative phosphorylation dysfunction, impaired $Ca^{2+}$ homeostasis and signaling, and oxidative stress (Belenguer et al., 2019). Impaired mitochondria dynamics resulting in mitochondrial fragmentation was observed in the hippocampus of mice exposed to HFD (Ruegsegger et al., 2019), which might result in reduced energy production (Schmitt et al., 2018). Park et al. (2018) showed that mitochondrial activity is affected in mice fed with HFD. More specifically, they suggested enhanced mitochondrial production of $H_2O_2$, impaired $O_2$ consumption, and lower $Ca^{2+}$ retention capacity in the hippocampus of HFD-exposed mice compared to controls. Moreover, the hippocampus of mice fed an HFD for 6 months showed deficits in the respiratory chain and oxidative phosphorylation (at the level of complexes I, II, III, and IV), as well as reduced levels of key proteins for mitochondrial health, such as PGC-1α and TFAM (Petrov et al., 2015). Decrease in mitochondrial respiration, membrane potential, and energy levels was also observed in the cerebral cortex and hippocampus of mice exposed to high sucrose (20%) in the drinking water, defects that are associated to reduced levels of key proteins for mitochondrial function, such as ATG7, LAMP1, ND1, and NRF2 (Carvalho et al., 2015). Although not all diet-induced diabetic phenotypes comprise baseline fed hyperglycemia, increased glucose levels in the brain might contribute to mitochondrial defects (Hinder et al., 2012). Unfortunately, studies of mitochondria from diabetes models have not been designed to distinguish between the different cellular compartments, that is, whether mitochondria originate from neurons or other brain cells. It is plausible that neuronal mitochondria, especially those locate within or near synapses, are key in the process of synaptic deterioration. On the other hand, altered metabolism within processes of reactive astrocytes is likely to contribute for poor support of neurons and synapses.

### Neuron–Astrocyte Metabolic Interactions

There is abundant knowledge on the plethora of molecular events in neurons that define synaptic activity and the electrophysiological basis of memory. By contrast, mechanisms by which other brain cells regulate synaptic functions are less understood. Astrocytes are brain cells that surround synapses, and are well equipped to modulate neuronal functions, namely, those involved in memory formation: they are excitable through $Ca^{2+}$ fluctuations when responding to neurotransmitters released at synapses, synchronize to nearby astrocytes by $Ca^{2+}$ waves, release gliotransmitters that influence

synaptic plasticity, communicate to blood vessels thus coupling neuronal activity to nutrient supply from circulation, and regulate energy metabolism in support of neurons (Sonnay et al., 2017). Indeed, there is a tight coupling between oxidative metabolism in astrocytic mitochondria and excitatory glutamatergic neurotransmission, defined by the rate of the glutamate-glutamine cycle (Sonnay et al., 2016, 2018), which is crucial for brain function and memory (Alberini et al., 2018). Notably, astrocytes are also the brain reservoir of glucose storage in the form of glycogen, which is nearly absent in neurons (Duran et al., 2019), and lactate produced by glycogenosis and glycolysis in astrocytes has been proposed to be necessary for fueling brain function and memory (Alberini et al., 2018). While healthy astrocytes support neurons, neuroinflammatory microglia release molecules that favor the formation of a neurotoxic subset of astrocytes called A1. A1 astrocytes lose their normal functions, and also secrete harmful factors that may damage neurons (Liddelow and Barres, 2017). Upon this astrogliosis process, the metabolic support from astrocytes to neurons is likely disrupted.

In non-obese, insulin-resistant GK rats, T2D is associated to impaired glucose utilization and glutamatergic neurotransmission in neurons, while astrocytes *in vivo* display exacerbated oxidative metabolism and impaired glutamine synthesis (Girault et al., 2019). According to increased mitochondrial metabolism in astrocytes, Liu et al. (2017) reported higher labeling incorporation from [$^{13}$C]acetate (an astrocyte-specific metabolic tracer) into glutamine in HFD-fed mice than controls, without substantial changes of labeling from [$^{13}$C]glucose. Astrocytic glutamine production is particularly importance for excitatory glutamatergic neurotransmission. Furthermore, work on GK rats shows that insulin resistance is associated with defects of astrocytic glycogen metabolism, namely, in the hippocampus that controls learning/memory (Soares et al., 2019). These observations suggest that energy metabolism in astrocytes is dysregulated in diabetes and might contribute to synaptic dysfunction (**Figure 2**). Such energy metabolism changes result in modified brain metabolic profiles in GK rats, as measured *in vivo* by $^{1}$H magnetic resonance spectroscopy (MRS), and are accompanied by astrogliosis, loss of synaptic proteins required for neurotransmission, and impaired synaptic plasticity (Duarte et al., 2019). Similar decreased density of proteins that depict synaptic degeneration was verified in the hippocampus of mice fed a HFD for 6 months (Lizarbe et al., 2019b), and obese NONcNZO10/LtJ mice (Duarte et al., 2012a).

## Brain Metabolic Profiles

Modifications of brain metabolism in diet-induced obesity and diabetes are likely to be reflected in brain metabolic profiles, which can be measured *in vivo* by MRS. In diabetes patients, studies have generally observed a reduction in the levels of the

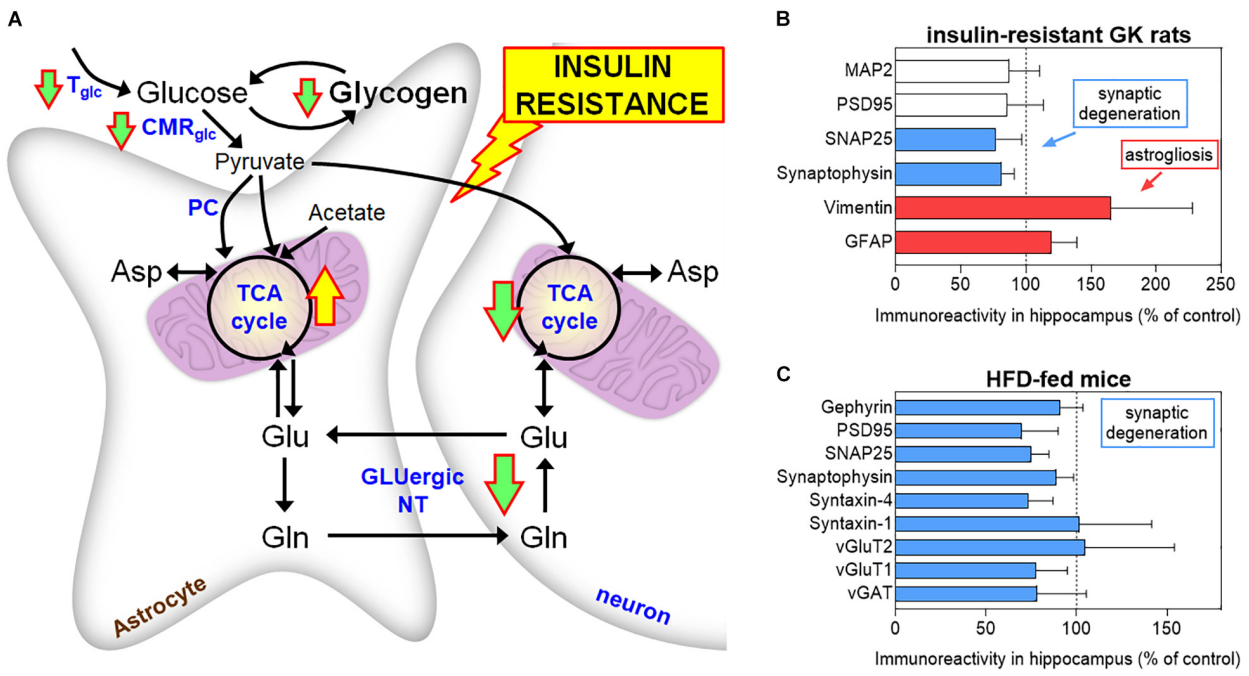


**FIGURE 2 |** Brain energy metabolism alterations in insulin resistant GK rats **(A)**. Down and up arrows indicate decreased and increased rate of pathways in insulin-resistant GK rats, respectively (Girault et al., 2019; Soares et al., 2019). These alterations are supported by findings of increased astroglial markers (GFAP and vimentin) and reduced levels of synaptic proteins in the hippocampus of GK rats, relative to control Wistar rats **(B)**. Mice exposed to 60% HFD for 6 months also show synaptic degeneration in the hippocampus, as suggested by reduced levels of synaptic proteins, versus 10% fat-fed control mice **(C)**. MAP2 is a neuronal marker, PSD95 and gephyrin are post-synaptic density markers, SNAP25, synaptophysin, and syntaxin-1/4 are located in the presynaptic button, and vGluT1/2 and vGAT are transporters in synaptic vesicles. Data in graphs of **B** and **C** are from Duarte et al. (2019) and Lizarbe et al. (2019a), respectively, and are shown as% of controls (mean ± SD). $T_{glc}$, glucose transport; $CMR_{glc}$, cerebral metabolic rate of glucose; PC, pyruvate carboxylase; TCA, tricarboxylic acid; GLUergic NT, glutamatergic neurotransmission.

putative neuronal marker *N*-acetylaspartate (NAA), as well as an increase in *myo*-inositol content (Duarte, 2016). Levels of *myo*-inositol in brain MRS are considered to reflect the size of the astrocytic metabolic pool (discussed in Duarte et al., 2012c). Alterations in the concentration of these two brain metabolites are generally patent in neurodegenerative disorders, namely, AD, Parkinson's disease, and Huntington's disease (Duarte et al., 2014). Moreover, concentrations of both NAA and *myo*-inositol were found to be associated with insulin sensitivity (Karczewska-Kupczewska et al., 2013).

Higher concentrations of *myo*-inositol were also observed in hypothalamus but not hippocampus or cortex of mice fed a 60% HFD during 6 months (Lizarbe et al., 2019a,b). However, rather than reduced NAA levels, these MRS experiments have found an increase of NAA content particularly prominent in the hippocampus. This NAA increase may be linked to changes of osmolarity since the concentration of other major osmolites such as taurine and creatine was also observed. In rats under 60% HFD for 5 months, Raider et al. (2016) have observed no changes in hippocampal NAA but reduced levels of *myo*-inositol, compared to controls. Hassan et al. (2018) also observed HFD-induced metabolic changes in extracts from the prefrontal cortex, namely, higher relative concentrations of lactate, alanine, taurine, and *myo*-inositol, and lower GABA levels. Some metabolic alterations were also observed in the mouse striatum, but not in the hippocampus and hypothalamus. Differences between metabolic profiles *in vivo* and *post mortem* might contribute to the differences in these studies. However, further work must be undertaken to understand the cause of metabolic profile changes in the hippocampus of mice under diabetogenic diets.

## CONCLUSION

Diet-induced metabolic syndrome or T2D in rodents show variable phenotypes depending on the employed diet. Nevertheless, all models show robust effects on memory performance, particularly in spatial tasks that rely on adequate hippocampal function. Across the available literature, one observes that metabolism alterations underlying memory impairment include alterations of glucose utilization in neurons and astrocytes, dysfunctional mitochondria in neurons but exacerbated oxidative metabolism in astrocytes, which is likely required to sustain T2D-induced astrocyte hyper-reactivity. Despite increased astroglial metabolism, the metabolic support from astrocytes to neurons is not adequate, and might contribute to synaptic dysfunction and memory derangements. The mechanisms by which insulin differentially regulates metabolism in neurons and astrocytes require further investigation, in order to understand brain insulin resistant development and how it leads to impaired cognition.

The interaction of insulin with other neuromodulation systems that regulate cell signaling and metabolism has been proposed but insufficiently investigated. For example, IRs interact with the endocannabinoid system (Dalton and Howlett, 2012; Kim et al., 2012) that modulates neuronal and astrocytic metabolism (Duarte et al., 2012b; Köfalvi et al., 2016), and with biliverdin reductase-A that modulates cellular stress responses (Barone et al., 2016). Such signaling interactions may be key for insulin to fulfill its glucose uptake-unrelated roles, and may reveal to be therapeutic targets against brain dysfunction.

Finally, while aging is a key factor on the development of insulin resistance, there is a major knowledge gap on the T2D-aging interaction leading to dysregulation of cerebral metabolism. Suggestions of the time complexity of brain insulin resistance mechanisms come from longitudinal studies in humans. For example, it is known that mid-life obesity is associated with an increased risk of incident dementia (see above), but late-life obesity was found to be negatively associated with incident dementia (Pedditzi et al., 2016). Moreover, insulin resistance was proposed to be associated with glucose cerebral hypo-metabolism in AD patients, but associated to hyper-metabolism in subjects with MCI that will later progress to AD (Willette et al., 2015b). Further research is needed to identify trajectories of insulin-dependent brain metabolism dysregulation leading to brain dysfunction.

## AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript.

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# Effects of Physical Exercise on Working Memory and Attention-Related Neural Oscillations

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Cognitive functions, such as working memory (WM) and attention, have been shown to benefit from physical exercise. Quantifying frequency-band-specific neural oscillatory patterns during the use of such cognitive functions can provide insight into exercise-induced benefits in the brain. Specifically, we investigated whether a 4-month physical exercise training influenced theta and alpha power measured in visual WM and attention tasks. The delayed match-to-sample (DMS) task required mnemonic discrimination of similar visual stimuli, akin to pattern separation, while the visual-attention search (VAS) task required detecting the presence of a specific object (i.e., target) in an image. Behavioral and electroencephalographic data were acquired during a DMS visual WM task and VAS task both before and after the intervention. Forty-three sedentary young adults (19–34 years) were pseudorandomly assigned to a training group (indoor treadmill, $n$ = 20) or to a control group ($n$ = 23). Compared to the preintervention baseline, the exercise group showed increased frontal alpha power (9–12 Hz) during the VAS task after the intervention. In addition, alpha power changes correlated positively with fitness changes. Behaviorally, there were no exercise-related effects on reaction times or accuracy in either task. Our findings substantiate that aerobic training of sedentary young adults may influence neural dynamics underlying visual attention rather than visual WM and mnemonic discrimination.

**Keywords: physical exercise, alpha power, visual attention, EEG, young adults**

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## INTRODUCTION

The beneficial effects of exercise on brain function are of widespread interest but remain elusive. Human research has demonstrated that physical exercise training has the greatest impact on spatial memory, working memory (WM), and executive attention (Cassilhas et al., 2016; De Sousa et al., 2018). More specifically, other studies showed higher fit participants performed better during visual figure recognition (Maass et al., 2015), spatial memory (Erickson et al., 2009, 2011), and attentional control (i.e., the flanker task; Verstynen et al., 2012). However, exercise studies have been quite inconsistent, and the number of studies on chronic (i.e., long-term) physical exercise, especially in young adults, is limited (Verburgh et al., 2014). Hence, it is essential to continue to study the cognitive benefits and neural plasticity associations with exercise, particularly in young adults. Notably, very few studies obtained measures of neural function in a controlled exercise intervention in addition to the commonly obtained behavioral and structural measures.

It has been suggested that neural oscillations in the high- and low-frequency range, which can be measured by electroencephalogram (EEG), relate to distinct cortical operations. For instance, theta-band activity has been implicated in WM processes (Yamagishi et al., 2008) and has been shown to increase at frontal sites with task difficulty (Gundel and Wilson, 1992; Gevins et al., 1997), with WM load (Jensen et al., 2002) and with successful memory performance (Klimesch et al., 1996; Fuentemilla et al., 2010). This frontal theta increase is known as the frontal midline theta (FMT) and may, in part, be related to hippocampal activity (Klimesch, 1999; Mitchell et al., 2008). During recollection, theta activity has also been shown to mediate dynamic links between hippocampal and neocortical areas (Guderian et al., 2009). Alpha-band activity, on the other hand, has been associated with corticocortical and thalamocortical networks that are thought to reflect alertness, memory performance, and attention demands (Jensen et al., 2002; Hsieh et al., 2011; Klimesch, 2012). Decreases in alpha power (i.e., desynchronization) with increasing task difficulty reflect an inverse relation to the number of cortical resources allocated to task performance (Gevins et al., 1997; Babiloni et al., 2010). Measuring these neural oscillations pre- and post-training may aid in understanding how physical exercise impacts cognitive functions.

In rodents, voluntary wheel running has been associated with hippocampal neurogenesis and was positively correlated with synaptic plasticity (Vivar et al., 2012) and spatial pattern separation (Creer et al., 2010). In older adults, Erickson et al. (2009) found a triple association between aerobic fitness levels, hippocampal volume and memory functions, where higher aerobic fitness levels were associated with the preservation of left and right hippocampal volume and better performance on their spatial memory task (Erickson et al., 2009). Together, these studies supported the idea that exercise-induced hippocampal plasticity may improve learning and memory.

Recently, a cross-sectional study showed a relationship between aerobic fitness and neural rhythms in a Posner visuospatial attention task in high- and low-fitness young adults (Wang et al., 2015). Interestingly, high-fitness participants had faster reaction times (RTs) as well as greater beta and theta power during target processing. These findings indicated that aerobic fitness could be positively related to visuospatial attention capacity through the modulation of attentional processes. Some researchers have argued that even single bouts of physical exercise may be sufficient to improve memory performance (Roig et al., 2016) and attentional processes (Hogan et al., 2013). However, it remains unclear whether these exercise-induced improvements in WM and attention are linked to changes in neural oscillations.

Hence, we sought to examine how theta and alpha activity is modulated by a 4-month physical training regimen and aimed to determine the exercise-induced effects on brain dynamics in the frontoparietal network. To that end, we acquired EEG data from sedentary young adults during a delayed match-to-sample (DMS) WM task, which measured mnemonic discrimination of visual stimuli, akin to pattern separation, and a visual-attention search (VAS) task that did not require any recognition memory. We focused on theta (5–7 Hz) and alpha (9–12 Hz) oscillatory activity due to their association with WM and attention, respectively. Based on previous findings, we expected that our training intervention would increase aerobic fitness, enhance mnemonic discrimination, and as an indication of hippocampal activity change, increase theta power in the EEG. Particularly, for the exercise group, we expected faster RT and/or higher accuracy. Only the exercise group was expected to present changes in EEG spectral power reflected as a greater increase in theta power during the DMS task.

# MATERIALS AND METHODS

## Subjects

Forty-three healthy, sedentary young adults (age range: 19–34 years, mean age: 25.33 ± 3.62 years, 23 females) were recruited for the study; one participant dropped out of the study, and two participants were excluded because of technical issues with the EEG recording. All subjects reported no signs of neurological or psychiatric illness and had a normal or corrected-to-normal vision. After providing informed consent, participants were pseudorandomly assigned either to an aerobic exercise group ($n$ = 18) or a control group ($n$ = 22), which were balanced in terms of age, sex, and fitness level (**Table 1**). Subjects received monetary compensation for their participation, and the experiment was carried out in accordance with the guidelines of the ethics committee of the Faculty of Medicine from the Otto von Guericke University Magdeburg (OVGU).

## Intervention Protocol

### Cardiovascular Training (Exercise Group)

The exercise group ran on a stationary treadmill ergometer three times per week for 16 weeks. Each participant received an individually optimized 45–75-min training set, including 5-min warm-up and 5-min cool-down periods. Under the supervision of sports scientists, participants monitored their heart rate (HR) during their workouts and exercised at intervals with an intensity range of 70–90% of maximum heart rate (HRmax). Individual training intensities were determined by target HRs, as estimated by the Karvonen method (Karvonen et al., 1957), and verified to HR levels at the individual anaerobic threshold, as indicated by lactate measures.

**TABLE 1** | Group demographics at baseline.

| Variables | Exercise group | Control group | One-way ANOVA |
|---|---|---|---|
| Age | 26.1 (4.0) | 24.7 (3.3) | $F(1,38) = 1.345$, $p = 0.253$ |
| Sex (f/m) | 10/8 | 13/9 | $X^2(1,39) = 0.051$, $p = 0.822$ |
| BMI | 25.33 (5.71) | 23.44 (3.29) | $F(1,38) = 1.345$, $p = 0.198$ |
| $VO_2$-RC | 28.12 (4.93) | 29.26 (4.02) | $F(1,38) = 0.656$, $p = 0.423$ |
| $(La^-)_b$ | 4.77 (1.34) | 5.12 (1.43) | $F(1,38) = 0.647$, $p = 0.426$ |

*Blood lactate [$(La^-)_b$ in g/mol]; oxygen uptake at respiratory compensation ($VO_2$-RC in ml/min/kg); body mass index (BMI in kg/m$^2$).*

### Walking (Control Group)

The control group walked on the treadmill twice a week, which maintained variables such as social interaction, scheduling, and motivation similar to the exercise group, while not affecting their aerobic fitness. The control group walked for 10–12 min, with breaks in between, and maintained a maximum HR of approximately 50–60% HRmax. The setting and HR monitoring procedures were kept constant between the groups. Due to the low HR training zone, the maximum incline of the treadmill was 3% at a maximum walking speed of 4.5 km/h.

## Fitness Assessment

We assessed the consumption of oxygen (CPET Quark, COSMED, Italy) at the respiratory compensation point ($VO_2$-RC) by graded maximal exercise testing on a treadmill ergometer. The initial speed of 3 km/h was increased every 2 min to a maximum of 6.5 km/h. During this time, the slope of the treadmill also increased from 0 to 18%. This testing occurred until a respiratory exchange ratio (RER) of 1:1 was reached, indicating exhaustion near the limit of the cardiorespiratory system. Here, the oxygen exhaustion criterion was defined by the uptake at respiratory compensation ($VO_2$-RC) to better control for volitional effects. To assess lactate levels (Biosen C-Line, EKF Diagnostic, Magdeburg, Germany), capillary blood samples were taken from the earlobe during the resting state, at 2-min intervals during the fitness test, and 2 min after the maximum intensity. This fitness assessment was repeated after 4 weeks and again after 16 weeks, at the end of the intervention.

## Task Procedure

Electroencephalography recordings were acquired a day before and 2–3 days after the end of the 4-month intervention, while participants performed 120 trials of a DMS WM task (**Figure 1A**) and 60 trials of a VAS task **Figure 1B**). The trials from both tasks were divided into 4 and 2 blocks, respectively, which were presented in a random sequence. During the DMS task, a sample stimulus and a probe stimulus were presented in succession for 3 s, separated by a 5-s delay (i.e., the maintenance phase). Subjects were instructed to answer as correct and quickly as possible whether the sample stimulus was a public or a private place and to spend the remaining time memorizing the image. The maintenance phase is critical for sustaining the WM item and is susceptible to interference from other external factors. Afterward, participants had to decide whether the probe image was identical to (i.e., repeat) or a modification (i.e., lure) of the sample image. All of the DMS stimuli were computer-generated indoor scenes, including 50% lure images. During the VAS task, participants were shown a similar indoor scene and were instructed to detect whether a target (i.e., spherical object) was present. The background image for the VAS remained the same throughout the different blocks, so neither encoding nor maintenance was required for this task.

## Data Acquisition and Processing

Continuous electroencephalogram (EEG) was acquired from 32 active electrodes mounted in an elastic cap (Brain Products GmbH, Germany) with a bandpass filter of 0.1–250 Hz and digitized at a rate of 500 Hz. We placed the electrodes according to the international 10–20 system. From the 32 electrodes, 2 electrodes were positioned at the external ocular canthi of each eye, and a vertical electrooculography (EOG) was placed below the left eye to measure horizontal and vertical eye movement, respectively. The left mastoid was used as the online reference, and all electrode impedances were kept below 5 kΩ. The skin under the electrodes was slightly abraded with a blunt needle, which was used to fill each electrode with electrolyte gel. MATLAB (MATLAB and Statistics Toolbox Release, 2012b, MathWorks, Inc., Natick, MA, United States) and its EEGLAB toolbox (Delorme and Makeig, 2004) were used for offline EEG data processing. The continuous EEG data were high-pass filtered at 0.1 Hz, low-pass filtered at 50 Hz, and referenced to the right mastoid. The data were segmented into 13-s epochs, including 1-s pre-stimulus as the baseline and 1-s post trial. Next, independent component analysis (ICA) was performed to correct for eye-related artifacts and excessive muscle activity. In addition, all trials were visually inspected, and those containing additional artifacts were excluded from further analysis. Participants included in the data analysis had less than 20% of their trials excluded after artifact correction.

## Time-Frequency Analysis

Event-related spectral perturbation (ERSP) was computed using algorithms from EEGLAB (Delorme and Makeig, 2004) and custom MATLAB scripts. Artifact-free data comprising 13-s segments were used for time-frequency analysis (TFA). Trial-by-trial event-related spectral power was calculated using Hamming window tapering with five cycles and 100 logarithmically spaced frequencies ranging from 3 to 20 Hz. For each frequency, event-related spectrum power at each time-frequency point was divided by the average spectral power in the pre-stimulus baseline period at the same frequency. These measures were normalized by taking the log value of the percentage of baseline activity ($ERSP_{\%}$) (Grandchamp and Delorme, 2011). By definition, the unit of $ERSP_{log}$ is the decibel (dB), which is commonly used in the literature (Makeig, 1993; Cohen and Donner, 2013; Jiang et al., 2015).

The mean ERSPs were calculated for two frequency bands of interest: theta (5–7 Hz) and alpha (9–12 Hz). These bands were chosen because of their involvement in visuospatial attention with the frontoparietal network (Wang et al., 2015). Because of frequency smearing after time-frequency decomposition, we used a width slightly different from the typically defined theta (4–8 Hz) and alpha (8–12 Hz) frequency bands (Klimesch, 1996). Narrower frequency bands were chosen to better characterize the changes in neural oscillations at those frequency bands (Hsieh et al., 2011). Additionally, electrodes were grouped into two different clusters with three electrodes each: frontal cluster (F3, Fz, and F4) and posterior cluster (P3, Pz, and P4). To measure if there is an exercise-induced effect, we focused on the sample probe. That is, power estimates from the frontal and posterior clusters were averaged across a section spanning the frequency band (see **Figures 2A,B**) and the

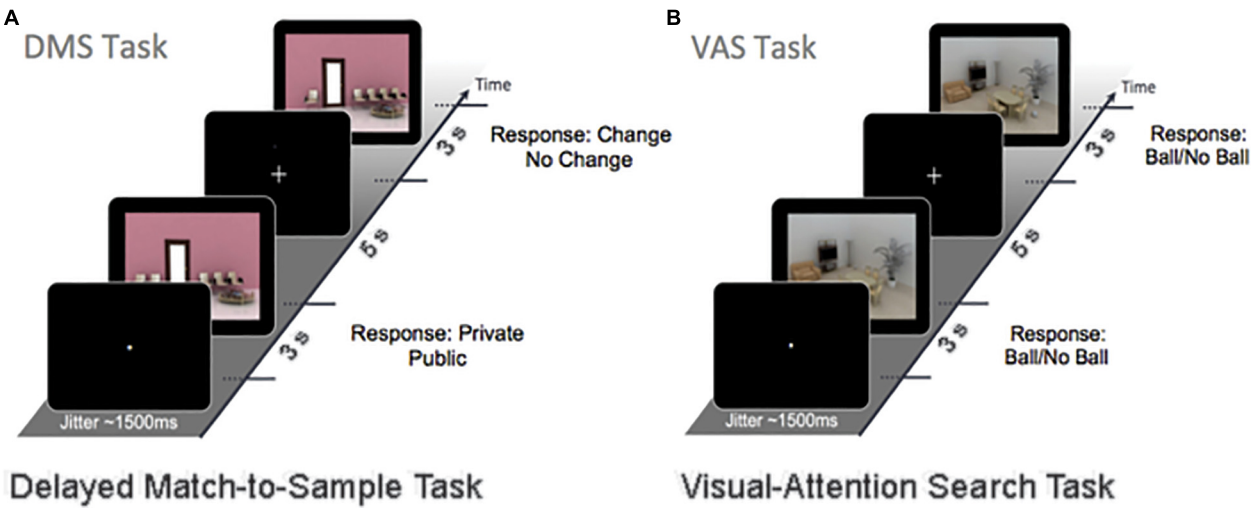


**FIGURE 1 |** The working-memory experimental paradigm **(A)** is a DMS task (120 trials). A sample stimulus (encoding) and a probe stimulus (retrieval) were presented with a stimulus delay of 5 s (maintenance). Participants were instructed to respond to whether the sample stimulus was either private or public and to memorize the image. The probe stimulus was either the same (repeat item) or had small changes in the image (lure items) that needed to be detected. **(B)** The VAS task (60 trials). During this task, subjects were instructed to detect whether a target (i.e., ball) was present. The background image remained the same throughout the visual-attention paradigm.

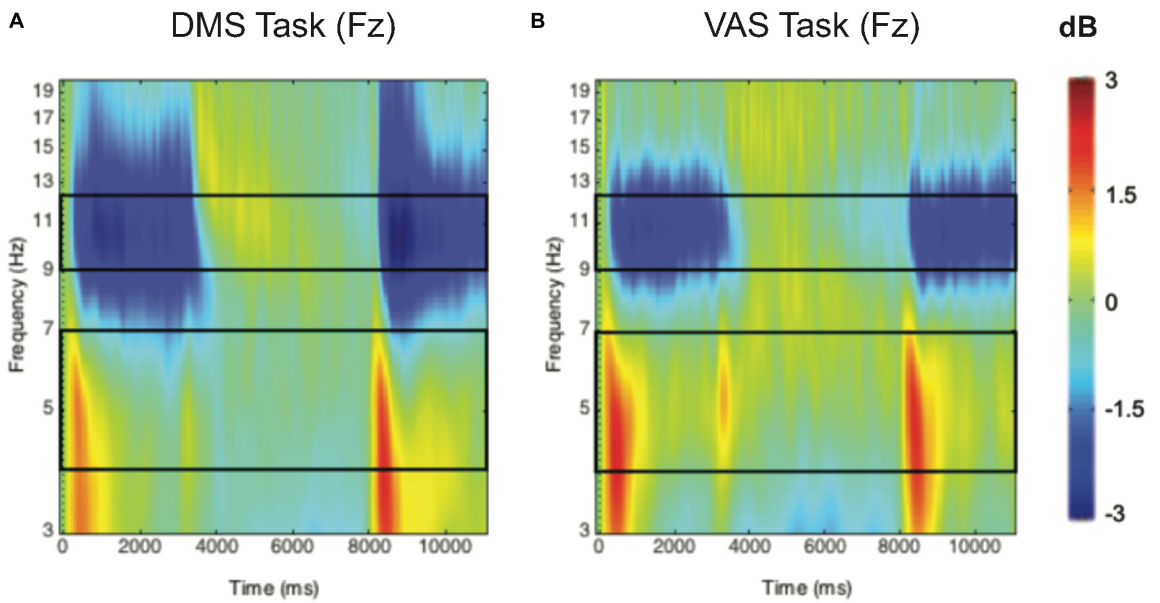


**FIGURE 2 |** Group-averaged time-frequency 2D spectra (in dB) during a DMS task **(A)** and a VAS task **(B)** for the Fz electrode. Time (in ms) is indicated on the *x*-axis, with 0 ms defined as the onset of the sample stimuli and 8000 ms for the onset of the probe stimuli. Frequency (in Hz) is shown on the *y*-axis and is logarithmically scaled from 3 to 20 Hz. The event-related desynchronization (ERD) corresponds to negative values and is shown in blue, while the event-related synchronization (ERS) appears in red.

time period of interest (0–3000 ms), and then subjected to statistical analysis.

## Statistical Analysis

All statistical analyses were performed using the statistical software SPSS (IBM Corps., IBM SPSS Statistics, V24, Armonk, NY, United States, 2016). First, we ran independent-samples *t*-tests to assess whether there were any differences between the groups at baseline (preintervention). To compare differences in categorical variables such as sex and age, we used the Chi-square test. There were no differences observed between the groups at baseline (**Table 1**).

Furthermore, to examine the possible effect of training on cognition, the behavior data were submitted to separate

rmANOVAs (one ANOVA per condition), where the within factor was *time* (pre, post), and the between factor was *group* (exercise, control). As for the EEG data, these were submitted to two separate repeated measures ANOVAs (one rmANOVA per frequency band of interest). The factors in the rmANOVAs included *task* (DMS, VAS), *cluster* (frontal, posterior), and *time* (pre, post intervention) as within-subject factors and *group* (exercise, control) as a between-subjects factor. We did not include any variables as covariates since groups did not differ in demographics. When appropriate, the Greenhouse–Geisser correction was used to adjust the degrees of freedom when the sphericity assumption was violated. All alpha levels for significance were set at 0.05. When significant interactions were found with rmANOVA, *post hoc* independent *t*-tests were conducted.

## Correlations

To assess the possible relationship between exercise and cognition, we first calculated the intervention-related changes in performance, spectral power values, and fitness. In particular, accuracy, RT, and EEG spectral power differences were obtained by subtracting pre from post measures of EEG power. For a better estimation of aerobic fitness increase, a composite fitness score was calculated separately for pre- and postintervention fitness test data as a mean of the inverse *z*-scores of blood lactate level changes (%) and the *z*-score of $VO_2$-RC changes (%). The bivariate (Pearson) correlations were calculated between changes in ERSP values, fitness scores, and behavioral performance to examine how fitness changes affect cognitive functioning after 4 months of exercise training.

# RESULTS

## Participant Characteristics and Aerobic Fitness Assessment

Demographics and fitness levels for the exercise and control groups are reported in **Table 1**. As shown in **Table 1**, the groups were matched for age and sex and did not differ at baseline in body mass index (BMI), average blood lactate, and $VO_2$-RC [$F(1,38) < 1.35$; $p > 0.198$].

To measure exercise-induced changes in fitness, we performed rmANOVAs with *time* (pre- and postintervention measures) as the within-subject factor and *group* (exercise and control) as the between-subjects factor. As seen in **Table 2**, rmANOVA revealed a time × group interaction for lactate [$F(1,38) = 36.93$; $p < 0.001$] as well as for $VO_2$-RC [$F(1,38) = 86.50$; $p < 0.001$]. *Post hoc* paired *t*-tests showed an increase in fitness represented as a decrease in lactate [$t$ (17) = −9.94; $p < 0.001$] and an increase in $VO_2$-RC [$t$ (17) = 2.27; $p < 0.001$] for the exercise group but not the control group [$t$ (21) = −0.25; $p = 0.809$ and $t$ (21) = −1.62; $p = 0.121$, respectively].

## Behavioral Data

**Table 3** illustrates the pre- and postintervention measures for accuracy and RT for each group. We considered performance values that were greater than 2.2 SDs from the mean as outliers, and these values were excluded from the statistical analysis. rmANOVAs revealed a main effect of time for all behavioral performance measures, for the exception of the correct response [$F(1,36) = 2.00$; $p = 0.166$; see **Table 3**]. Although the time × group interactions were all not significant ($F < 1.96$; $p > 0.172$), meaning that the difference in behavioral performance between the pre and post measures was not due to our intervention. **Figures 3A–D** further illustrates this point by showing the similar exercise-induced before and after performances between the exercise and the control group.

## Brain Dynamics and Behavioral Performance (Preintervention Measures)

To test the fundamental relationship between spectral EEG power and behavioral performance, Pearson's correlation analyses were performed on baseline measures across participants. For the DMS task, we did not find a correlation between FMT and behavioral performance during the encoding phase although there was a negative correlation between FMT and accuracy (corrected hit rate = hit−false alarm) during the maintenance phase [$r$ (36) = −0.344; $p = 0.040$; **Figure 4A**], where better performance was associated with a greater decrease in theta power relative to baseline. Also, FMT correlated with RT, where faster detection of a lure was associated with greater decrease in theta power [$r$ (39) = 0.431; $p = 0.006$; **Figure 4B**]. Notably, **Figure 4B** seems to have a high leverage point on the far right, although after obtaining the Mahalanobis distance, it was not excluded. Together, these correlations indicate that a greater decrease in theta during the maintenance conveys a more accurate and faster RT. As for the VAS task, correlations were performed between behavioral performance and posterior alpha power, which is where alpha power is most prominent and known

**TABLE 2 |** Aerobic fitness measures pre and post intervention by group.

| | Exercise | | Control | | |
|---|---|---|---|---|---|
| Variables | Pre M (SD) | Post M (SD) | Pre M (SD) | Post M (SD) | rmANOVA |
| $(La^-)_b$ | 4.77 (1.34) | 3.05 (1.32) | 5.13 (1.43) | 5.50 (1.38) | $F(1,38) = 36.926$, $p < 0.001$ |
| $VO_2$-RC | 28.12 (4.93) | 32.60 (5.07) | 29.26 (4.02) | 29.32 (4.12) | $F(1,38) = 86.496$, $p < 0.001$ |

*Fitness measure: blood lactate [$(La^-)_b$ in g/mol]; oxygen uptake at respiratory compensation ($VO_2$-RC in ml/min/kg).*

**TABLE 3 |** Group mean (SD) for behavioral performance values pre and post intervention.

| | Exercise | | Control | | rmANOVA | |
|---|---|---|---|---|---|---|
| | Pre M (SD) | Post M (SD) | Pre M (SD) | Post M (SD) | Main effect (Time) | Interaction (Time × group) |
| **DMS** | | | | | | |
| CHR | 0.65 (0.12) | 0.60 (0.12) | 0.72 (0.11) | 0.64 (0.17) | $F_{(1,32)} = 8.956$, $p = 0.005$ | $F_{(1,32)} = 0.305$, $p = 0.585$ |
| Hit RT | 1576 (320) | 1688 (321) | 1448 (259) | 1562 (306) | $F_{(1,37)} = 10.099$, $p = 0.003$ | $F_{(1,37)} = 0.000$, $p = 0.990$ |
| Lure RT | 1335 (221) | 1517 (321) | 1306 (234) | 1394 (258) | $F_{(1,37)} = 16.224$, $p < 0.001$ | $F_{(1,37)} = 1.938$, $p = 0.172$ |
| **VAS** | | | | | | |
| CR | 0.92 (0.04) | 0.93 (0.02) | 0.91 (0.03) | 0.92 (0.03) | $F_{(1,36)} = 1.996$, $p = 0.166$ | $F_{(1,35)} = 0.022$, $p = 0.883$ |
| Target present RT | 1288 (216) | 1349 (247) | 1142 (223) | 1266 (214) | $F_{(1,35)} = 7.945$, $p = 0.008$ | $F_{(1,35)} = 0.311$, $p = 0.581$ |
| Target absent RT | 1328 (233) | 1397 (260) | 1171 (234) | 1328 (231) | $F_{(1,36)} = 12.549$, $p = 0.001$ | $F_{(1,36)} = 1.902$, $p = 0.177$ |

*Included in **Table 3** are the descriptive statistics and the results from a rmANOVA during the DMS and the VAS task. For the DMS task, accuracy was measured using hits minus false alarms, known as the corrected hit rate (CHR) and for the VAS task accuracy was measured by averaging the correct responses (CR) for both conditions (target present and absent).*

to be related to attention (Frey et al., 2015). Our results showed a positive correlation between alpha power and RT during target absent trials [$r$ (38) = 0.333; $p$ = 0.041] and a marginal positive correlation for RT during target present trials [$r$ (38) = 0.284; $p$ = 0.084] but not for accuracy [$r$ (40) = 0.020; $p$ = 0.902].

## Exercise-Induced Spectral Power Changes (Pre- and Postintervention Measures)

Repeated measures ANOVAs performed for the sample stimuli presentations (0–3 s) for theta power revealed a main effect of task [$F$(1,38) = 18.87; $p < 0.001$], no main effect of cluster [$F$(1,38) = 1.15; $p$ = 0.290], and no main effect of time [$F$(1,38) = 0.01; $p$ = 0.921]. The interaction between time and group was not significant [$F$(1,38) = 0.51; $p$ = 0.480], as well as the task × cluster × time × group interaction [$F$(1,38) = 0.22; $p$ = 0.645]. For the alpha band during the sample stimuli presentations (0–3 s), there was a main effect of *task* [$F$(1,38) = 25.09; $p < 0.001$], a main effect of *cluster* [$F$(1,38) = 17.76; $p$ = 0.000], and no main effect of time [$F$(1,38) = 2.45; $p$ = 0.126]. There was however, a significant *task* × *cluster* × *time* × *group* interaction [$F$(1,38) = 4.26; $p$ = 0.046]. *Post hoc* independent $t$-tests showed no group difference for the posterior cluster [$t$ (38) = 32; $p$ = 0.751], while the frontal cluster showed a significant group difference [$t$ (38) = 2.34; $p$ = 0.025] during the VAS task, with increased alpha power after the intervention (see **Table 4**). This effect was specific to attention, as it appeared during the VAS task but not the DMS task [$t$ (38) < 1.52; $p > 0.136$]. This EEG finding is further illustrated in **Figures 5A,B** in a histogram and a topographic map.

Furthermore, given the significant *post hoc* results, we sought to examine a direct link between anterior alpha power changes with aerobic fitness changes and behavior performance changes (during the VAS task). Such correlations were performed irrespective of the group (i.e., across all subjects) since fitness changes were also present in the control group. Specifically, there was a positive correlation between changes in frontal alpha power and changes in aerobic fitness [$r$ (40) = 0.379; $p$ = 0.016; see **Figure 6A**]. Additionally, changes in frontal alpha power were also positively correlated with changes in accuracy [VAS task – see **Figure 6B**, target absent, $r$ (37) = 0.336, $p$ = 0.042; target present, $r$ (39) = 0.302, $p$ = 0.066]. Although a correlation between changes in aerobic fitness and changes in accuracy ($p > 0.423$) was absent. However, changes in aerobic fitness did correlate negatively with RT for the target present condition [$r$ (38) = −0.360; $p$ = 0.026] and marginally for the target absent condition [$r$ (37) = −0.297; $p$ = 0.074], yet failed to correlate with changes in alpha power ($p > 0.345$).

# DISCUSSION

As anticipated, we observed an increase in aerobic fitness after a 4-month exercise intervention. The exercise intervention led to an increase in oxygen consumption at ventilatory anaerobic threshold, and a decrease in blood lactate threshold at maximum intensities. Contrary to our expectations, we did not observe intervention-related improvements in mnemonic discrimination or visual attention over time. Additionally, there were no effects of the exercise intervention on theta oscillations. However, the TFA revealed an increase in amplitude for frontal alpha power in the exercise group compared to that in the control group during the visual-attention-search (VAS) task. This overall change in alpha power positively correlated with aerobic fitness changes and changes in accuracy during the VAS task across all participants. Changes in fitness were further associated with RT behavioral performance in the VAS task as well.

## Preintervention Measures

A cross-sectional correlation analysis derived from baseline measures revealed that greater event-related desynchronization (ERD) in theta power was negatively correlated with memory performance (in the DMS task; **Figure 4**). Thus, stronger theta desynchronization in the maintenance phase was associated with better mnemonic discrimination and faster RT (in lure trials), which is in line with previous findings in the literature (Greenberg et al., 2015). Notably, the correlation

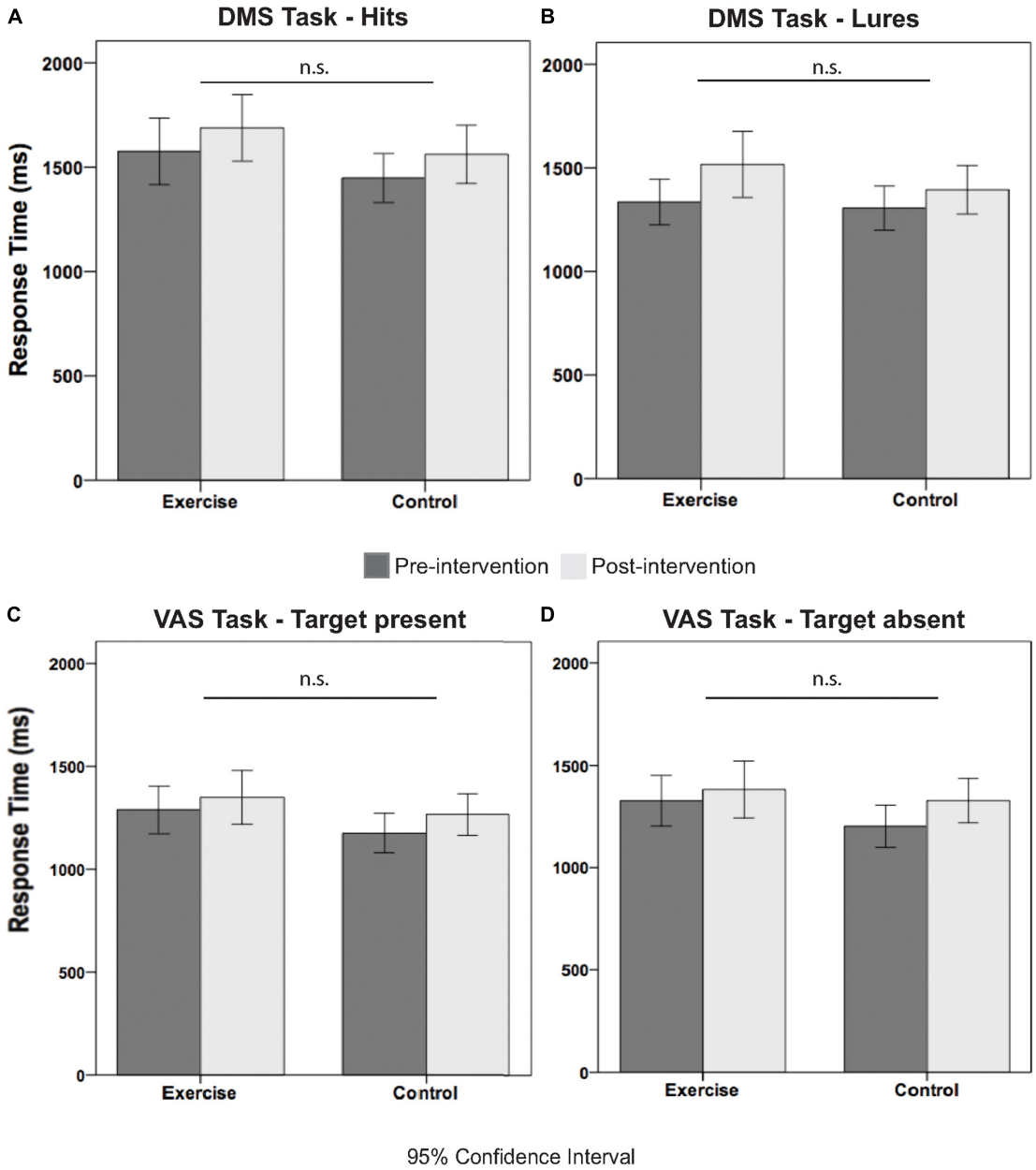


**FIGURE 3 |** Illustrates the response time measures for pre and post intervention separated by group and condition. Specifically, **(A)** shows the RT pre and post intervention measures for hits, **(B)** for lures, **(C)** for target present, and **(D)** for target absent trials.

between frontal theta power and performance was specific to lures (RT), potentially indicating that a decrease in frontal theta power associates with trials that require greater brain resources. In addition, the time-frequency representations (TFRs) for alpha power are in accordance with previous studies stating that alpha ERD occurs during a broad range of cognitive tasks, where harder tasks elicit a more negative ERD (Klimesch, 1999). Also, our findings revealed a positive correlation between alpha power and RT during target absent trials, where faster RT had greater ERD at posterior sites, further illustrating the importance of alpha desynchronization in attention processing. And, even though alpha power is most prominent in the parietal region, alpha ERD has also been observed in the frontal cortex during the performance of a selective attention task (i.e., Stroop task; Chang et al., 2015), indicating that alpha ERD reflects a top-down process.

## Exercise-Induced Changes

We observed an exercise-related increase in alpha power (i.e., weaker ERD), predominantly at the frontal electrodes during the VAS task (see **Figure 5**). Note that an increase

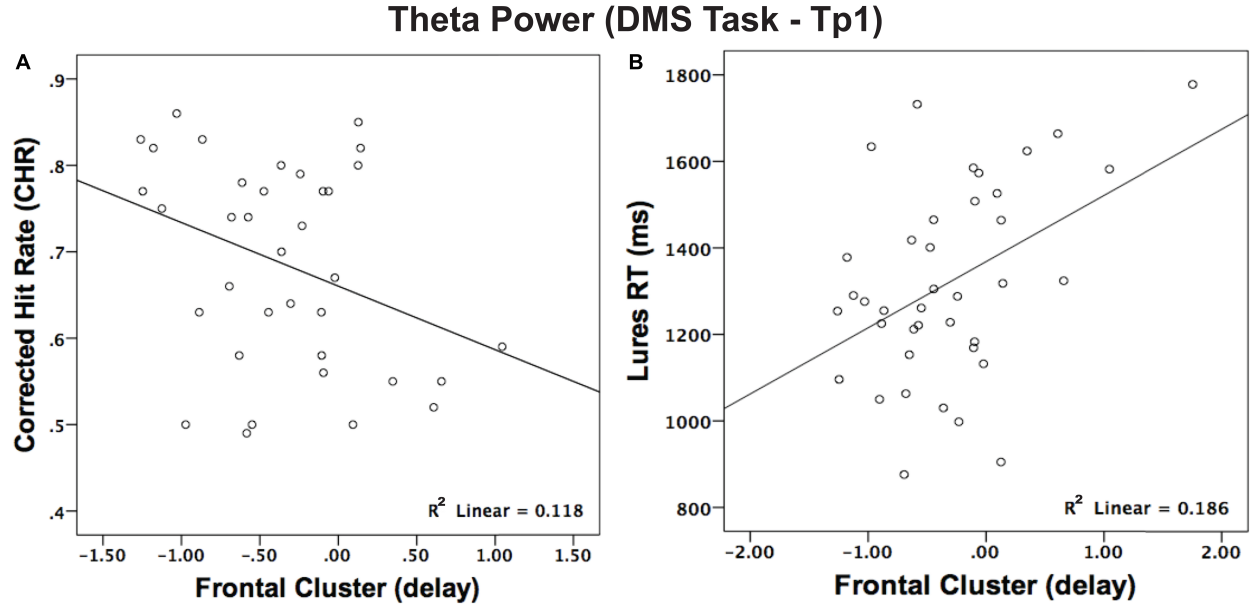


**FIGURE 4 | (A)** Correlation between theta power in frontal regions and performance (corrected hit rate) during the maintenance phase (delay). Higher scores for corrected hit rates revealed a more prominent event-related desynchronization (ERD) for theta. **(B)** Correlation between theta power in frontal regions and RT (lure stimuli) during the maintenance phase. Shorter RTs for detecting lure items revealed greater ERD for theta during the maintenance phase (delay).

**TABLE 4 |** Group mean (SD) values for theta and alpha power for frontal and posterior sites pre and post intervention.

| | Exercise | | Control | | |
|---|---|---|---|---|---|
| | Pre M (SD) | Post M (SD) | Pre M (SD) | Post M (SD) | Independent *t*-test |
| **Theta** | | | | | |
| DMS (frontal) | −0.46 (0.83) | −0.34 (1.19) | −0.13 (0.76) | −0.08 (0.96) | $t$ (38) = 0.068, $p$ = 0.795 |
| DMS (posterior) | −0.56 (1.44) | −0.33 (1.39) | −0.17 (1.46) | −0.39 (1.51) | $t$ (38) = 2.315, $p$ = 0.136 |
| VAS (frontal) | 0.20 (0.60) | 0.14 (1.04) | 0.33 (0.86) | 0.28 (0.95) | $t$ (38) = 0.000, $p$ = 0.988 |
| VAS (posterior) | −0.04 (1.38) | −0.03 (1.41) | 0.21 (1.34) | 0.01 (1.34) | $t$ (38) = 0.253, $p$ = 0.618 |
| **Alpha** | | | | | |
| DMS (frontal) | −1.85 (1.63) | −1.89 (1.52) | −2.38 (1.79) | −2.39 (1.75) | $t$ (38) = 0.005, $p$ = 0.945 |
| DMS (posterior) | −2.74 (2.73) | −2.46 (1.92) | −3.86 (2.29) | −3.62 (2.37) | $t$ (38) = 0.013, $p$ = 0.910 |
| VAS (frontal) | −1.77 (1.62) | −1.10 (1.39) | −1.80 (2.16) | −2.04 (1.59) | $t$ (38) = 5.465, $p$ = 0.025 |
| VAS (posterior) | −2.30 (2.66) | −1.81 (2.03) | −3.45 (2.33) | −3.11 (2.01) | $t$ (38) = 0.102, $p$ = 0.751 |

*Table 4 shows the frontal and posterior mean (SD) ERSP values for frontal and posterior theta- and alpha-band power. Both frequency-band power values are separated by group and further separated by task and time (pre and post intervention measures). Independent t-tests were conducted on differences in theta and alpha power (pre minus post intervention) between groups.*

in alpha power stems from a reduction in alpha ERD in the postintervention session. That is, overall subjects had similar performance levels, but the exercise group was characterized by reduced alpha desynchronization (i.e., less cortical activity) to accomplish the same task. Tentatively, we attribute this increase in alpha power to neural efficiency, specifically to an enhanced capacity for resource allocation involving visual attention. Our findings are compatible with the Ludyga et al. (2016) study in which they measured activity before and after a month of a cycling intervention, and also observed improvements in fitness performance and decreased cortical activity during exercise (Ludyga et al., 2016).

Furthermore, neuroimaging studies have demonstrated that subjects with higher WM and spatial skills have weaker frontoparietal activation during cognitive tasks (Rypma and D'Esposito, 1999). Similar results have been found in EEG studies where some have indicated that elite athletes (e.g., shooters, karatekas, and gymnasts) require "less" cortical activation (weaker ERD) in task-relevant brain areas than novices during sport-specific tasks (Ludyga et al., 2016) and a judgment-allocating task (Del Percio et al., 2009; Babiloni et al., 2010). This observation has been coined "neural efficiency" (Del Percio et al., 2010; Maffei et al., 2017). Studies that have taken aerobic fitness into consideration have come

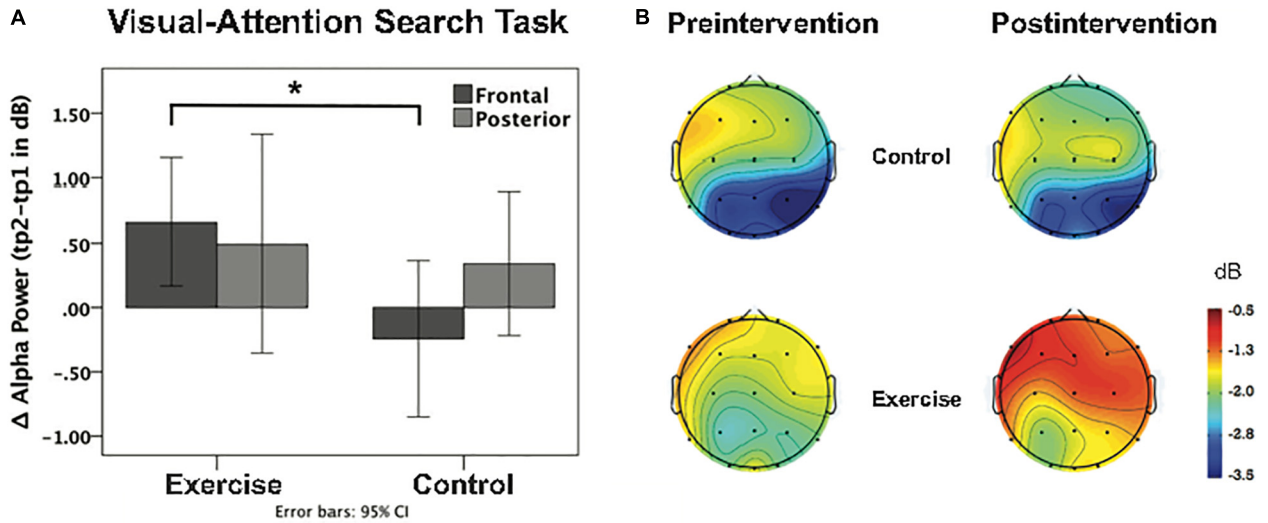


**FIGURE 5 | (A)** Bar graph shows the intervention-induced alpha power change during the VAS task at frontal and posterior clusters for each group. Asterisk indicates $p < 0.05$ (uncorrected, 2-tailed *t*-test). **(B)** Topographical distribution of ERSP values for alpha-band activity, including the first 3 s of the sample stimuli presentation in both the pre and post intervention groups.

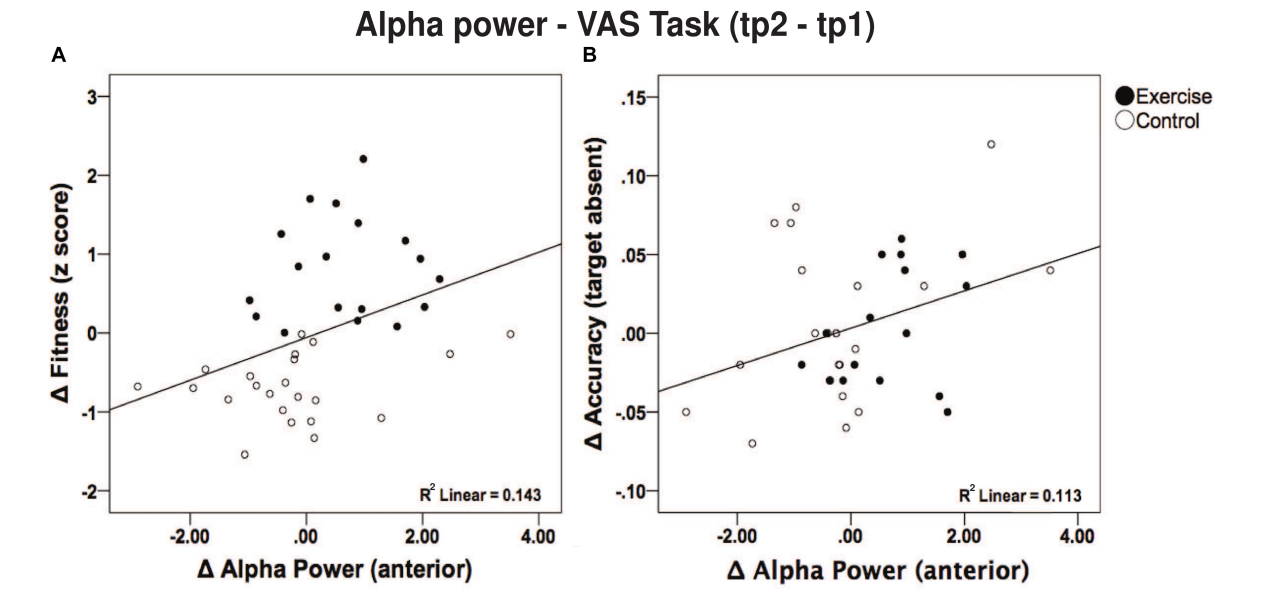


**FIGURE 6 | (A)** Correlation between changes in alpha power in frontal regions and fitness score. A higher fitness score was related to a greater increase in alpha power. **(B)** Correlation between change in alpha power in frontal regions and correct response during the VAS (target absent trials) task.

to similar conclusions (Hogan et al., 2015; Ludyga et al., 2016). For instance, a cross-sectional study performed by Hogan et al. (2013) found that preadolescents with lower fitness require more neural resources for a given task, which was reflected by an increased EEG coherence in comparison to the fit subjects.

Moreover, our results showed that participants with greater improvements in fitness had a larger increase in alpha power in the anterior regions (**Figure 6A**). Other cross-sectional studies have also found comparable correlations (Weinstein et al., 2012; Chu et al., 2016; Hyodo et al., 2016). An exercise intervention study in older adults found that higher aerobic fitness derived from the walking program was associated with greater changes in white matter integrity in the frontal and temporal lobes (Voss et al., 2013). Given these previous findings, it is congruent that we observed an increase in alpha power reflected in the anterior electrodes, which also correlated with changes in accuracy (**Figure 6B**). Notably,

such correlations (alpha-fitness and alpha-performance) were existent across groups, implying that alpha power modulations may also be sensitive to other physiological changes. Fitness changes, for instance, might not only be driven by the intervention, but also other parameters such as daily routine activities, adaptability in training (Bonafiglia et al., 2016), and a genetic disposition to physical activity could also be playing a role (Bouchard, 2012). Nonetheless, only our exercise group showed less cortical activation to performed the VAS task compared to the control group, as shown by the increased alpha power after the intervention, which we attribute to neural efficiency.

Our study has some limitations. First, we had a limited sample size of 18 subjects comprising the exercise group. Further intervention studies will have to confirm our findings in larger samples, and it would be beneficial to lengthen the intervention duration to determine whether the training benefits transfer to hippocampal-dependent tasks and oscillations in young adults. On the other hand, our sedentary group was matched in age, sex, and baseline fitness level. Second, we did not have a tracker to measure outside activity; however, we encouraged all participants to avoid changes to their lifestyle outside the laboratory for the time of the intervention. The sedentary group of young adults who underwent our vigorous exercise training showed improvements from our 4-month training intervention, which we measured with not one but two fitness markers providing a more precise indication of the aerobic fitness change. Hence, the negative findings that we observed here were not due to an ineffective intervention.

Taken together, our study provides tentative evidence in support of cardiovascular exercise modulating oscillatory brain activity. Also, our findings support the possibility that aerobic training of sedentary young adults may influence neural dynamics underlying visual attention. However, we did not confirm our hypothesis that aerobic exercise would enhance theta oscillations and improve WM performance in a mnemonic discrimination task.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Otto von Guericke University Magdeburg. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AC performed the EEG experiments, analyzed EEG data, and wrote the manuscript. AB designed the study, performed the fitness research, analyzed fitness data, and edited the manuscript. ED designed the research and edited the manuscript.

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# Ketosis After Intake of Coconut Oil and Caprylic Acid—With and Without Glucose: A Cross-Over Study in Healthy Older Adults

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**Introduction:** Medium-chain-triglycerides (MCT), formed by fatty acids with a length of 6–12 carbon atoms (C6–C12), constitute about two thirds of coconut oil (Coc). MCT have specific metabolic properties which has led them to be described as ketogenic even in the absence of carbohydrate restriction. This effect has mainly been demonstrated for caprylic acid (C8), which constitutes about 6–8% of coconut oil. Our aim was to quantify ketosis and blood glucose after intake of Coc and C8, with and without glucose intake. Sunflower oil (Suf) was used as control, expected to not break fasting ketosis, nor induce supply-driven ketosis.

**Method:** In a 6-arm cross-over design, 15 healthy volunteers—age 65–73, 53% women—were tested once a week. After a 12-h fast, ketones were measured during 4 h after intake of coffee with cream, in combination with each of the intervention arms in a randomized order: 1. Suf (30 g); 2. C8 (20 g) + Suf (10 g); 3. C8 (20 g) + Suf (10 g) + Glucose (50 g); 4. Coc (30 g); 5. Coc (30 g) + Glucose (50 g); 6. C8 (20 g) + Coc (30 g). The primary outcome was absolute blood levels of the ketone β-hydroxybutyrate, area under the curve (AUC). ANOVA for repeated measures was performed to compare arms.

**Results:** β-hydroxybutyrate, AUC/time (mean ± SD), for arms were 1: 0.18 ± 0.11; 2: 0.45 ± 0.19; 3: 0.28 ± 0.12; 4: 0.22 ± 0.12; 5: 0.08 ± 0.04; 6: 0.45 ± 0.20 (mmol/L). Differences were significant (all $p \leq 0.02$), except for arm 2 vs. 6, and 4 vs. 1 & 3. Blood glucose was stable in arm 1, 2, 4, & 6, at levels slightly below baseline ($p \leq 0.05$) at all timepoints hours 1–4 after intake.

**Conclusions:** C8 had a higher ketogenic effect than the other components. Coc was not significantly different from Suf, or C8 with glucose. In addition, we report that a 16-h non-carbohydrate window contributed to a mild ketosis, while blood glucose remained stable. Our results suggest that time-restricted feeding regarding carbohydrates may optimize ketosis from intake of MCT.

**Clinical Trial Registration:** The study was registered as a clinical trial on ClinicalTrials.gov, NCT03904433.

**Keywords: ketosis, β-hydroxybutyrate, aged, glucose, medium-chain fatty acids, coconut oil, ketogenic diet, fasting**

## INTRODUCTION

The interest for ketosis as a potentially beneficial metabolic state for health started in the epilepsy field in the 1920's (1), and in more recent decades the interest has expanded to a broad range of neurological conditions (2), including Alzheimer's disease (3), and also to weight loss (4), type 2 diabetes (5), and high performance in sports (6). Fasting or carbohydrate-restricted diets are typically used to induce ketosis, which is a condition where the liver uses fatty acids to produce the ketone bodies ("ketones") β-hydroxybutyrate (BHB) and acetoacetate (AcAc). Ketones are transported through the blood and provide energy for various tissues, which is essential for the brain, due to its limited ability to extract energy directly from fatty acids (7). Ketones also have a function as substrate for lipid synthesis (8), and in recent years BHB has been identified as an epigenetic signal molecule related to brain health (9).

Medium-chain fatty acids (MCFA)—which form together as medium-chain triglycerides (MCT)—have been described as providing a shortcut to ketosis, by their specific metabolic properties compared to long-chain fatty acids (LCFA), which dominate human diets. However, as pointed out by Dayrit (10), the concept MCT is used inconsistently and sometimes without definition in the literature. Sometimes it refers to a triglyceride formed by fatty acids with a carbon chain of 6–12 atoms, which is the chemical definition, including caproic (C6), caprylic (C8), capric (C10), and lauric (C12) acid. However, often MCT refers to just C8 and C10, which is the content of many commercialized "MCT-oils," used as dietary supplements since the 1960's. This has led to an ambiguity on whether C12 share the ketogenic properties of the shorter MCFA. As coconut oil is constituted by almost 50% C12 and just ≈6–8% each of C8 and C10, the ketogenic effect of coconut oil has also been unclear. A recent intervention study investigated the acute ketogenic response to coconut oil and different MCFA-combinations, including ≥91% concentrations of C8, C10, and C12. Comparisons of the arms have been reported in two articles (11, 12), concluding that C8 increased blood ketones during a few hours, while the ketogenic response to the other MCFA and coconut oil was weak. In the current article the concepts MCFA/MCT will refer to C6–C12, but while C6 only constitutes <1% of coconut oil, and is rarely used in supplements, it will not be further discussed.

Fasting and caloric restriction in different forms have been associated with a broad range of health and longevity pathways. Several of the same pathways have also been associated with ketogenic diets, and in some of them BHB has been identified as a mediator (9, 13, 14). Time restricted feeding is a form of intermittent fasting, where total energy intake is not necessarily reduced, but intake is concentrated to e.g., 8 h per day, leaving a 16-h daily fasting period. The combination of MCT intake and a time restricted eating pattern could potentially enhance ketosis, as carbohydrates inhibit ketosis through an increase in insulin secretion and a decrease in glucagon (15). In ketogenic diet protocols, the daily carbohydrate limit to avoid breaking ketosis is often set in the range 20–50 g (16). The ketogenic response to fasting is much quicker in children compared to adults (17), but whether the ketogenic response is influenced by aging is not well studied in humans. Here we study a sample of older adults, with the purpose of optimizing future ketogenic interventions in the field of cognitive health.

The first aim of this study was to compare the short-term ketogenic effect of coconut oil vs. C8 for 4 h, and to investigate how this response is affected by previous intake of 50 g of glucose. In contrast to previous studies, which have mainly investigated change in ketones vs. baseline levels, our main interest is the absolute level of ketosis. We expect this outcome to give clarity on the interaction between ketogenic and anti-ketogenic processes. The second aim was to investigate the response in blood glucose to these interventions. The third aim was to assess satiety and tolerance, to evaluate the feasibility of investigating a similar administration of the fatty acids in long-term interventions. Additionally, we will analyze inter-individual differences in the BHB responses to C8.

**Abbreviations:** BHBv, β-hydroxybutyrate in venous whole blood; BHBp, β-hydroxybutyrate in plasma; AcAc, acetoacetate; Glu, glucose; Coc, coconut oil; C8, caprylic acid; C10, capric acid; C12, lauric acid; Suf, sunflower oil; AUC, area under the curve; MCFA, medium-chain fatty acids; MCT, medium-chain triglycerides; LCFA, long-chain fatty acids; BMI, body mass index.

## MATERIALS AND METHODS

### Study Sample

Fifteen healthy volunteers, 53% women, were recruited by advertising in a daily newspaper. Inclusion criteria were age 65–75 years, written informed consent during a screening visit, and daily coffee consumption, as coffee was used as a

**TABLE 1 |** Characteristics of the participants.

| **Age (years)** | **69.2 ± 2.4** |
|---|---|
| BMI (kg/m$^2$) | 23.9 ± 4.0 |
| Glucose (mmol/L) | 5.2 ± 0.6 |
| Insulin (mIE/L) | 5.5 ± 3.3 |
| Glucagon (pmol/L) | 40.3 ± 4.4 |
| BHBv (mmol/L) | 0.15 ± 0.14 |
| BHBp (mmol/L) | 0.12 ± 0.08 |
| Acetoacetate (mmol/L) | 0.18 ± 0.03 |
| Total ketones (mmol/L) | 0.30 ± 0.10 |

*Mean values and standard deviations. BHBp, β-hydroxybutyrate in plasma; BHBv, β-hydroxybutyrate in venous whole blood.*

**TABLE 2 |** Description of the intervention arms, and introduction of abbreviations.

| | **Intervention Arms (intake in combination with 2.5 dl coffee & 15 g cream)** | | |
|---|---|---|---|
| Suf | 1: Sunflower oil (30 g) | low-caloric | 0% MCT |
| C8 | 2: C8 (20 g) + Sunflower oil (10 g) | low-caloric | 67% MCT (C8) |
| C8+Glu | 3: Glucose (50 g)—[15 min]==> C8 (20 g) + Sunflower oil (10 g) | high-caloric | =Arm 2 + glucose |
| Coc | 4: Coconut oil (30 g) | low-caloric | ≈62% MCT (mainly C12) |
| Coc+Glu | 5: Glucose (50 g)—[15 min]==> Coconut oil (30 g) | high-caloric | =Arm 4 + glucose |
| C8+Coc | 6: Coconut oil (30 g) + C8 (20 g) | high-caloric | MCT-content of arm 2 + 4 |

*Low-caloric ≈ 300 kcal; high-caloric ≈ 500 kcal.*
*Arm 1: Black was selected to represent the "control" arm.*
*Arm 2 and 4: Red and sand were arbitrarily chosen colors for C8 and Coc.*
*Arm 3 and 5: These arms correspond to 2 and 4, respectively (with glucose added). Colors are therefore the same as 2 and 4, but with lower opacity (in **Figures 2**, **5** they are striped, instead of changing opacity).*
*Arm 6: Blue was chosen arbitrarily as a contrasting color.*

vehicle. Exclusion criteria were weight <50 kg, current smoking, diagnosed diabetes (type 1 or 2), history of heart disease, history of disease related to internal organs or metabolism, experience of "sensitive gut" or known intolerance to coconut oil or sunflower oil, medication expected to affect glucose- or lipid-metabolism, fasting during the study or one month before, high intensity physical activity >3 times/week, dementia, severe psychiatric conditions, Hb <125 g/L, and participation in a lifestyle intervention during the last 6 months. Baseline characteristics of the participants are described in **Table 1**. Body mass index (BMI) and age were assessed at the screening visit. The other values are from the first study day, after a 12 h overnight fast. Participants were informed that fatty acids from coconut oil and sunflower would be used in the study, but were blinded from further details. The sample size was calculated based on effect sizes reported in previous studies (12), and was supposed to be sufficient to detect differences of clinical significance.

## Study Design

In a cross-over design, participants were exposed to six different intervention arms, which are described and labeled with abbreviations in **Table 2**. Participants were randomized to receive the intervention arms weekly in one of the following sequences: 421653, 216435, 164253, 642135. The test oils were mixed with 2.5 dl coffee—containing ∼170 mg caffeine—and 15 g full-fat cream, containing 0.4 g carbohydrates, 0.3 g protein and 6 g fat (≤0.6 g MCT). Caffeine has been reported to have a mild ketogenic effect, mainly 3–4 h after intake when consumed at levels comparable to those in our study (18). We expect the relative ketogenic contribution from caffeine in our study to be small, and equally distributed between arms. Cream was added for the purpose of masking any nuances of the test oils, and it may also give some contribution to satiety. At the given dose we expect cream to have a negligible effect on ketosis. Sunflower oil is not assumed to be an active substance of arm 2 & 3 (19), but is added to balance caloric content, and potential competition in uptake of MCFA by LCFA. Therefore, the labeling will be just C8. Arm 1, 2, 4, & 6—referred to as the non-glucose arms—include a 16-h non-carbohydrate window, which can be described as time-restricted feeding regarding carbohydrates. Although this technically also could be labeled a non-protein window, our labeling is based on the fact that carbohydrates are the main inhibitors of ketosis (16, 20). Arm 1, 2, & 4 will be referred to as low-caloric, and 3, 5, & 6 as high-caloric.

## Test Oils

The test ingredients were bought in local food or health stores, and were from arbitrarily chosen brands: Sunflower oil, constituted by 100% LCFA. C8 (100%) from highly refined coconut oil, which means 100% MCFA. Deodorized coconut oil with fatty acid composition in the following ranges, according to the manufacturer: C8 4.6–10%, C10 5.5–8.0%, C12 45.1–50.3%. By assuming the midpoint of the ranges, we calculated MCFA content to ≈62%, and thus LCFA content to ≈38%.

## Sampling Procedure and Laboratory Analyses

Recruitment and data collection took place between August and October 2018. The study was conducted at the Clinical Pharmacology Trial Unit (CPTU) at the Karolinska University Hospital, Huddinge, Sweden. One participant, exposed to the Coc arm, dropped out during the first study week due to reporting severe diarrhea which occurred several hours after leaving the study center. That participant was replaced by another from the waiting list, who was exposed to all intervention arms. Participants were instructed to keep their usual habits regarding diet and exercise, and to especially avoid deviation from habits on days preceding a study day. Instructions were given to consume nothing but water after 8 P.M. the day before testing, to not consume alcohol the day before testing, and to avoid physical activity exceeding 20 min stroll in the morning of a study day.

On each study day participants arrived at 7.30 A.M. They were at rest during the study session, and water was allowed *ad*

*libitum*. A venous catheter was applied for repeated collection of blood. Baseline assessments were performed 20–40 min after arrival, and within the following 0–5 min participants received the drink in a covered cup, at a time point defined as T0. They were instructed to ingest the drink during 5–7 min. In arm 3 & 5, participants were served 50 g glucose dissolved in a glass of water, 15 min before the blood test preceding T0. The decision to place the glucose intake prior to T0 (without a baseline assessment) was based on considerations of tolerance and logistics, and it should have negligible effect on the interpretation of our primary outcome, which is area under the curve (AUC) of absolute ketone levels. However, it limits dynamic comparisons between glucose and non-glucose arms.

Details of the sampling and analyses of ketones has been described previously (21), and a summary of the procedure is given here. Our main outcome, BHB assessed in venous whole blood (BHBv), was measured with a point-of-care meter (Statstrip Xpress®) at time points T0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 (minutes). Whole blood was drawn by a syringe through the venous catheter, and then applied to the test strip within ∼10 s. BHBv was validated against a laboratory assay on total ketones, calculated from plasma BHB (BHBp) + AcAc, at T0, 30, 60, & 120, which showed that the correlation was high (Pearson's r = 0.91, $p < 0.0001$; $n = 360$) for BHBv vs. BHBp, as well as for BHBv vs. total ketones). Also, the agreement between BHBv and BHBp was satisfactory (Lin's concordance coefficient of absolute agreement = 0.91) (21). A divergence in the ratio between BHB and AcAc has been reported after intake of C8 vs. coconut oil (11, 12), which could potentially induce bias when only BHB is measured. We therefore analyzed if a divergence could be observed in our data. Glucose was assessed at T0, 60, 120, 180, and 240. Insulin and glucagon were assessed at T0 and 240, and additionally at T0, 30, 60, and 90 in the glucose-arms (3 & 5).

Glucose, insulin, glucagon, BHBp, and AcAc were analyzed according to routine procedures at two different university hospital laboratories. Glucose was analyzed from venous blood by enzymatic photometry and potentiometry. Insulin was analyzed from serum by immunochemistry, electrochemilumiscent. Glucagon was analyzed from plasma by immunochemistry (RIA). BHBp levels were measured using the Randox D-3 Hydroxybutyrate (Ranbut) reagent kit (Randox, Crumlin, UK). Quantification of BHBp was performed with an automated clinical chemistry analyzer (ABX Pentra C400, Horiba, Montpellier, France) using a spectrophotometric endpoint assay at 340 nm and 37°C. AcAc was measured by mixing 8 μl of plasma diluted in 5 μl $H_2O$ with 153 μl working reagent containing 2.3 U 3-Hydroxybutyrate dehydrogenase (Sigma-Aldrich, MO, USA), 96.5 mmol/L calcium phosphate buffer, pH 7.0 (Merck, NJ, USA) and 0.18 mmol/L NADH (Sigma-Aldrich, MO, USA). The conditions and analyzers used for BHB were also used for AcAc samples. In the calculations, we used raw data for values below the ranges that had been validated at the lab—AcAc <0.20 mmol/L and BHBp <0.10 mmol/L. Storage and transportation followed the laboratory guidelines, with the exception of 9% of the AcAc samples, which were analyzed 1–2 days after the proposed 5 days at −20°C limit. However, by storing at −80° most of the time—which is expected to extend stability (22)—we assume this minor delay to be negligible. In contrast to BHBp, which was measured with two decimals, BHBv was measured with one decimal. BHBv was selected as the main outcome due to its much lower cost compared to BHBp, which enabled more frequent monitoring of ketosis.

A questionnaire, designed for this study, on satiety and tolerance was given immediately after T240 on each study day. Tolerance was assessed by this question: "Did you experience any inconvenience (e.g., nausea, upset stomach), which you attribute to the beverage you were served today?" (a. No / b. Yes, minor inconvenience. / c. Yes, moderate inconvenience. /d. Yes, major inconvenience). If they selected answers b, c, or d, they were asked to give a short description. Participants were informed that they could contact the research assistant and report inconveniences occurring after leaving the study center. Satiety was assessed by one question with five alternative answers, described in **Table 4**. The scales of the questionnaires were considered ordinal.

Our study was approved by The Regional Ethical Review Board in Stockholm, and complies with the Declaration of Helsinki. The study was registered as a clinical trial on ClinicalTrials.gov, NCT03904433.

## Statistical Analyses

The software used for all analyses was STATA 15. Calculations of AUC were performed according to the trapezoidal method. Arms were compared with ANOVA for repeated measures. When comparing specific time points we used a mixed model with *arm* × *minute* as categorical variables, controlling for subject. Correlations were determined by Pearson's *r*. Results are reported in tables and graphs with a significance level of $p < 0.05$, without correction for multiple comparisons. Bonferroni corrected *p*-values are additionally reported in the results text. Log transformation of AUC for the ketone measures increased normality, but the effect on the calculations was very small, so we kept the untransformed variables. After normality check of the other variables we decided to use log transformed values for the BHBp/AcAc-ratio, and the insulin/glucagon-ratio. Test of homogeneity of the variance for our primary outcome indicated no difference between arms 1, 2, 3, 4, & 6. The variance was lower in arm 5, possibly due to a floor effect. For glucose, there was a difference in variance between the glucose and non-glucose arms, but not between arms within those categories. We only report comparisons between the non-glucose arms.

# RESULTS

Data was collected without missing values, with the exception of a questionnaire on tolerance and satiety missing from one participant in arm 6, and a value of AcAc in arm 5, which was replaced by the average of the values before and after (0.15 & 0.18). Only 7 out of 90 values of BHBv at T0, divided among 5 participants, was higher than 0.3 mmol/L. Five of those values were 0.4 and two were 0.6. At T0 there were no significant differences between the non-glucose arms in any of the ketone measures, glucose or the insulin/glucagon-ratio (data not shown).

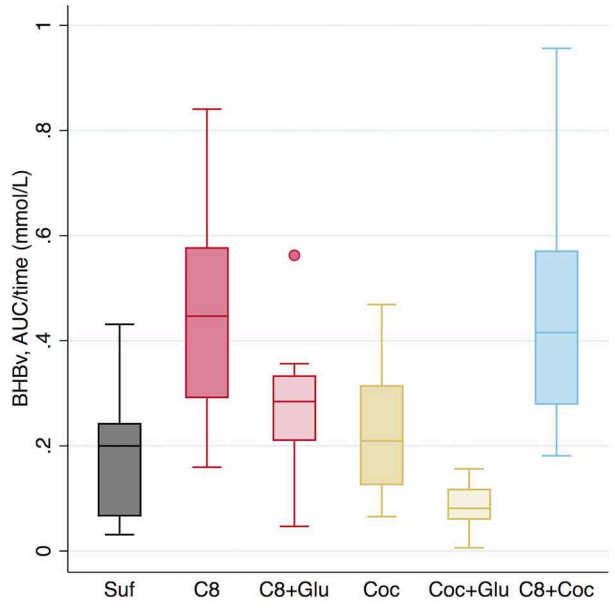


**FIGURE 1 |** BHBv concentrations in blood, AUC/time, (mmol/L) during T0–T240 (minutes). Values are absolute and not adjusted for baseline levels. Suf, Sunflower oil (30 g); C8, Caprylic acid (20 g) + Sunflower oil (10 g); C8+Glu, Caprylic acid (20 g) + Sunflower oil (10 g) + Glucose (50 g); Coc, Coconut oil (30 g); Coc+Glu, Coconut oil (30 g) + Glucose (50 g); C8+Coc, Caprylic acid (20 g) + Coconut oil (30 g). BHBv, β-hydroxybutyrate in venous whole blood.

Our primary outcome, which was absolute levels of AUC (T0–T240) for BHBv, is reported in **Figure 1**. Values can be interpreted as mean concentrations during the 4 h, as AUC is divided by time in the graph. Means and standard deviations of BHBv, AUC/time, for the different arms were as follows: Suf: 0.18 ± 0.11; C8: 0.45 ± 0.19; C8+Glu: 0.28 ± 0.12; Coc: 0.22 ± 0.12; Coc+Glu: 0.08 ± 0.04; C8+Coc: 0.45 ± 0.20 (mmol/L). A description of the effect sizes, using Coc+Glu as a reference (100%), gives the following results: Suf: 216%, C8: 544%, C8+Glu: 336%, Coc: 273%, C8+Coc: 547%. Differences between arms are presented with 95% CI, without correction for multiple comparisons, in **Table 3**. All significant results remained significant ($p \leq 0.003$) after Bonferroni correction was performed, with the exception of Suf vs. C8+Glu and Suf vs. Coc+Glu, for which the significance level was $p < 0.10$. A descriptive graph of the dynamics of BHBv is available in **Figure 2**. A mixed model with *arm* × *minute* as categorical variables, controlling for subject, revealed that C8 was not different from C8+Coc at any time point (all $p > 0.15$). Big individual differences in the ketogenic response to C8 were observed, which is exemplified in **Figure 3** by four individuals selected to illustrate this variation.

To investigate potential bias from differing BHBp/AcAc-ratio in Coc vs. C8, we made a graphical comparison (**Figure 4**) of arms including C8 (2 & 3) vs. Coc (4 & 5) vs. neither or both (1 & 6). Our interpretation of the graph is that there is no difference between the tested oils in their paths, and this was also confirmed by comparing C8 and Coc in a linear regression with the BHBp/AcAc-ratio as the dependent variable. Although the ratio was significantly higher in C8 vs. Coc ($p < 0.001$), this difference disappeared ($p = 0.88$) when BHBp was added as a covariate, to adjust for the fact that the arms have their values distributed in different ranges of ketosis. There was a high correlation between BHBp and the BHBp/AcAc-ratio ($r = 0.81$, $p < 0.0001$; $n = 360$).

Glucose levels in plasma are described in **Figures 5**, **6**. We compared the arms Suf and Coc (low ketosis) vs. C8 and C8+Coc (high ketosis) in a mixed regression model, controlling for repeated measures in the same individual, to investigate if glucose levels were different at any time point between the two groups. Slightly lower levels were observed in the high ketosis group at T240 (−0.17 mmol/l, 95% CI −0.33 • −0.01, $p = 0.04$), but this was not significant after Bonferroni correction. There was no difference at the other time points. Glucose was lower at all time-points T60-T240 compared to T0 in all non-glucose arms ($p < 0.05$, uncorrected). After Bonferroni correction, significance remained at all time points T60-T240 compared to T0 for C8 and C8+Coc, and at T60-T120 compared to T0 for Suf. On an individual level, the change in glucose T0–T60 had a negative association with the simultaneous change in BHBv concentrations ($p = 0.006$).

Sufficient satiety—defined as answer a/b/c in **Table 4**—was reported by 80–93% in the low-caloric arms at T240. Satiety was not higher in the high-caloric arms. Tolerance was in general good according to the questionnaire (**Table 5**). Absence of inconvenience was reported by 80–93% in the low-caloric arms, and by 60–73% in the high-caloric arms. The one report of major inconvenience, and two of the three cases of moderate inconvenience, referred to nausea. The third case of moderate inconvenience was more diffuse, and associated with tiredness. Three cases of diarrhea occurring at a later stage of a study day were reported by participants to the test leaders, and these were not captured by the questionnaire. Two of these were in the Coc arm, and one in the C8+Coc arm. Answers were missing from one participant in the C8+Coc arm.

## DISCUSSION

We observed that ketosis was higher after intake of C8 compared to Coc or Suf. The difference between C8 and Coc was significant both with and without previous glucose intake, but glucose intake attenuated ketosis for both. This attenuation was not observed in C8+Coc (high-caloric) vs. C8 (low-caloric), indicating that it was not driven by increased energy content in general, but specifically by carbohydrates. When Coc was compared to C8+Glu or Suf, the relatively small difference in ketosis was not significant. Blood glucose was mainly stable in the non-glucose arms during the 4 h of testing. Most participants reported good tolerance and sufficient satiety.

Our results are in line with previous findings on changes in ketosis after intake of different fatty acids (11, 12), but adds information on their relative contribution in relation to fasting

**TABLE 3 |** Differences (row-column) in BHBv (AUC/time) between arms during T0–T240 (minutes).

| | Suf | | C8 | | C8+Glu | | Coc | | Coc+Glu | | C8+Coc | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(mmol/L)** | **Difference (95% CI)** | ***p*** | **Difference (95% CI)** | ***p*** | **Difference (95% CI)** | ***p*** | **Difference (95% CI)** | ***p*** | **Difference (95% CI)** | ***p*** | **Difference (95% CI)** | ***p*** |
| Suf | | | −0.27 (−0.35 • −0.19) | <0.001 | −0.10 (−0.18 • −0.02) | 0.02 | −0.05 (−0.13 • 0.03) | 0.25 | 0.10 (0.02 • 0.17) | 0.02 | −0.27 (−0.35 • −0.19) | <0.001 |
| C8 | 0.27 (0.19 • 0.35) | <0.001 | | | 0.17 (0.09 • 0.25) | <0.001 | 0.22 (0.14 • 0.30) | <0.001 | 0.37 (0.29 • 0.44) | <0.001 | 0.00 (−0.08 • 0.08) | 0.97 |
| C8+Glu | 0.10 (0.02 • 0.18) | 0.02 | −0.17 (−0.25 • −0.09) | <0.001 | | | 0.05 (−0.03 • 0.13) | 0.20 | 0.19 (0.11 • 0.27) | <0.001 | −0.17 (−0.25 • −0.09) | <0.001 |
| Coc | 0.05 (−0.03 • 0.13) | 0.25 | −0.22 (−0.30 • −0.14) | <0.001 | −0.05 (−0.13 • 0.03) | 0.20 | | | 0.14 (0.06 • 0.22) | 0.001 | −0.23 (−0.30 • −0.15) | <0.001 |
| Coc+Glu | −0.10 (−0.17 • −0.02) | 0.02 | −0.37 (−0.44 • −0.29) | <0.001 | −0.19 (−0.27 • −0.11) | <0.001 | −0.15 (−0.22 • −0.06) | 0.001 | | | −0.37 (−0.45 • −0.29) | <0.001 |
| C8+Coc | 0.27 (0.19 • 0.35) | <0.001 | 0.00 (−0.08 • 0.08) | 0.97 | 0.17 (0.09 • 0.25) | <0.001 | 0.23 (0.15 • 0.30) | <0.001 | 0.37 (0.28 • 0.45) | <0.001 | | |

*Arm 1: Black was selected to represent the "control" arm.*
*Arm 2 and 4: Red and sand were arbitrarily chosen colors for C8 and Coc.*
*Arm 3 and 5: These arms correspond to 2 and 4, respectively (with glucose added). Colors are therefore the same as 2 and 4, but with lower opacity (in **Figures 2**, **5** they are striped, instead of changing opacity).*
*Arm 6: Blue was chosen arbitrarily as a contrasting color.*

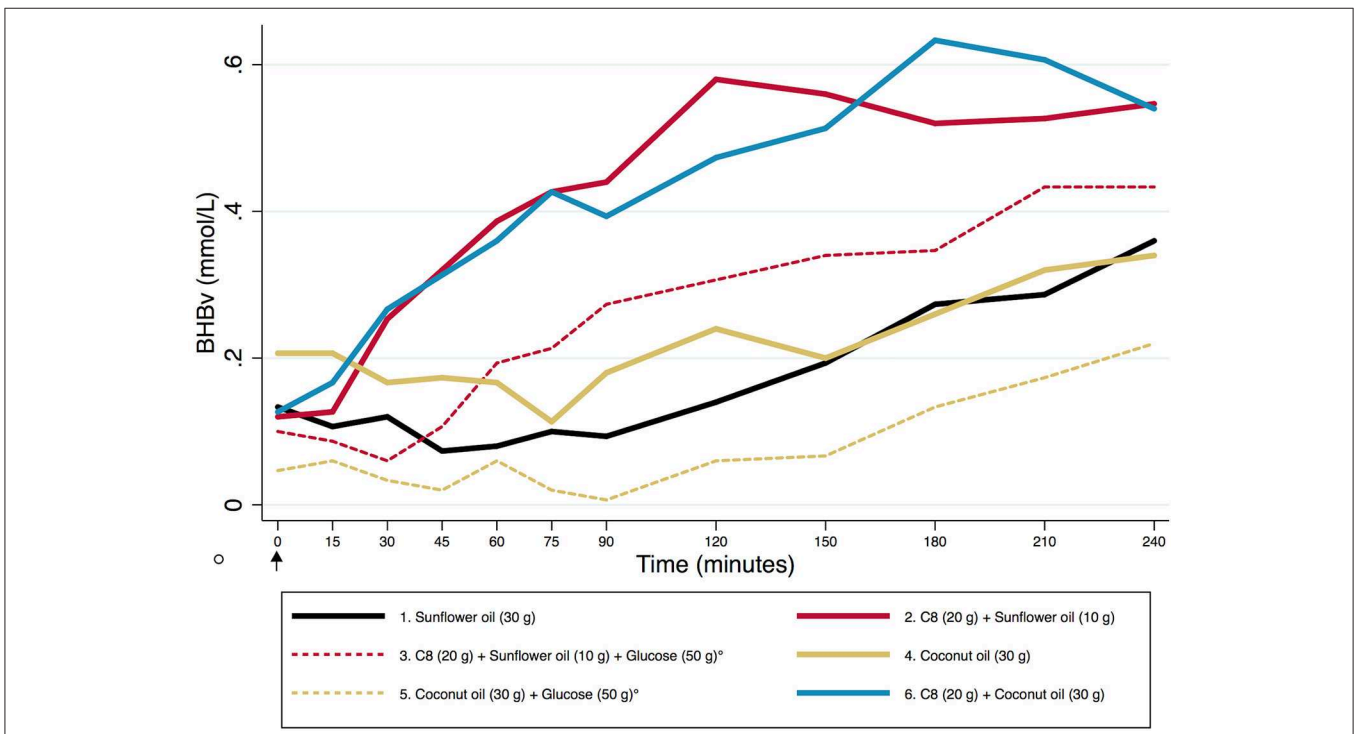


**FIGURE 2 |** The dynamics of BHBv during T0–T240 (minutes). A descriptive graph based on absolute mean values. The arrow at T0 indicates the start of ingestion of coffee with fatty acids. The circle indicates intake of glucose (arms 3 & 5 only, striped lines), 15 min before the blood test performed immediately before T0. BHBv, β-hydroxybutyrate in venous whole blood.

ketosis, and also expands the evidence to include an older sample. Extension of the non-carbohydrate window of an overnight fast—by replacing breakfast with coffee plus any of the tested fatty acids—induced a mild ketosis in the same range as the additional effect of 20 g C8 (on AUC/time). Our results suggest that consumption of MCT-supplements or coconut oil for the purpose of achieving ketosis, is optimized by combining it with time restricted feeding regarding carbohydrates. Whether a 16-h

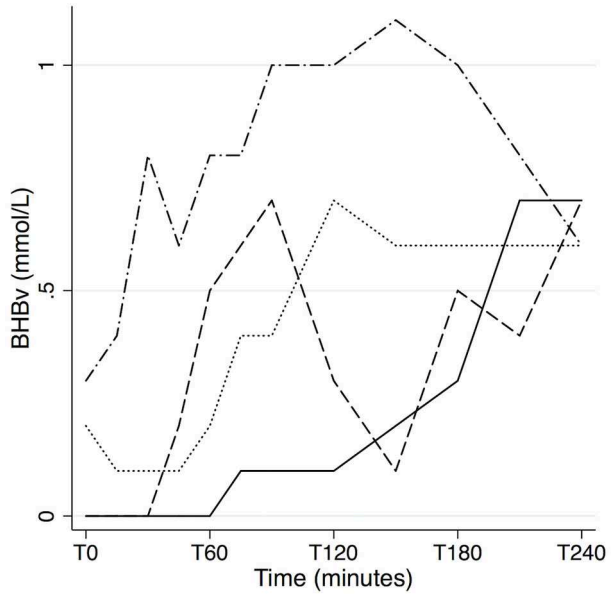


**FIGURE 3 |** Individual differences in the ketogenic response to C8. The paths of four subjects in the C8 arm (20 g Caprylic acid + 10 g Sunflower oil), selected to illustrate the big individual differences in BHBv response, and the potentially transient nature of ketosis. BHBv, β-hydroxybutyrate in venous whole blood.

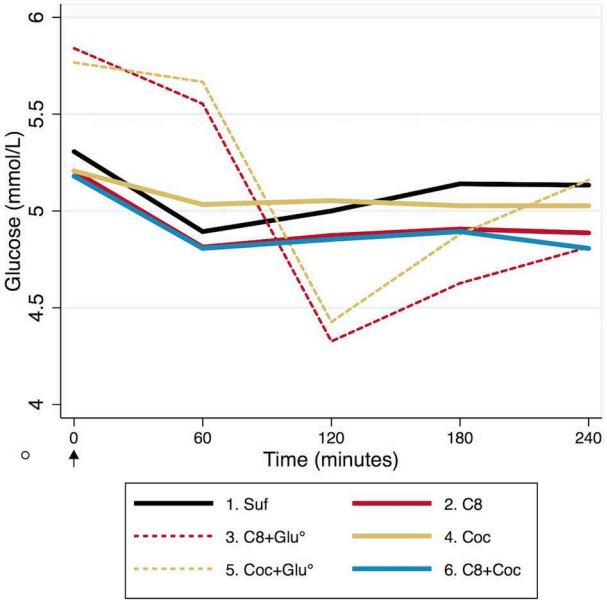


**FIGURE 5 |** Descriptive dynamics of mean blood glucose during T0–T240 (minutes). T0 represent 12 h fasting values, except for arm 3 & 5 (striped), where glucose was ingested 15 min before the blood test performed immediately before T0, indicated by a circle. The arrow at T0 indicates the start of ingestion of coffee with fatty acids (5 mmol/L = 90 mg/dL).

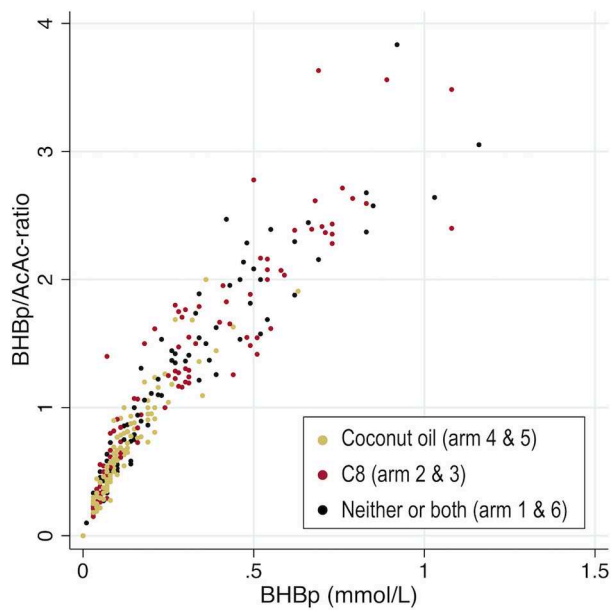


**FIGURE 4 |** The ratio between the two plasma ketones (BHBp/AcAc), plotted against BHBp. Comparisons of arms including coconut oil vs. caprylic acid (C8) vs. neither or both. For enhanced scaling, one case in arm 2 with a ratio of 11 (0.11/0.01), is excluded from the graph. BHBp, β-hydroxybutyrate in plasma; AcAc, Acetoacetate.

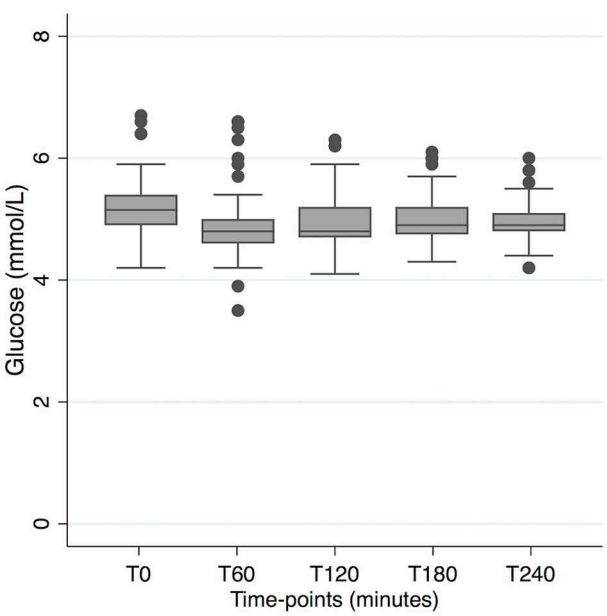


**FIGURE 6 |** Distribution of blood glucose levels in the four arms with a 16-h non-carbohydrate window. T0 represents time for start of ingestion of fatty acids, preceded by 12 h fasting. (5 mmol/L = 90 mg/dL).

non-carbohydrate window distributed at another part of the day would give a similar response on ketosis and satiety, is a question for further studies.

The very similar BHBv paths of C8+Coc and C8 (**Figure 2**) suggest that the metabolization of C8 does not slow down by increased competition from 30 vs. 10 g of other fatty acids.

Whether the observed difference between C8 and C8+Glu is a result of slower metabolization of C8 cannot be determined based on our study design. An alternative interpretation of the observed difference between those arms would be that anti-ketogenic effects (of the glucose intake) on pre-existing ketosis interact with the ketogenic effect of C8, i.e. the metabolization of C8 is not necessarily slower. In our data, such counteracting forces would be in the same range. Also, by comparing Coc with Coc+Glu, a similar anti-ketogenic response to the glucose intake can be observed, although not counteracted by a ketogenic effect by Coc (**Figure 2**). Simplified, three levels of ketosis (BHBv, AUC/time) can be identified in our data: low (0.08 mmol/L), mid-level (0.18–0.28 mmol/L), and high (0.45 mmol/L). At the low level we have Coc+Glu, characterized by including neither C8 nor a 16-h non-carbohydrate window. The observed level corresponds well with what is expected on a "normal" diet that is not carbohydrate-restricted (17), and would be interpreted as absence of nutritional ketosis. At the mid-level we find arms including either C8 or a 16-h non-carbohydrate window: Suf, C8+Glu, and Coc. In those arms BHBv was 120–240% higher compared to low. Finally, at the high level we find arms including both C8 and a 16-h non-carbohydrate window: C8 and C8+Coc. In comparison with low, BHBv here was increased by 450%. Ketosis may ideally be described as a continuous variable, but certain cut-off points may be useful in clinical or scientific settings. BHB $\geq$0.5 mmol/L, measured by capillary finger pricks, has been used as a cut-off for ketosis clinically (23) and as a research outcome (5, 24). As previously reported (21), exploratory analyses on our data revealed that a cut-off at 0.5 for capillary blood corresponded to 0.3 in venous blood (the outcome of the current article). Accordingly, at the mid-level some participants are above the threshold, and at the high level most of them would be defined to be in ketosis. Courchesne-Loyer et al. (25) estimated ketones to supply 8–9% of brain energy at a concentration of 0.29 mmol/L for total ketones, which roughly should correspond to the mid-level arms in our study, with C8 and C8+Coc being somewhat higher. In comparison, after adaptation to a ketogenic diet, BHB may be in the range 0.5–3 mmol/L (6).

A slight decrease in blood glucose between T0 and T60 was observed in all non-glucose arms (**Figure 5**). This response was not stronger for the arms where ketosis was high (C8 and C8+Coc) vs. low (Suf and Coc), suggesting that it was not solely driven by increased ketosis, although a negative association on an individual level was observed between change in BHBv and glucose during the first hour. Thereafter, blood glucose remained stable—slightly below baseline levels—illustrating an expected buffering capacity from liver glycogen, in combination with gluconeogenesis, where the glycerol back-bone from the metabolized triglycerides may have provided substrate (26). In the arms with glucose intake, an expected pattern was also observed: a rise in blood glucose, followed by an under-shoot reaching its minimum at T120, and a subsequent gradual increase back to normal levels at T240. When stable blood glucose is a target of interest, in combination with mild ketosis, coconut oil used in the absence of carbohydrates might be considered as a preferred alternative to C8-supplementation together with carbohydrate intake, as ketosis is not significantly different but blood glucose is more stable. And consequently, C8 in the absence of carbohydrates would be the optimal alternative to combine ketosis with stable blood glucose.

Almost every participant reported sufficient satiety, and interestingly satiety was not higher in the three high-caloric arms. More than half of the participants in the low-caloric arms responded "not hungry" or "lunch can wait" at the end of testing, suggesting that extended ketosis may be easily achieved. Concentrations of BHBv were on the rise—or at a plateau—at T240, which means that if a strict low-carbohydrate lunch had been served at this point, it is likely that a mild ketosis would have continued. Quantification of ketosis in such an 8-h study day could be a target for future studies. If a mild/moderate ketosis is achievable during a substantial part of the day even when dinner is composed *ad libitum*—by time restricted feeding regarding carbohydrates, and the optional supplementation with ketogenic MCT—it would increase flexibility in the implementation of ketogenic diets. In the low-caloric arms, 80–93% of the participants reported "no inconvenience," while the remaining participants reported mild or moderate inconvenience, mainly related to gut tolerance or nausea. In a previous 90-day trial (27), 23% of the participants receiving an MCT-supplement discontinued the study due to tolerance problems, mainly gastrointestinal symptoms. A similar dropout rate was reported in a 6-months trial (19), and both these studies targeted patients with mild cognitive impairment. Daily MCT doses were 20 g and $2 \times 15$ g respectively, and intake was recommended to be with food. In comparison, our results do not suggest a lower level of tolerance in the administration context of our study, but whether this would be sustained in longer-term trials requires further studies. To minimize discontinuation due to tolerance problems, and to avoid a potentially stronger decrease in blood sugar than observed in our study, it may be advisable to limit the dose of C8 to 15–20 g per intake. Other oils and/or cream might be added for satiety, but should not be expected to make a substantial contribution to ketosis. Also, the metabolic responses observed may be different following long-term administration, and this is worth further investigation. Although C8 increased ketone levels, its relative contribution to ketosis would likely decrease in the context of a carbohydrate restricted ketogenic diet, where basal ketone levels are expected to be substantially higher.

Our study has some limitations, which should be considered in the interpretation and generalization of our results. The majority of the participants had normal BMI, glucose and insulin levels. A different ketogenic response might be found in a metabolically unhealthy population. Our choice of coffee as a vehicle was motivated by an intention to study the fatty acids in a real-life context, and not primarily with the goal of investigating the effects of coffee itself. Further studies should investigate possible additive or synergistic effects from caffeine. Increased levels of circulating free fatty acids could be one mechanism, by which caffeine may have boosted the ketogenic response, especially during the last hour investigated in our study, as shown by Vandenberghe et al. (18). Self-reported "sensitive gut" was an exclusion criterion, suggesting that tolerance in the general population of this age might be slightly lower than reported here.

**TABLE 4 |** Self-reported satiety, assessed by a questionnaire directly after T240 (minutes).

| "What is the most suitable description of your hunger?" | Suf | C8 | C8+Glu | Coc | Coc+Glu | C8+Coc |
|---|---|---|---|---|---|---|
| a. Not hungry at all. | 27% | 27% | 13% | 33% | 20% | 13% |
| b. Modestly hungry. Lunch can wait. | 13% | 33% | 33% | 20% | 27% | 33% |
| c. It feels like the right time for lunch. Hunger appeared during the last hour. | 53% | 27% | 33% | 40% | 40% | 27% |
| d. Very hungry. Hunger appeared during the last hour. | 0% | 7% | 13% | 0% | 13% | 13% |
| e. Hungry or very hungry. Hunger has been palpable for more than one hour. | 7% | 7% | 7% | 7% | 0% | 7% |

*Arm 1: Black was selected to represent the "control" arm.*
*Arm 2 and 4: Red and sand were arbitrarily chosen colors for C8 and Coc.*
*Arm 3 and 5: These arms correspond to 2 and 4, respectively (with glucose added). Colors are therefore the same as 2 and 4, but with lower opacity (in **Figures 2**, **5** they are striped, instead of changing opacity).*
*Arm 6: Blue was chosen arbitrarily as a contrasting color.*

**TABLE 5 |** Self-reported tolerance, assessed by a questionnaire directly after T240 (minutes).

| "Did you experience any inconvenience (e.g., nausea, upset stomach), which you attribute to the beverage you were served today?" | Suf | C8 | C8+Glu | Coc | Coc+Glu | C8+Coc |
|---|---|---|---|---|---|---|
| a. No | 93% | 87% | 73% | 80% | 60% | 67% |
| b. Yes, minor inconvenience. | 7% | 13% | 27% | 13% | 20% | 27% |
| c. Yes, moderate inconvenience. | 0% | 0% | 0% | 7% | 13% | 0% |
| d. Yes, major inconvenience. | 0% | 0% | 0% | 0% | 7% | 0% |

*Arm 1: Black was selected to represent the "control" arm.*
*Arm 2 and 4: Red and sand were arbitrarily chosen colors for C8 and Coc.*
*Arm 3 and 5: These arms correspond to 2 and 4, respectively (with glucose added). Colors are therefore the same as 2 and 4, but with lower opacity (in **Figures 2**, **5** they are striped, instead of changing opacity).*
*Arm 6: Blue was chosen arbitrarily as a contrasting color.*

Participants were instructed to keep their usual diet, but we did not collect data on their actual food intake. However, baseline ketones (with a small number of possible exceptions) indicated that they were not on a low-carbohydrate diet. Our main outcome, BHBv, only show concentrations with one decimal, but considering its high agreement with the laboratory test (21), this shortcoming should be outweighed by benefits of the large number of time-points to capture the transient dynamics of ketosis. Another strength of our study is the complete collection of rich data, assessed under well-controlled conditions.

Previous studies (11, 12) have reported that coconut oil, C12 and C8 were associated with different ratios between the two blood ketones BHB and AcAc, raising interesting questions on potential variations in their metabolism. If such a difference is present, interpretations may be biased when only BHB is used to assess ketosis, as for the primary outcome of our study. However, our data does not support the hypothesis that the diverging ratios are caused by properties of the oils. Rather, we interpret the differing ratios as a secondary consequence of varying distribution along the range of ketosis, which in itself correlates strongly ($r = 0.81$, $p < 0.0001$) with the BHB/AcAc-ratio. This view is also supported by a visual interpretation of **Figure 4**, and consequently we do not consider our comparisons of Coc and C8 to be biased.

Differences between MCFA and LCFA have been shown at different stages of their uptake and metabolization. After intestinal uptake LCFA are incorporated into chylomicrons and transported through the lymphatic system, while MCFA can be transported to the liver via the portal vein. Ketogenesis predominantly takes place in liver mitochondria, and while MCFA can diffuse freely through the inner mitochondrial membrane, LCFA require enzymatic help from carnitine (28). The division between the alternative pathways mentioned above is best described as a proportional, rather than an absolute choice. Nevertheless, Dayrit (10) concluded after reviewing the evidence, that all MCFA in the range C6–C12 are metabolized differently from LCFA, both regarding intestinal uptake and mitochondrial entry. The specific ketogenic properties of C8—which in our data are distinct from C12, and according to previous findings (12) also from C10—thus calls for an explanation beyond the aforementioned metabolic shortcuts. Different pathways for beta-oxidation have been suggested to underlie these variations (29).

Our results should enhance the interpretation of previous and future studies on supplementation with MCT or coconut oil. We demonstrate, in line with Vandenberghe et al. (12), that it is not valid to generalize findings on C8 to coconut oil. Another important finding is that ketosis from C8 may differ substantially depending on timing and macronutritional composition of the overall food intake. The influence on brain health by MCT and coconut oil may not be limited to ketogenic pathways, as reviewed by Augustin et al. (30) and Fernando et al. (31) respectively. For the mechanistic interpretation of interventions, it therefore should be of great interest to determine to what degree participants actually achieve ketosis. Ideally this should be determined by blood testing, but when such data are not available our results can provide a rough estimation of ketosis under different conditions. For example, we show that it is unlikely that

coconut oil, especially when added to a carbohydrate rich diet, would induce significant ketosis. Acute effects on cognition after intake of MCT (mainly C8) have been studied with conflicting results (32, 33). The large individual differences in magnitude and timing of the ketogenic response in our study (illustrated by **Figure 3**), illuminate the difficulty to detect potential acute effects. The observed opposing effect on blood glucose by BHB further complicates interpretation.

In combination with the observed signaling functions of BHB (9), the hypothesis that ketones could compensate for glucose hypometabolism in Alzheimer's disease and normal aging (3) provides a theoretical framework to motivate ketogenic interventions in the field of cognitive health. As small ketogenic diet interventions (34, 35), as well as interventions with MCT-supplements (19, 27), have shown promising results, the next step to advance the field would be to conduct larger clinical trials in the range of 3–6 months. Combining different ketogenic mechanisms like carbohydrate restriction, scheduled eating windows and MCT-supplementation, could be a strategy to enhance flexibility and adherence.

In summary, we conclude that C8-supplementation and time restricted feeding regarding carbohydrates provide two, optionally additive, strategies to achieve a mild ketosis in the context of otherwise food intake *ad libitum*. Our results on satiety and tolerance suggest that the strategies can be further investigated in longer-term ketogenic diet interventions, where they could potentially enhance compliance, by allowing a less strict carbohydrate restriction in total. In the absence of carbohydrate intake during 16 h, we observed that blood glucose remained stable in older adults. And finally, the heterogenic ketogenic properties of different MCFA highlight the importance of not using the terms MCT/MCFA without definition.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The study involving human participants was reviewed and approved by the Regional Ethical Review Board in Stockholm, Sweden. The participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JN, SS, AS-M, UA, KN, SR, and MK conceived and designed research. KN performed experiments. JN, SS, AS-M, IK, and MD analyzed data. JN, SS, AS-M, IK, TN, and MK interpreted results of experiments. JN prepared figures and drafted manuscript. All authors edited and revised the manuscript, and approved the final version.

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REVIEW
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# Feeding Rhythms and the Circadian Regulation of Metabolism

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The molecular circadian clock regulates metabolic processes within the cell, and the alignment of these clocks between tissues is essential for the maintenance of metabolic homeostasis. The possibility of misalignment arises from the differential responsiveness of tissues to the environmental cues that synchronize the clock (zeitgebers). Although light is the dominant environmental cue for the master clock of the suprachiasmatic nucleus, many other tissues are sensitive to feeding and fasting. When rhythms of feeding behavior are altered, for example by shift work or the constant availability of highly palatable foods, strong feedback is sent to the peripheral molecular clocks. Varying degrees of phase shift can cause the systemic misalignment of metabolic processes. Moreover, when there is a misalignment between the endogenous rhythms in physiology and environmental inputs, such as feeding during the inactive phase, the body's ability to maintain homeostasis is impaired. The loss of phase coordination between the organism and environment, as well as internal misalignment between tissues, can produce cardiometabolic disease as a consequence. The aim of this review is to synthesize the work on the mechanisms and metabolic effects of circadian misalignment. The timing of food intake is highlighted as a powerful environmental cue with the potential to destroy or restore the synchrony of circadian rhythms in metabolism.

**Keywords: circadian, metabolism, time-restricted feeding, fasting, high fat diet, ketogenic diet, peripheral clock**

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## INTRODUCTION

The rotation of the Earth produces daily and seasonal changes in the environment to which organisms have become adapted. The self-sustaining oscillations in physiology produced in approximation of the 24 h daily cycle that evolved to exploit these changes are referred to as circadian rhythms (from the Latin *circa* and *diem*, about a day). The anticipation of reliable patterns in the environment evolved through over two trillion day–night cycles (1). Circadian biology is pervasive from protozoans, cyanobacteria and algae to plants, fungi, and animals (2), and the molecular mechanisms that regulate it are similar among the kingdoms of life, indicating more than 500 million years of positive selection (3). The present review focuses on the mammalian system. The selective pressures under which the circadian rhythms evolved were fundamentally linked to the period of specific environmental oscillations, such as light/dark, which became cues for the *entrainment* of the endogenous clock period. It is the reliable prediction of regular environmental changes that make circadian biology advantageous. Rhythms misaligned to the environment are not only neutral but detrimental to organismal fitness (4). For example, mice with an endogenous period substantially shorter than 24 h (due to the *tau* mutation in casein kinase 1ε) have greatly reduced fitness (5).

Within an organism, circadian rhythmicity is pervasive at all levels of organization, from intracellular molecular networks of transcription and translation to the neuronal networks that produce rhythms at the behavioral level and even the coordination of social activities and reproduction. The most important feature of an endogenous clock is the *phase relationship* between the rhythms it generates and those of the external environment. In peripheral tissues with primarily metabolic functions, the most important phase relationship is between their rhythmic processes and environmentally dictated daily fluctuations in energy input and requirement. Cellular energy demands vary temporally, which requires regulators to orchestrate the oscillations in metabolism (6). Moreover, rhythmic cellular respiration produces metabolites that become damaging if they accumulate, which necessitates synchronized compensation mechanisms, such as reactive oxygen species (ROS) scavengers (7, 8). Opposing biochemical pathways must also be kept separate, and in addition to sequestration in subcellular compartments, this is achieved through temporal separation with inhibitory feedback loops (9). For example, the hepatic clock directly regulates a daily switch between dark phase glycogenesis and light phase glycogenolysis in mice (10).

Most food consumption and physical activity occur during the animal's active phase of the 24-h cycle. For humans and other diurnal species this corresponds to the light phase, and for nocturnal species such as mice, to the dark phase. In both nocturnal and diurnal mammals, the molecular architecture of the circadian system involves the same components, which oscillate in a similar phase with an approximately 24-h period; the opposite phase of activity is the output of homologous circadian physiology. The core molecular clock is also the same across tissues, although the outputs and regulation of the clock are tissue-specific. Food intake is an important synchronizing cue for the clocks of many tissues with essential metabolic roles, and these will be the focus of the present review.

## THE MAMMALIAN MOLECULAR CLOCK

In mammals, the autonomous oscillator that produces the circadian rhythms observable at all levels of biology is found at the molecular level. The molecular clock is a highly conserved transcription–translation feedback loop in which transcriptional activators drive the production of their own repressors in an ∼24 h cycle (11). The core clock feedback loop is described briefly in **Figure 1** and was recently reviewed in detail by Takahashi (13).

Epigenetic mechanisms make the metabolic interactions with this core clock highly plastic. Exposing or condensing E-box regions control CLOCK:BMAL1 binding and the ability to regulate entire networks (14). Numerous clock-controlled genes (CCGs) are key metabolic regulators, a topic that has been extensively reviewed (9, 15, 16). Among the CCGs involved in metabolism, a subset directly feeds back into the clock, producing the bidirectional regulation of metabolism and circadian rhythms, which includes all members of the PPAR family [α, β/δ, and γ; (17)], PGC1α, and AMPK (15). The interaction of the clock and nutrient-dependent metabolic regulators is so tight that a distinction between their rhythmic transcriptional outputs is somewhat arbitrary (18). Other direct circadian output genes that act as non-essential modulators of the clock rhythm and have known metabolic functions include the PARbZip transcription factors Dbp, Hlf, Tef, and the repressor Nfil3 (19) as well as Dec1 and Dec2 (20).

## ORGANIZATION AND ENTRAINMENT OF THE CIRCADIAN SYSTEM

The molecular oscillator maintains a phase that closely ∼24 h in free-running conditions (without external input), but its *free-running period* does not perfectly match the external day. What allows the circadian system to keep accurate time with the external environment is its plasticity: the clock undergoes daily resynchronization to rhythmically occurring environmental cues called *zeitgebers* ("time givers"). The most reliable daily rhythm in the environment, and therefore a universal zeitgeber across all phylogenetic levels, is the cycle between light and dark.

### The Central Circadian Clock

The mammalian circadian system is classically depicted as having a hierarchical organization with a "master clock" oscillator in the suprachiasmatic nucleus (SCN) of the hypothalamus that synchronizes the phases of all the other molecular clocks in the body (21). Specialized melanopsin-expressing photoreceptor cells in the retina, known as "intrinsically photosensitive retinal ganglion cells," transduce incoming light and relay this information to the SCN via the retinohypothalamic tract (22). Light causes phase delays in the clock when it is present early in the night, or phase advances when it occurs late at night (23, 24), resulting in effective synchronization of the SCN clock to the external light/dark cycle.

### Synchronization of Peripheral Clocks by the SCN

In addition to the master clock in the SCN, nearly every cell in the body has an autonomous clock (25) which has the components described in **Figure 1**. The network of pacemaker neurons in the SCN acts as the orchestrator of these self-sustaining oscillators throughout the body, relaying the light-entrained phase by various mechanisms (**Figure 2**). These include the regulation of core body temperature as well as direct communication through autonomic innervation and endocrine signaling (26), primarily through adrenal glucocorticoids and pineal melatonin (27). As outputs of the master clock, fluctuations in body temperature, plasma cortisol, and melatonin rhythms are used to gauge its phase.

The other important mechanism by which the central and peripheral clocks are aligned is through SCN-generated behavioral rhythms (28), including wake/sleep, activity/rest, and feeding/fasting (**Figure 2**). Indeed, the SCN is necessary to drive behavioral and feeding rhythms. Early studies of SCN lesions (29) and recent SCN-enriched selective knockouts of clock genes (30, 31) confirmed that without a functional central clock, animals in constant darkness are arrhythmic in their

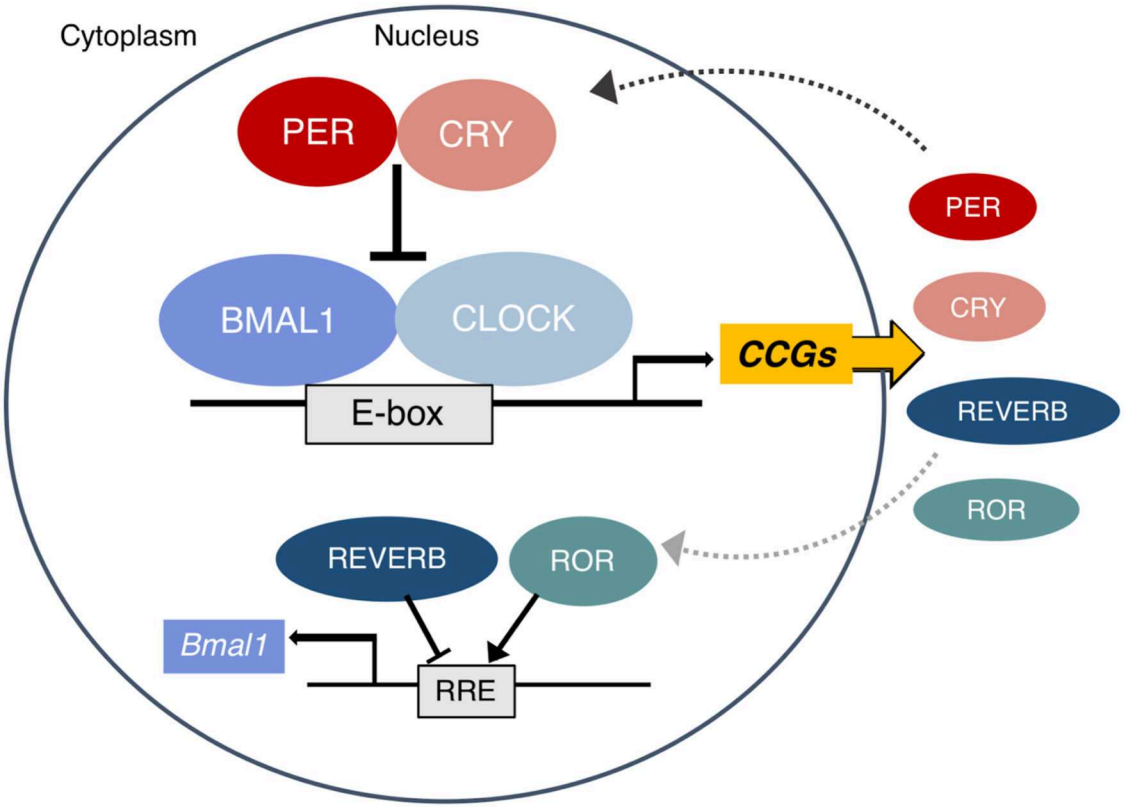


**FIGURE 1 |** The mammalian molecular clock. The complex of BMAL1 and CLOCK rhythmically binds E-box elements to activate the transcription of clock-controlled genes (CCG), including other parts of the core clock which form the negative arm of the feedback loop. The PER and CRY proteins translated from *Period* (1–3) and *Cryptochrome* (1, 2) translocate to the nucleus and inhibit the activity of CLOCK:BMAL1. Eventually they are tagged for proteasomal degradation and the cycle begins again approximately every 24 h. In a secondary loop, the protein products of the *Rev-erbs* (α and β) and *RORs* (α, β, and γ) inhibit and activate *Bmal1* transcription, respectively. Other CCGs are involved in the pathways of metabolic regulation. A subset of these (including PPARs, PGC1α, AMPK, and NAD+ dependent SIRT1) in turn also regulate the clock. Figure adapted with permission from Gaucher et al. (12).

activity and consume similar proportions of food during both light and dark phases. Along with lost activity rhythms, SCN lesioning flattens rhythms in energy expenditure which disturbs energy balance even without differences in total expenditure (32). The SCN-generated rhythms in activity and food intake produce feedback that strongly entrains the clocks of other tissues. When adrenal hormone output (direct SCN control) and food intake rhythms (indirect behavioral control) are eliminated, rhythmic clock gene expression in the liver and white adipose is lost, along with metabolic output genes and adipokines (33, 34).

## Entrainment of Central and Peripheral Clocks

Light and dark cycles are the dominant zeitgeber of the master clock in the SCN, whereas the rhythm of feeding behavior is arguably the most powerful zeitgeber of the peripheral tissues. In a classic study, Damiola et al. (35) showed that restricting food access to the inactive phase in mice (i.e., the light phase) caused a phase shift in the peripheral tissue clocks of the liver, pancreas, heart, skeletal muscle, and kidney. In contrast, the central clock in the SCN was unaffected. This uncoupling between the central and peripheral clocks occurred equally when the animals lived with normal light–dark cycles or in constant darkness (35). Feeding is therefore an ineffective zeitgeber for the SCN even in the absence of light, its primary zeitgeber. At the same time, feeding is a potent zeitgeber for the periphery regardless of opposing cues from the light-entrained SCN clock. Further studies confirmed that the food exerts a dominant zeitgeber effect on peripheral clock phase without affecting the SCN (36–40). Furthermore, phase advancing the light–dark cycle does not alter the phase of the liver clock if accompanied by a fixed restricted feeding schedule, and restricted feeding entrains the liver even in SCN-lesioned mice (37).

Nevertheless, the peripheral clock phase is still indirectly controlled by the light-entrained SCN. This is demonstrated by the fact that when feeding rhythms and the light phase are inverted, peripheral clock resetting is slowed by opposing signaling from the SCN. In nocturnal rodents who normally consume most food during the dark phase, restricting the feeding schedule to the day produces slow phase resetting of the liver and kidney to match feeding rhythms, with comparatively rapid resetting when returning from day to nighttime eating; this effect of slowed switching in the 'wrong direction' does not occur

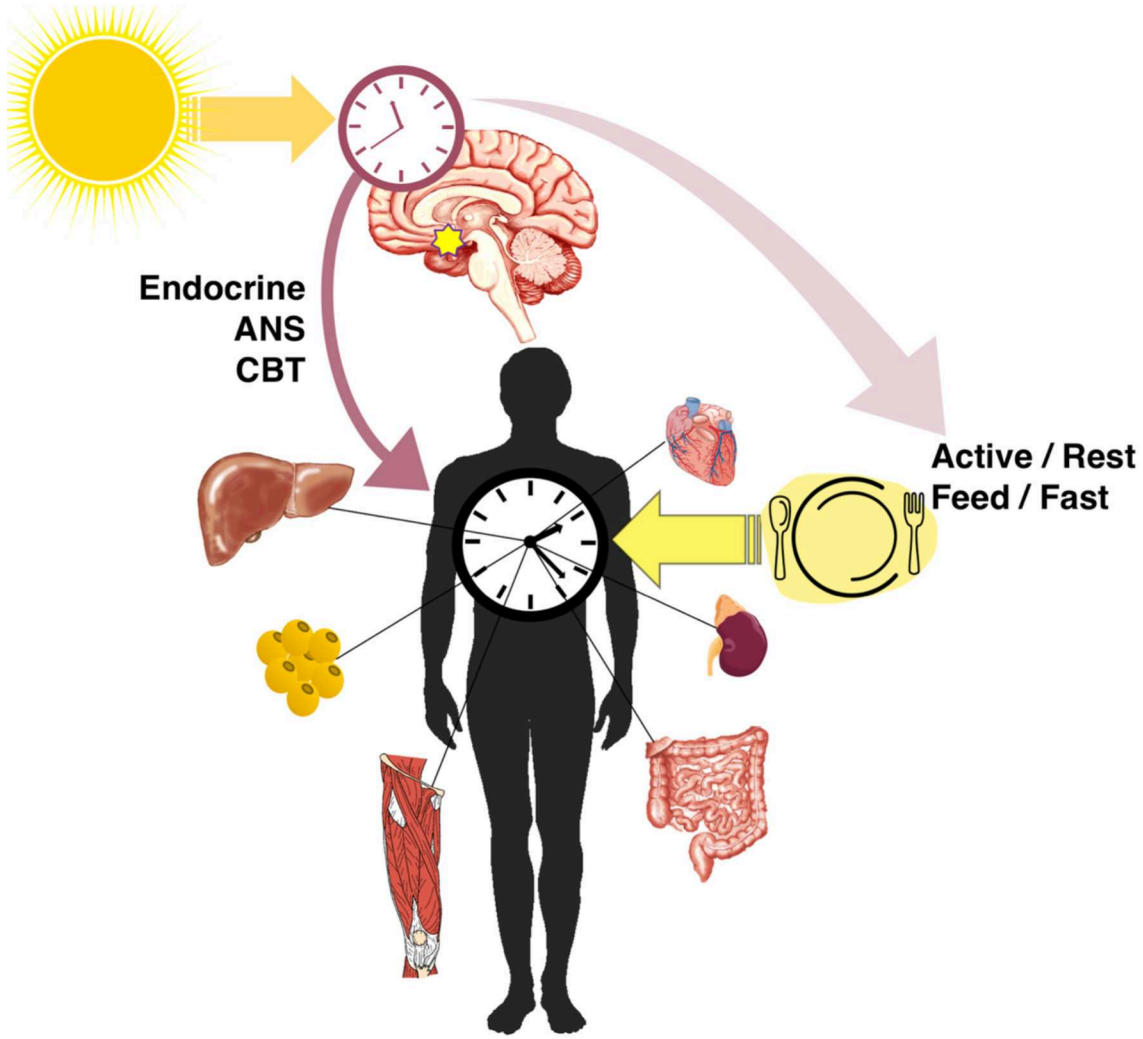


**FIGURE 2 |** Entrainment and alignment of the SCN and peripheral clocks. The primary zeitgeber of the SCN oscillator is light, whereas peripheral clocks are strongly entrained by feeding/fasting rhythms. The SCN synchronizes peripheral rhythms directly through endocrine and autonomic nervous system (ANS) signals and the regulation of the core body temperature (CBT), as well as indirectly through behavioral feedback from rhythms in activity and feeding.

in adrenalectomized or glucocorticoid insensitive mice (41). That the master clock acts to counteract discordant entrainment of peripheral clocks by light phase feeding was confirmed by *in vivo* recording of *Bmal1*-driven luciferase activity in the hepatocytes of SCN-lesioned mice. After only 6 days of daytime restricted feeding, the hepatocytes in the SCN-lesioned mice had undergone a steady and complete phase shift to match the food intake rhythm, in contrast to the sham-operated mice where the hepatocyte clock had only partially phase-shifted this short time (42).

The differential responsiveness of tissues to a particular zeitgeber makes evolutionary sense because the component of an endogenous clock presented to natural selection is its phase relationship with respect to the environmental events it anticipates. As tissues become specialized in their function, the relevance of environmental cues as zeitgebers would be aligned accordingly. For example, in a metabolically active organ such as the liver, regularly timed food intake is an important event with which metabolic processes are synchronized. Among the peripheral organs studied in the literature, the liver clock phase shifts the most rapidly in response to restricted feeding (35), corresponding to its primary metabolic function. It is interesting that while SCN is not sensitive to time-restricted feeding, it does respond to long-term fasting and caloric restriction. The SCN phase shifts in response to caloric restriction (43) and synchronizes to a single hypocaloric meal given at the same time each day in constant darkness (44). Whereas, acute differences in food intake timing are not relevant zeitgebers to the SCN, perceived starvation certainly is.

## CIRCADIAN DISRUPTION AND METABOLIC DYSFUNCTION

Under normal physiological conditions, the light-driven SCN rhythm and feeding-driven peripheral rhythms are aligned. However, light generally has only indirect effects on peripheral clocks through signals from the SCN, and feeding behavior resets the phase of peripheral clocks without shifting the SCN clock phase, which produces the possibility of misalignment between the central and peripheral rhythms. The sustained discordance of feeding time with the activity phase governed by the central pacemaker was shown to result in peripheral uncoupling in rodents (35). As we will see, the modern food environment and socially enforced behavioral rhythms produce similar effects in humans today with profound metabolic consequences.

### Mouse Models

The causal relationship between the disfunction of the clock and metabolic disease is clearly demonstrated in the phenotypes of circadian mutant mice. It was first observed that deletion of *Bmal1* led to severe glucose dysregulation (45), and perturbation of metabolic homeostasis was subsequently found in mice mutated in other clock components. There is significant phenotypic variation depending on the clock component mutated, but what all have in common discernable metabolic dysregulation (46). It is likely that the clock function, rather than any other function of these particular genes, is at the root of the metabolic dysregulation because a similar result is observed repeatedly in the ablation of various clock components (18). The converse case of circadian disruption in models of metabolic disease is also observed: the loss of feeding rhythms precedes the development of obesity in leptin deficient *ob/ob* mice (47, 48) and in diet-induced obesity models (49, 50). Moreover, the effects of genetically "breaking" the clock by knocking out a key component in mice are in many cases similar to the effects of chronic stress on the circadian system through behavioral misalignment in humans.

### Humans

#### Shift Work

There is now a substantial body of evidence for the role of disrupted circadian rhythms in metabolic disease pathogenesis. Epidemiological studies on long-term shift workers were the first to demonstrate the connection between circadian misalignment (51) and metabolic disease (52–54). Mechanistic support in animal models of shift work was reviewed by Opperhuizen et al. (55). In a classic demonstration of the effects of misalignment in humans, a 10-day laboratory protocol forced desynchrony with a 28-h day, resulting in widespread alterations in the rhythms of plasma leptin, glucose, insulin, and cortisol levels (56). In 3 out of 8 subjects, just 10 days of misalignment induced a prediabetic state.

Indeed, acute circadian disruption in a laboratory setting reliably alters glucose metabolism and induces a diabetogenic state in humans [reviewed in (57, 58)]. In a randomized trial simulating night shift work, 3 days of a phase inversion (i.e., awake and eating during the inactive phase) showed significant impairment in insulin sensitivity and greater postprandial glucose rise in response to the same meal (59), and after 6 days of phase inversion, plasma proteomics revealed alterations in the rhythms of many proteins known to regulate glucose homeostasis, along with much higher postprandial glucose and insulin (60). The plasma glucagon rhythm was inverted to match feeding schedule, but importantly was also elevated under nighttime food intake, which itself is a risk factor for diabetes (61). Elevated glucagon is potentially explained by the stimulatory action of melatonin (which peaks at night) on pancreatic alpha cells (62). Finally, observational evidence supports that chronic circadian disruption impairs glucose homeostasis and increases disease risk, with shift work increasing risk of developing Type 2 diabetes (63, 64).

The composition of the diet and energy intake during night shifts is unlikely to be the major contributor to metabolic disruption. A recent survey of eating patterns in night shift workers showed that, except for slightly higher sugar and lower saturated fat consumption during the night shift, there was no difference in caloric intake or adherence to dietary guidelines compared with day shifts or days off (65). This minor shift toward carbohydrate intake compared with fat intake during night shifts is consistent with data showing that improperly timed feeding in mice resulted in inefficient energy utilization and greater reliance on carbohydrate oxidation (66), an unfortunate combination with the impaired glucose handling induced by circadian misalignment.

The greatest change in nutrient input during nightshifts though is in its timing, and this can in itself contribute to metabolic disruption. During nightshifts meals are spread out over the 24 h period, as compared to dayshift or off days where the fasting window generally approximates sleep time (65, 67). Therefore, like in laboratory simulations of shift work with controlled isocaloric meals, the main variable with respect to food intake is its timing with respect to the endogenous clock. Notably, the eating pattern of shift workers on their days off, where fasting duration approximates sleep duration, has also been observed in the general population. Data collected from a smartphone application showed that the majority of healthy adult participants ate at random intervals in a window of 14–15 h (68). The tendency in modern humans is to shorten the overnight fast by continuing consumption into the inactive phase of the circadian period; when food is constantly available, the only constraint on eating is to be awake. Impressively, even modestly condensing the feeding window to 10–11 h led to weight loss and lower plasma insulin levels, as self-reported through the application (68). The effects of meal timing and time-restricted feeding (TRF) are explored in greater detail in section Effects of Meal Timing.

#### Social Jetlag

Shift workers perhaps represent the extreme end of circadian misalignment, but they are not alone in their vulnerability to its deleterious effects. Constant food availability, reduced overall sleep, and longer active hours are common to the modern lifestyle [reviewed in (69)]. Many people experience a chronic mismatch between their endogenous circadian rhythms and

socially dictated rhythms of behavior, such as those enforced by work or school start times. The discrepancy between internal and imposed rhythms can be quantified by the difference between the sleep midpoint on work and work-free days. This phenomenon, termed *social jetlag*, is experienced by as much as 87% of the day-working population (4). Distinct from shift work, social jetlag has also been independently associated with obesity (70), the risk of developing T2D (71), abdominal adiposity, and metabolic syndrome (72).

## Common Disruptive Factors

At least three factors are common to the circadian disruption seen with shift work and social jetlag; (1) light exposure during the dark phase; (2) food intake during the inactive phase; (3) sleep disruption. Indeed, sleep duration alone is strongly associated with the development of metabolic syndrome and accounts for a large proportion of the cardiometabolic risk in shift workers (73). Sleep deprivation has also been shown experimentally to reduce insulin sensitivity (60, 74) and has been strongly linked to development of obesity (75, 76) and diabetes (77). Although the harms of sleep deprivation are manifold, these will not be discussed in detail in the present review. Sleep is not itself a zeitgeber, but rather an output of the SCN clock (78) as well as a modulator of responsiveness to zeitgebers such as light (79). Importantly, while sleep deprivation alone induces insulin resistance in controlled laboratory experiments, circadian misalignment causes additive impairment of insulin sensitivity even with sleep time kept constant at a meager 5 h (80). When considering circadian misalignment, it is what we do when awake at the wrong time, i.e., eating and light exposure, that matters for clock entrainment.

### *Light at night*

As previously described, light is the most powerful zeitgeber of the master clock in the SCN. In a population of healthcare workers, the phase shift of the master clock after 3–4 consecutive nightshifts could be 71% accounted for by intensity of light exposure according to the individual's baseline phase response curve (81). The SCN clock in turn regulates system wide energy metabolism as well as activity and food seeking behavior in alignment with these light-entrained rhythms (82). This finding helps to explain why, independent of other lifestyle factors (e.g., sleep duration, physical activity, and smoking), light at night (LAN) has been correlated with the increased risk of developing obesity in humans (83) and has been shown to acutely induce glucose intolerance in rats (84).

### *Mistimed food intake*

Mechanistic studies in animals point to shifts in food consumption as a potential underlying cause of this correlation between LAN and obesity. Mice exposed to LAN gained weight and had impaired glucose tolerance, despite equivalent calorie intake and daily activity, because their food consumption was redistributed into the rest phase (85). This mistimed food intake results in peripheral misalignment. After 8 weeks of a rotating light schedule consisting of 3 days of normal light/dark (L:D 12:12) followed by 4 days of reversal (D:L 12:12), mice lost daily rhythms in fuel utilization and energy consumption, leading to greater weight gain, elevated blood glucose and lipids, and hepatic steatosis (86). In sum, mistimed food intake can account for a large proportion of the deleterious metabolic effects of light-induced circadian misalignment.

Metabolomics studies support the strong effects of food intake on rhythmic metabolism in humans. In constant conditions of enforced posture, dim light, sleep deprivation, and hourly isocaloric meals, ∼15% of the metabolites in saliva and plasma were found to have a circadian rhythm, with most in saliva being amino acids, and most in plasma being lipid metabolites (87). Among these were prominent rhythms in free fatty acids and triglycerides, which peaked during the light phase; since they were independent of feeding or rest-activity cycles, this suggested endogenous circadian oscillators control lipid metabolism. In contrast to the approximately 15% metabolites that were rhythmic independent of feeding rhythms, when participants were fed normal meals (i.e., three daily meals plus a snack), 60–70% of metabolites became rhythmic, and most of these retained rhythmicity during constant wakefulness (88). Thus, rhythmicity is gained in as much as half the human metabolome through rhythmic feeding, while sleep/wake rhythms have comparatively little effect.

It is worthwhile to note that it is possible some metabolite rhythms newly observed with meals are properly thought of as being disinhibited by the introduction of fasting periods in this schedule compared to the hourly meals in constant conditions. Nutrient-sensing pathways active during periods of fasting, such as AMPK and the Nampt/NAD+/Sirt1 feedback loop (89), are intertwined with the core clock feedback loop and thereby affect widespread outputs of circadian metabolic regulatory mechanisms (15). Constant conditions were used to observe free-running endogenous rhythms in the absence of zeitgeber cues, but both food intake and fasting can serve as cues to the clock. The light-entrained SCN clock is well-understood to have a different free-running period under constant dark (DD) or constant light (LL) conditions (90). Similarly, it would be worthwhile to investigate the rhythms in metabolites thought to be outputs of food-entrained peripheral clocks under constant fasting as well as constant feeding conditions.

Behavioral rhythms are the predominant drivers of metabolite rhythms, which is reflected by phase shifts in metabolite rhythms during shift work. Targeted plasma metabolomics showed phase shifts in 95% of rhythmic metabolites when measured under constant routine following 3 days simulated shiftwork, with many metabolites following the 12 h phase inversion of behavior (activity and feeding) from the preceding 3 days (91). In another metabolomics investigation of simulated shift work with a 10-h delay of behavior, 75% of oscillating plasma metabolites were phase-shifted with an average phase delay of 8.8 h, although there was high interindividual variability (92). Urine metabolite rhythms were also altered in night shifts, particularly in individuals with an early chronotype [defined by the midpoint of sleep; (93)], as was the plasma proteome (60). Importantly, SCN clock outputs (i.e., melatonin and cortisol) were consistently found *not* to be phase-shifted in laboratory-simulated shift work (91, 92, 94) or in most permanent shift workers (95), thereby

producing the anticipated misalignment with the periphery. The timing of food intake is unquestionably a powerful stimulus for peripheral circadian entrainment, having the potential to cause misalignment of metabolic processes. Of the deleterious modern lifestyle factors, it is also the most amenable to intervention.

# EFFECTS OF MEAL TIMING

## Potential Benefits of Early Meals

Due to the differing responsiveness of tissue clocks to zeitgebers, it is possible for phase misalignment to occur when these cues are not presented together at their usual times. This is one way by which food intake during the dark phase can result in systemic metabolic dysregulation. An additional mechanism by which food intake timing can alter metabolic function comes from the circadian regulation of homeostatic responses to nutritional challenge. The same meal consumed at different times of day can produce distinct responses because of circadian variations in energy uptake and utilization (58, 96), and the master clock of the SCN controls the endocrine response to nutrient intake. For instance, the same meal eaten at 8 a.m. instead of 8 p.m. produced greater postprandial epinephrine and norepinephrine and less acylated ghrelin (97). These observations indicate that because of the circadian regulation of food intake response, the timing of the feeding period is a decisive factor in nutrient handling.

In this respect, recent evidence has reinforced the advice of Maimonides, the Jewish philosopher and doctor (also called Rambam, ca.1135): "Eat like a king in the morning, a prince at noon, and a peasant at dinner." Indeed, those who consume most of their daily calories at dinner are at the increased risk of developing obesity and metabolic syndrome (98, 99). In a 20-week weight loss trial, subjects with an overall food intake later in the day lost less weight and had slower weight loss despite similar caloric intake, energy expenditure, and sleep duration (100). Similarly, a randomized control trial of calorie timing during weight loss found the "big breakfast group" (200/500/700 kcal rather than 700/500/200 kcal in three daily meals) lost more weight and inches around their waist. The big breakfast group also had a greater decrease in fasting glucose, insulin, and insulin resistance, while reporting greater satiety, lower hunger, and having concordantly lower ghrelin levels (101). Moreover, skewing energy intake toward breakfast rather than dinner in patients with T2DM reduced postprandial hyperglycemia and average glucose while increasing insulin and incretin levels (102). Taken together, caloric intake weighted toward the beginning of the active phase (i.e., a big breakfast and small dinner) may confer a metabolic advantage, especially in the context of weight loss, by coordinating food intake to coincide with the circadian timing of nutrient handling.

## Entrainment of Peripheral Clocks to Meal Timing

Similar benefits of early meal timing have also been observed in healthy subjects. In a highly controlled in-lab crossover experiment, healthy young men ate identical meals at 5-h intervals beginning either 0.5 h (early schedule) or 5.5 h (late schedule) after waking. The participants were acclimated to early meals for 3 days and then switched to late meals for 6 days. The circadian rhythms of the participants were measured after each meal schedule in a 37-h constant routine (i.e., no sleep, dim light, and hourly isocaloric meals) to observe the effects of the eating schedule without acute effects of consumption. Previous exposure to the late meals decreased average glucose without altering insulin rhythms, corroborating the benefits of earlier meals observed in the context of weight loss (101). Glucose rhythms were also delayed (6 h) in accordance with meal timing (delayed 5 h) in the prior days without changes in the levels of central clock markers, indicating the entrainment of peripheral clocks but not the SCN (103). Indeed, a smaller delay (∼1 h) was observed in adipose tissue PER2, serving as proof of principle that meal schedule can entrain the peripheral clock phase in humans. A recent study in mice suggests that postprandial rises in insulin and IGF-1 may directly induce PER protein translation in peripheral tissues (40).

Studies in rodents suggest that food entrainment of the adipose clock, as compared to the more rapidly adapting liver clock, may require longer-term exposure to the new meal schedule (104). The rapid entrainment of the liver clock could explain the disproportionate effect of meal timing on glucose compared to lipid levels, the latter having been unaffected by 6 days of entrainment to earlier meal timing (103). Lipid levels are known to be under circadian regulation in humans, and a large proportion of the circadian metabolome under constant conditions is comprised of lipid species (87). However, their regulation involves multiple peripheral tissues, such as adipose, whose clock may be entrained gradually. On the other hand, glucose homeostasis has been demonstrated in mice to be the direct output of the liver-independent circadian clock. Koronowski et al. (10) examined the circadian metabolome and transcriptome of the liver in the absence of all other tissue clocks using a hepatocyte-specific reconstitution of Bmal1 in whole-body Bmal1 knockout mice (Liver-RE). The reconstituted livers showed the restoration of 10% of transcript and 20% of metabolite oscillations, and glucose metabolism pathways were among the most represented in these independent hepatocyte clock outputs. The rhythms in hepatic glycogen synthesis and turnover were almost completely restored as well as the rhythmic recruitment of Bmal1 to the rate-limiting enzyme *Gys2* (glycogen synthase 2). Moreover, regulation of hepatic *Glut2* by BMAL1 drove active phase peaks in glucose levels that were otherwise absent in the full knockout. Previous studies on mice showed that the liver was the most rapidly entrained by feeding time (35) and that its clock autonomously drove rhythmic plasma glucose levels (10). Together, studies in mice have shown that the liver clock is most sensitively entrained by feeding time (35, 104) and autonomously drives rhythmic plasma glucose levels (10), which could explain the relative sensitivity of human plasma glucose but not lipid rhythms to the acute changes in feeding schedule observed by Wehrens et al. (103).

## Components of Meal Timing

The timing of meals as a potential zeitgeber is comprised of multiple distinguishable variables: meal frequency, the length of eating or fasting window, and its timing with respect to

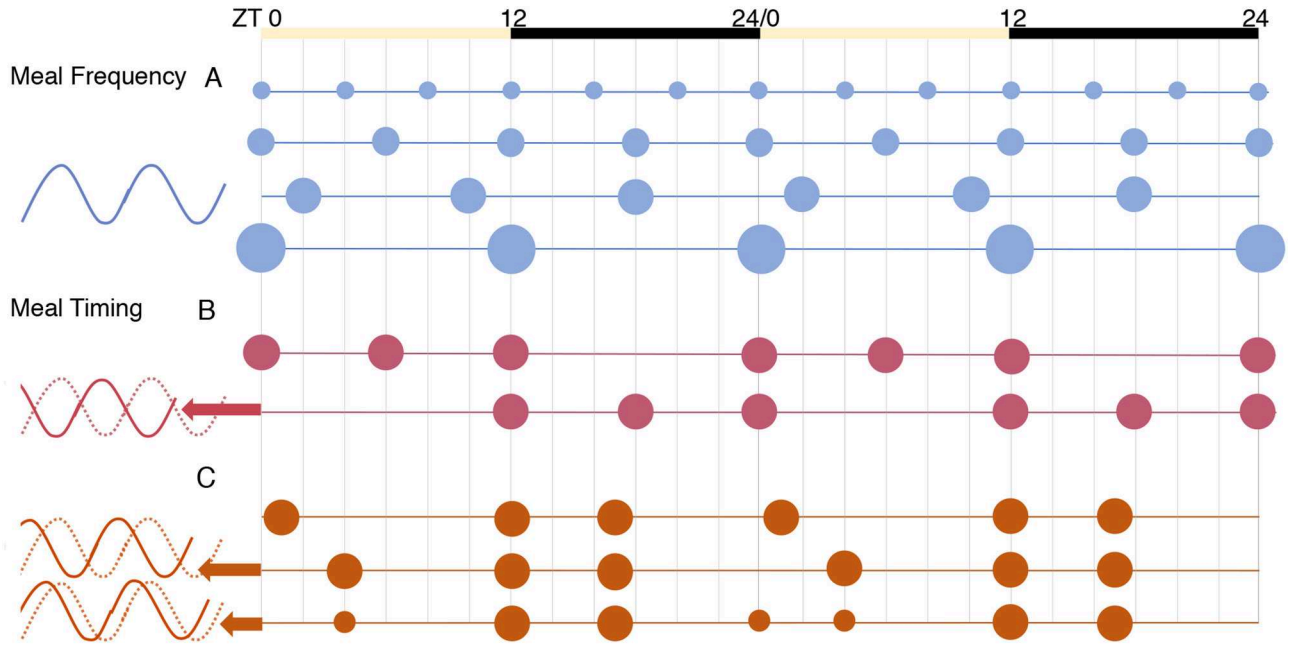


**FIGURE 3 |** The effects of meal frequency vs. timing on peripheral tissue clocks [from results of (105)]. The phase of Per2 expression was similar in all conditions for the tissues tested: liver, kidney, and submandibular gland. **(A)** Meal frequency, spreading calories equally into 2, 3, 4, or 6 meals per day, did not alter clock phase. **(B)** Daytime (inactive phase) feeding significantly advanced phase. **(C)** Using a three-meal schedule designed to mimic human eating habits, a "late dinner" meal (in the early inactive phase) caused a dramatic phase advance, which was partially reversed by splitting the dinner meal to include a "pre-dinner snack".

the day/night cycle. The effect of meal frequency vs. timing on the core clock was explored by Koruda et al. (105) using *in vivo* recording of Per2 activity in the kidney, liver, and the submandibular gland of circadian reporter (Per2:Luciferase) mice. Meals of varying frequencies (2, 3, 4, or 6 meals) given at equal intervals throughout the 24 h period did not affect the phase of clock genes in these peripheral tissues [**Figure 3A** and (105)]. In contrast, a constant frequency of three meals per day given in different temporal patterns resulted in significant changes in the clock phase. A "late dinner" (ZT4) caused a dramatic phase advance in peripheral clocks, which was partially reversed when the dinner was split into two smaller meals to include a "pre-dinner snack" (ZT0) in the period before the late dinner (**Figure 3C**). Viewed from another perspective, the late dinner created a shorter fast before breakfast and a longer fast after lunch, the latter being filled when a pre-dinner snack was added. This illustrates the importance of feeding intervals or periods of fasting in peripheral clock entrainment, which is a plausible mechanism given the interaction between the core clock and nutrient-sensing pathways (e.g., AMPK) that are activated during fasting (9).

The importance of the fasting period is relevant to the interpretation of research in chrono-nutrition. For instance, a recent observational study found no association between BMI and the timing of meals in the self-reported data of 125 adults collected from a smartphone app (106), a result that at first glance is contradictory. However, the food timing variables analyzed were the "time of first eating episode" and "time of last eating episode," neither of which by itself is indicative of fasting period. As a positive example of the relevance of fasting period, a randomized controlled trial in patients with T2DM found advantages of two meals/day compared with an isocaloric regimen of six meals/day. Eating only breakfast and lunch resulted in reduced body weight, fasting glucose, glucagon, and increased insulin sensitivity (107). From the above study in rodents (**Figure 3**), these results were likely due to the extended fasting window rather than reduced meal frequency. Observational work in humans is also in agreement with this. Individuals who ate one or two meals per day showed a BMI reduction over a one-year period, while increased BMI was observed in those who ate more than three meals a day; a greater reduction in BMI was observed in individuals who fasted at least 18 hours overnight compared to a medium overnight fast of 12–17 hours, as well as in those who consumed more calories early in the day (108). The authors concluded that a practical strategy for BMI reduction would be to consume the majority of calories early in the day and extend the overnight fast to 18 hours. Thus, both the length of the fasting period and the timing of meals with respect to the circadian day appear to be decisive in the maintenance of a healthy weight.

## Time-Restricted Feeding

The practice of limiting daily food intake to a finite time window, thereby extending the overnight fast, is referred to as time-restricted feeding (TRF). This mode of eating has received considerable attention in recent years as a strategy

for aiding weight loss and improving metabolic health (109) and as an alternative to caloric restriction to promote longevity (110). Despite isocaloric intake, numerous benefits are observed simply by restricting the intake window. However, important questions remain regarding (1) the length of the feeding window permitted required to observe these benefits, and (2) the timing of the feeding window with respect to the 24-h day. Although controlled comparisons of early and late TRF in humans have not yet been conducted, the inference drawn from rodent models is clear: it is imperative that restricted feeding windows occur during the active phase. Mice in inactive phase TRF were hyperphagic and gained more weight than the active-phase restricted feeders within only 9 days; they showed reduced metabolic flexibility with greater reliance on carbohydrate, and as previously outlined, peripheral clock gene expression in metabolic organs was perturbed with near complete phase inversion in the liver and strongly dampened rhythms in epididymal adipose, skeletal muscle, and the heart (39). In rats, active phase TRF improved whereas inactive phase TRF worsened glucose tolerance (111), and inactive phase TRF caused desynchrony of muscle and liver clocks (112).

The shifts in peripheral clocks were both consequences and causes of metabolic dysregulation under rest-phase feeding (113), producing a vicious circle of misaligned processes. By contrast, active phase TRF does not appear to alter the peripheral circadian phase compared with *ad libitum* (AL) feeding (114), somewhat expected given that AL fed mice consumed ∼80% of their food during the active phase when fed normal chow (35). The lack of phase shift during active phase TRF could also reflect the nature of the clock's response to zeitgebers: the same input produces phase changes that vary in magnitude and direction depending on time of day when exposure to the zeitgeber occurs (115). The master SCN clock is well-understood to be responsive to light entrainment during the dark phase, whereas the response to non-photic cues, such as arousal, is greater during the inactive phase (116). The presence of a zeitgeber outside its usual time induces phase shifts; peripheral clocks are therefore likely to have a characteristic phase response curve for food entrainment with greatest response coinciding with the inactive phase.

## Early Time-Restricted Feeding (eTRF)

In humans, restricting food intake to early in the day is associated with reductions in bodyweight, but the mechanism of this association was not agreed upon until recently. Some researchers have speculated from work in rodents that it was the result of greater energy expenditure early in the day (117). Others reported reduced caloric intake due to decreased appetite during early meals. From a clinical standpoint, natural appetite reduction is, of course, a viable method of weight loss. The findings in a recent series of controlled feeding studies in humans support that a natural reduction in food intake is the primary mechanism behind weight loss during early time-restricted feeding (eTRF). In three trials, the participants in the eTRF groups ate during a 6-h window beginning at 8:00 or 9:00 a.m., and the controls ate an equal number of calories during a 12-h window beginning at the same time. After 5 weeks on isocaloric eTRF, men with prediabetes had improved insulin sensitivity accompanied by increased pancreatic ß-cell responsiveness and lower fasting glucose, blood pressure, and oxidative stress, as well as reduced subjective hunger (118). However, there were no differences in body weight compared to the controls; weight loss did not occur in early restricted feeding alone when caloric intake was matched, although metabolic improvements were observed.

This conclusion was supported by the results of two shorter term trials of eTRF. Overweight adults were randomized to 4 days of eTRF and studied using whole room indirect calorimetry on the final day. The results confirmed that energy expenditure was not significantly affected by the feeding time (119). The thermic effect of food was greater in the morning eaters, which was consistent with the results of previous work (59, 120), however, this effect was predicted to increase energy expenditure by only 20–40 kcal/day. This daily change is small, but over time it could be clinically significant. Importantly, other beneficial effects that produce weight loss in practice were observed in the early TRF group: levels of the satiety hormone peptide YY (PYY) and subjective "stomach fullness" were higher, while ghrelin, perceived "hunger" and the "desire to eat" were all significantly reduced (119). Metabolic flexibility, that is the ability to switch between carbohydrate and fat oxidation, was also increased in the TRF group, who more effectively burned fat during their fasting period. In a final trial using the same eTRF schedule, this eating pattern was shown to decrease mean 24-h glucose and glycemic excursions (121). Intriguingly, many of the clock genes appeared to be upregulated in the eTRF group, although the interpretation of rhythm was not possible because only two timepoints were sampled. Taken together, the results of these trials supported that eTRF can cause weight loss primarily through reducing hunger and partly through minor increases in the thermic effect of food, while also independently improving metabolic parameters. Human clinical trials aimed at better defining the contribution of endogenous circadian physiology to these effects are currently underway as part of the Big Breakfast Study (122).

## Time-Restricted Feeding in Shift Workers

In addition to weight loss, a highly promising application of TRF is to maintain the cardiometabolic health of shift workers. The predominant effect of properly timed feeding in the presence of the factors of light exposure, sleep disturbance, and activity during the rest phase was clearly demonstrated by Salgado-Delgado et al. (123). Shift work was simulated by keeping rats on slow rotating wheels that forced wakefulness and a low level of activity from 9:00 to 17:00 (ZT2-ZT10), which is the inactive phase in nocturnal rodents, on weekdays for 5 weeks. On weekends, all rats were left undisturbed in their home cages with AL food access. During the work week, shift workers and controls were assigned to AL feeding, active phase TRF, or inactive phase TRF. Unsurprisingly, the AL-fed shift workers ate most of their food during the inactive phase when they were kept awake. They and the inactive TRF groups gained more weight and had greater visceral adiposity compared with the AL controls, active phase TRF controls, or active phase TRF shift workers, despite similar food intake across all groups. Even though the work schedule disrupted sleep and activity patterns, only *mistimed feeding* explained the differences in weight and fat gain. The

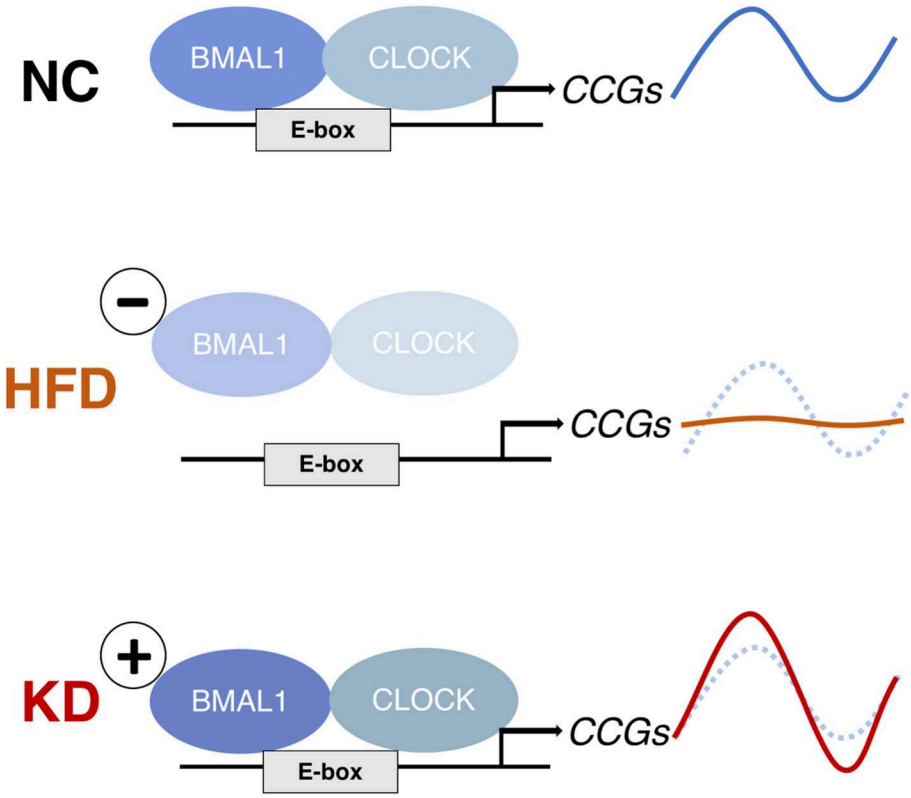


**FIGURE 4 |** The effects of a high-fat diet (HFD) and ketogenic diet (KD) on clock-controlled gene (CCG) expression in mouse liver. The HFD abolishes rhythms in CCGs by reducing CLOCK:BMAL1 chromatin binding (136), whereas the KD increases rhythmic CLOCK:BMAL1 binding and thereby CCG expression amplitude (147). Both diets induce *de novo* rhythms in other metabolic genes because of the rhythmic nuclear accumulation of non-clock transcription regulators (PPARg and SREBP-1 in liver under HFD; PPARα in intestine and rhythmic histone deacetylation by serum BHB under KD).

corticosterone rhythms remained unchanged, corroborating that SCN clock outputs are resilient to behavioral feedback, which contributes to internal desynchrony during shiftwork. However, disturbances in glucose and TAG rhythms and the accumulation of abdominal fat were all prevented when shift work was combined with active phase TRF. Similar protection by active phase TRF is also observed in simulated jetlag. Mice exposed to a 6-h phase advance in the light/dark cycle twice weekly with AL food access became overweight despite isocaloric intake, but this was prevented by active phase TRF (124).

## DIET COMPOSITION

In addition to the timing of food intake, the composition of the diet alters peripheral circadian alignment and acutely induces phase shifts. An important mechanism by which this occurs is through feedback from altered behavioral rhythms in feeding and activity. Other mechanisms include impaired or enhanced inter-tissue communication as well as interactions of the core clock with particular nutrient-sensing pathways. The following section focuses on the effects of the well-studied high-fat diet and the increasingly studied ketogenic diet on circadian metabolism.

### High-Fat Diet

The modern obesogenic food environment is most commonly modeled in animals with the high-fat diet (HFD). Mice given AL access to the HFD reliably develop obesity and a phenotype similar to the metabolic syndrome in humans, characterized by insulin resistance, hepatic steatosis, hypercholesterolemia, and dyslipidemia (125). As also occurs in humans, this metabolic disruption is preceded by circadian disarrangement.

#### Altered Rhythms in Feeding Behavior

In animals, an immediate alteration in rhythmic behavior is observed upon the start of the HFD. Whereas, normal chow fed animals are active and consume ∼80% of their food during the dark period, HFD fed mice become hyperphagic and eat continuously through the inactive phase, such that total food intake becomes indistinguishable between day and night (49, 104). The rapid onset of behavioral arrhythmicity indicates that it is likely to occur by a mechanism independent of clock gene regulation, which would take longer to adapt. Highly palatable foods or particular nutrient compositions can directly and rapidly signal to orexigenic centers (126). The HFD in particular directly affects appetite regulating centers in the arcuate nucleus as well as the regions associated with hedonic/reward stimulation that

influence food-seeking behavior (127–129). It appears that the homeostatic feeding circuits acutely responsive to the HFD are independent of clock regulation. One week of HFD did not alter clock gene expression in the SCN, arcuate nucleus, or pituitary (104). This is despite reciprocal connections between the arcuate and SCN (130). Moreover, 6 weeks of HFD did not alter clock gene expression in the mediobasal hypothalamus (49), a region containing AgRP neurons known to possess an autonomous clock controlling the rhythms of hunger and satiety (131). The HFD therefore appears to acutely alter feeding behavior by clock-independent central mechanisms.

Although the rapid shift in feeding behavior upon beginning high-fat feeding is likely to be clock-independent, the HFD does also interfere with the master clock in the SCN. As compared to normal chow (NC)-fed animals, in those fed the HFD the induction photic entrainment in the SCN (marked by *c-fos*) is impaired, as is locomotor activity phase resetting, and re-entrainment following "jetlag" (a 6 h advanced) (50). Interestingly, hypocaloric feeding also alters photic entrainment (132, 133), but in contrast to the HFD, enables a more rapid phase shift response to light (134). In summary, diet composition can alter photic entrainment of the SCN, but the most important effects of the HFD on the circadian system are likely to occur indirectly through phase shifts in peripheral clocks in response to altered feeding behavior.

## Altered Metabolic Rhythms in Liver

Shortly following the altered feeding rhythm, the clock genes of peripheral tissues are altered in amplitude and phase. The liver clock is the most widely studied and, as previously mentioned, perhaps the most sensitive to feeding rhythms. Long-term HFD feeding was shown to dampen hepatic clock gene expression (135, 136). After just 1 week on HFD, the hepatic clock was found to phase advance by 5 h (104). A similarly sized phase advance of 4 h was confirmed by Branecky et al. (137). Interestingly, they found that similar to the liver's rapid response at the beginning of HFD, it rapidly reverted to its normal phase within 7 days of the return to normal chow. This response lagged behind the feeding rhythms, which were restored within 2 days. As the changes in amplitude and phase are consistently found to occur after the changes in feeding rhythm, they are likely to be the result behavioral feedback. Diet composition may act as a zeitgeber in the liver by altering food intake timing through initially clock-independent mechanisms.

The phase advance in the liver clock is comparatively mild compared with the large-scale changes to downstream clock-controlled genes (CCG) observed with the HFD. In a comprehensive study of the effects of HFD compared with normal chow (NC) on the circadian transcriptome and metabolome in mouse liver, Eckel-Mahan et al. (136) found that large proportions of all oscillatory metabolites and transcripts either gained or lost rhythms under the HFD. Moreover, most of those that were oscillatory under both diet conditions exhibited phase shifts and overall advance under high-fat feeding. Another common effect of the HFD was to reduce the amplitude of oscillating metabolites and transcripts, and the transcripts that lost oscillations on the HFD had a robust peak at ZT8, coinciding with the greatest activity of CLOCK:BMAL1 at target genes. For instance, during NC feeding, the levels of NAD+ oscillated because of the CLOCK:BMAL1 regulation of *Nampt* which encodes the rate-limiting enzyme in the NAD+ salvage pathway. The HFD impaired CLOCK:BMAL1 chromatin binding to this and other metabolic CCGs, leading to the loss of oscillation in this key enzyme and the flattening of hepatic NAD+ levels (136). In other cases, the HFD produced *de novo* oscillation of the metabolites and transcripts encoding their regulatory enzymes, such as in the methionine cycle. These *de novo* oscillations appeared to be downstream of the oscillations in the nuclear accumulation of transcription factors outside the core clock, especially PPARγ and SREBP-1. Because of the relation between the methionine cycle and epigenetic methylation reactions (138), its newly oscillatory status may be one mechanism in the large-scale reprogramming observed under an HFD. Importantly, many of these changes were observable after an acute HFD exposure of just 3 days and were reversed with 2 weeks on NC, confirming these effects were the result of the HFD rather than diet-induced obesity (136).

Corroborating and expanding on these results, the HFD was again recently shown to induce the rhythmic binding of non-core clock transcription factors to metabolic target genes in the liver, including SREBP-1. Guan et al. (139) found that rhythmic lipogenesis under SREBP-1 control produced endogenous ligands for PPARα, which then activated rhythmic fatty acid oxidation. As is usual under high-fat feeding, the mice developed insulin resistance, hyperlipidemia, and fatty liver. The latter condition is targeted in humans by PPAR agonist drugs. Impressively, treatment with the PPARα agonist in the HFD fed mice at the peak of its activity (ZT8) resulted in a greater reduction of hepatic lipid accumulation and serum triglyceride levels than when it was given at the nadir of PPARα activity (ZT20) (139). This strategic administration of drugs in coordination with circadian rhythms is an example chronotherapy, which is a promising direction for translational work. It is known that lipid profiles in humans have a robust circadian rhythm (88). There is also some evidence that levels of clock genes in circulating monocytes, particularly the PERs, are altered in switching from a high-carb/low-fat to a high-fat/low-carb diet (140). In this study, however, the samples were taken only at three timepoints within an 8-h period, which was insufficient to extrapolate 24-h oscillations. The effects of dietary fat on clock gene expression and circadian regulation in humans will require further investigation.

## Impaired Inter-Tissue Communication and Misalignment

In addition to altering the liver clock and causing widespread alterations in rhythmic metabolism, the HFD impairs inter-tissue communication, thereby further exacerbating the misalignment of circadian rhythms between key metabolic tissues. For instance, adiponectin from mature adipocytes regulates hepatic lipid metabolism by activating AMPK, leading to the phosphorylation and inactivation of the fatty acid synthesis enzyme ACC, the activation of PPARα to increase FA oxidation, and the potentiation of insulin signaling to inhibit gluconeogenesis (141).

In the livers of mice fed the HFD, rhythms in AMPK and ACC transcripts were abolished, and a 3-h phase delay and dramatic dampening were observed in key components of the adiponectin signaling pathway (AdipoR1, Pepck, and PPARα) as well as the core clock gene Per1 (141). A similar phenotype during HFD feeding was observed in skeletal muscle and adipose (142). In addition to adiponectin, normal daily rhythms in plasma insulin, ghrelin, and leptin have been shown to be disrupted in rats fed an HFD (143).

Along with endocrine system interference, the HFD also directly misaligns tissues by differentially regulating their clocks. The HFD causes rapid phase shifts in the liver within 1 week, whereas the clocks of the lung, spleen, aorta, gonadal white adipose were unchanged in phase (104). It is possible that these tissues rely less on food intake as a zeitgeber, or that they adapt more gradually to HFD exposure. Whether the liver responds more strongly or just more rapidly to the HFD, this discrepancy in responsiveness is important because the liver then quickly becomes misaligned with other tissue clocks.

The induction of peripheral misalignment by HFD was strikingly demonstrated in recent circadian metabolomics studies. Abbondante et al. (144) showed that a large proportion of serum metabolite rhythms are lost under the HFD and become uncoupled from liver rhythms. Dyar et al. (145) expanded this finding, performing metabolomics on serum, liver, white and brown adipose tissue, muscle, the medial prefrontal cortex (mPFC), SCN, and sperm, following either HFD or NC feeding. They found tissue-specific alterations in circadian metabolism that caused the large-scale disruption of the temporal cohesion between the tissues. Positive and negative correlations in time of tissue metabolites are indicative of shared metabolic networks and the temporal gating of incompatible metabolic processes, respectively. The major source of metabolite correlations on NC were through those circulating in serum. An astounding 98% of these metabolite correlations were lost in serum under the HFD, in addition to 74, 39, and 34% in brown adipose tissue (BAT), muscle, and liver, respectively (145). Lipids in particular were highly correlated in time under NC and they lost this inter-tissue alignment under the HFD.

Similar untargeted metabolomics were undertaken in human blood samples and muscle biopsies from overweight/obese men after 5 days of a high-fat or high-carbohydrate diet [65/20/15% vs. 15/20/65% calories from carbohydrate/protein/fat, respectively; (20)]. Unfortunately, however, samples were taken at 7:30 a.m. in the fasted state and at 7:30 p.m. after dinner, making it impossible to separate the effect of time of day from acute effects of the evening meal; indeed, among the metabolites heightened in the evening (after dinner) on the HFD were fatty acids and ketone bodies, while xenobiotics that are known food additives were higher in the evening on both diets. The effect of diet composition on the human circadian metabolome requires further investigation.

## The Ketogenic Diet

The term HFD has been used to refer to rodent chow of varying compositions, but a typical diet used to model human obesity is by calorie 45% fat, 15–20% protein, and 35–40% carbohydrate (146). Hence, the term HFD is somewhat of a misnomer that perhaps more accurately designates a high-fat, moderate-carbohydrate diet. This is to be distinguished from the even higher fat and very low carbohydrate ketogenic diet (KD). Ketogenic rodent chow is nearly devoid of carbohydrates; it is comprised of more than 90% fat and the remainder is largely protein (147). In humans, a KD typically restricts daily carbohydrates to under 50 g per day (148). Whereas, the HFD is used experimentally to induce obesity and metabolic disease, the KD is under investigation because of its therapeutic potential to ameliorate these states (149). Whereas, the HFD induces hyperphagia, appetite inhibition is thought to be an important part of KD efficacy (148). Similarly, the interaction between the KD and the circadian clock is quite distinct from the HFD case.

The KD drives metabolism toward the pathways induced under fasting or caloric restriction without the need to restrict energy intake. Gluconeogenesis, fatty acid oxidation, and ketogenesis are upregulated, while glycolysis and *de novo* lipogenesis are inhibited (150). The circulating levels of ketone bodies in healthy humans have long been recognized to follow daily rhythms, with overall higher levels occur during carbohydrate restriction as in the KD or in fasting (151). The induction of ketogenic genes in the liver during fasting is controlled by the mTOR–PPARα axis (152), and the PPAR family is known to be circadian-regulated (17, 153). Moreover, the hepatic expression of the clock component Per2 was shown to be necessary in ketogenesis. Per2 is a direct regulator of *Cpt1a* expression, a rate-limiting enzyme that transfers long-chain fatty acids to the inner mitochondrial membrane for ß-oxidation, and is an indirect regulator of *Hmgcs2*, the rate-limiting enzyme for ketogenesis from the resulting acetyl-CoA (154). The KD may also increase transcriptional activation of CCG's by the CLOCK:BMAL1 complex (peaking at ZT8-12); the KD diet has been shown to upregulate the clock output gene *Dbp* in the liver, heart, kidney, and adipose tissue (155).

The circadian transcriptome was recently analyzed in the liver and intestine (ileal epithelia) of mice fed the KD or isocaloric quantities of NC, revealing large-scale alterations in oscillating transcripts (147). In contrast to the HFD, which mildly dampened clock gene expression (136), the amplitude of clock genes were either unaltered or slightly increased under the KD. The rhythm in the respiratory metabolism was abolished by KD as it was in the HFD (147). The rhythm in respiratory metabolism was abolished by KD as in the HFD. An arrhythmic respiratory exchange ratio (RER) is considered evidence of metabolic impairment (156), as the body is unable to efficiently switch from burning carbohydrate to burning fatty acids during the overnight fast. However, in the context of the KD, this flattened RER is an expected outcome given that fatty acids are the predominant fuel available. Indeed, the RER remained at ∼0.7 on the KD, indicative of purely fat rather than carbohydrate oxidation (147).

Similar to the case of the HFD, the greatest alteration in the liver was not at the level of the clock genes themselves but in the chromatin recruitment of CLOCK:BMAL1 to clock-controlled genes (CCGs). However, the overall effects were opposite (**Figure 4**). In the HFD, the occupancy of CLOCK:BMAL1 at target gene promoters was attenuated, causing blunted

oscillations of CCGs (136). In contrast, the KD increased the amplitude of clock target genes, including *Dbp* and *Nampt,* following greater Bmal1 recruitment to these sites at the critical timepoints. This KD-induced amplitude increase was not seen in *Clock*-mutant mice, confirming it is the output of the clock (147).

In the intestine of KD fed mice, the dietary intake of fatty acids induced rhythmic nuclear accumulation of PPARα and the expression of its target genes (147). Of all oscillating transcripts in the intestine, ∼20% were expressed in phase with the nuclear accumulation of PPARα, including *Hmgcs2*. Corresponding to the HMGCS2 induction were oscillations of the ketone body ß-hydroxybutyrate (ßOHB) in the gut and serum. ßOHB has received recent attention as a signaling molecule with histone deacetylase activity (151), and it appears to also be a cofactor in a recently defined epigenetic marker, histone b-hydroxyl-butyrylation, which is associated with active gene expression (157). Therefore, transcriptional reprogramming under the KD may partly be through chromatin remodeling by ßOHB. Whether the overall effect of this large-scale remodeling is beneficial is not yet clear; however, a positive effect of intestinal ßOHB in maintaining the stemness and regenerative capability of intestinal stem cells was recently identified (158).

## INTERACTION BETWEEN DIET COMPOSITION AND TIMING

Experiments using circadian mutant mice fed the HFD illustrate the interaction between circadian clock function and the response to different diet compositions. The RAR-related orphan receptor alpha (RORα) is a nuclear receptor that functions as a ligand-dependent transcriptional factor in lipid metabolism and in circadian regulation (159). The natural loss of function occurs in *Rorα* mutants, which are called Staggerer mice ($ROR\alpha^{sg/sg}$ mice). These mice exhibit a lean and dyslipidemic phenotype when fed NC. When fed the HFD, they are resistant to weight gain and hepatic steatosis, although they also develop particularly severe atherosclerosis (160). This finding attracted attention to RORα and other circadian nuclear receptors (Reverb's) as drug targets for metabolic disease (161). Recent work has suggested that RORα agonists may be effective in preventing hepatic steatosis (162), and liver-specific *Rorα* deletion correspondingly worsens hepatic steatosis (163). However, RORα appears not to alter the susceptibility to obesity and metabolic disease in mice fed a Western-style diet [40% fat, 40% carbohydrate; (164)]. Thus, the particular phenotype of circadian mutant mice is dependent on diet composition.

Further illustrating this interaction between the circadian clock and diet composition, some circadian mutants have strikingly different phenotypes on NC compared to the HFD. The phenotype of Per2–/– animals fed NC is of lower bodyweight and fat due to overaction of PPARγ target genes (*Ucp1, Cidea*) in adipose (165), but, when fed HFD the Per2 knockouts are more prone to gain fat than WT littermates. Similarly, Cry1–/– Cry2–/– double knockout mice have lower body weight and adiposity on NC because of increased energy expenditure, but when fed the HFD, they gain fat dramatically more quickly than WT mice despite being comparatively hypophagic, in part due to hyperinsulinemia-stimulated lipogenesis (166). It is interesting that in both cases, the mutant mice exhibited disrupted feeding rhythms even on NC, but a disrupted core clock and feeding rhythm were not sufficient to produce weight gain. Instead, they strongly predisposed the mice to diet-induced obesity.

## Preventing Diet-Induced Obesity Using Time-Restricted Feeding

Both eating at the wrong time and the HFD independently lead to internal misalignment between metabolic organs (84, 167). The HFD also acutely alters food intake timing, and this appears to be at the root of some of its adverse effects on circadian metabolism. The time-restricted feeding (TRF) of HFD within 8 h during the active phase, despite isocaloric intake compared with AL-fed controls, protected mice against diet-induced obesity (DIO) and related metabolic dysfunction (117). Similarly, mice on a 12-h active phase TRF were able to maintain normal metabolic parameters on both low- and high-fat diets (168). The combination of this obesogenic diet with active phase TRF also restored the rhythms in liver clock gene expression (169) and reduced plasma insulin, leptin, and proinflammatory cytokines, suggesting protection against systemic inflammation (170).

Active phase TRF acts as a protective buffer against an array of nutritional challenges. Although the HFD is the most common diet used to induce obesity in rodent studies, it is one among multiple experimental diets that model the energy-dense and processed foods eaten by modern humans and produce characteristic metabolic disease. For instance, a high-fructose diet causes insulin resistance, cardiac damage, and hepatic steatosis (171, 172). To address the efficacy of TRF to combat weight gain and disease on different obesogenic diets, one large mouse study examined three different experimental diets: a high-fructose diet (13% fat, 60% fructose); a high-fat and sucrose diet (32% fat, 25% sucrose); and the HFD (62% fat, 20% carbohydrates); a control chow (13% fat, 59% carb). They found that restricting food access to 8–9 h during the active phase effectively prevented or even reversed the already established obesity and metabolic dysfunction caused by any of these diets when fed AL, and this was accompanied by the restoration of temporal dynamics in several key metabolic pathways (173). Relevant to humans, this active phase TRF provided effective protection against diet-induced metabolic disease even when mice fed freely on weekends.

To get the protective benefits of TRF it is imperative that the feeding window occur during the active phase. In fact, restricting access to the HFD to the "wrong time" (rest phase) caused a 2.5-fold greater weight gain compared with active phase feeding (174). Timing the food intake with the active phase appears to confer a metabolic advantage because physiological systems are more prepared for the nutritional challenge. For example, in the response to a high-fat meal at the beginning of the active period fatty acid oxidation was shown to be increased, but this metabolic flexibility was lacking in the

response to the same high-fat meal at the end of the active phase (168). Over time, improperly timed feeding can promote metabolic disease independently of the daily total number of fat-derived calories consumed (168). In addition, inactive phase TRF of either the HFD or NC for 6 days almost completely inverted the liver clock's phase (135), causing misalignment with other peripheral clocks that were not as responsive to this zeitgeber. The same peripheral misalignment was observed in mice on inactive phase TRF of a high-fat high-sucrose (HFHS) diet, along with hyperphagia due to central leptin resistance (175). Collectively, the inactive phase feeding of an array of diets (NC, HFD, or HFHS) impairs nutrient handling, induces peripheral clock misalignment, and disrupts signaling between metabolic tissues.

The HFD and other obesogenic diets interfere with normal feeding rhythms, but mice consuming isocaloric amounts of obesogenic diets stay slim and healthy as long as consumption is confined to the active phase. Mutations of clock genes also result in disrupted feeding and activity rhythms in gene-specific metabolic phenotypes. As the results of previous work have shown, the susceptibility of circadian mutants to obesity and various metabolic diseases depends on the composition of the diet they are fed in interaction with their particular genotype. Significantly, restoring feeding rhythms with time-restricted feeding in circadian mutant strains that are normally susceptible to HFD-induced obesity (a liver Bmal1 KO; liver Rev-Erb α/β DKO, and full-body Cry1/Cry2 DKO) was sufficient to prevent DIO and resolve some genotype-specific metabolic abnormalities (176). This result suggests that the metabolic disruption in circadian mutant animals and animals fed the HFD secondary to their altered feeding rhythms, and that imposing an active/inactive phase rhythm of feeding/fasting is sufficient to prevent the metabolic disease phenotypes of these circadian mutants. Such an interpretation is further supported by a recent study in mice with SCN-targeted ablation of Bmal1. In constant darkness, these mice develop arrhythmic activity, feeding, and peripheral clock gene expression (in the liver, pancreas, and adipose). With this arrhythmicity comes increased adiposity and impaired glucose tolerance. However, TRF was sufficient to restore peripheral tissue rhythms, body weight, and glucose utilization (177).

Disrupted peripheral clock rhythms, whether from circadian mutations or environmental perturbation (e.g., shiftwork), can lead to overweight and metabolic disease. TRF is a promising intervention in this context. Periods of fasting are likely to restore metabolic function in an otherwise obesogenic environment independent of 24-h calorie intake partly by permitting the expression of metabolic pathways inhibited by food intake. For instance, liver-specific AMPK overexpression (mimicking the fasting state) inhibited *de novo* lipogenesis and was sufficient to prevent hepatic steatosis in mice fed a high-fructose diet (178). The takeaway is therefore 2-fold: optimal nutrient intake is confined to a restricted time period during the animal's active phase and leaves a sufficiently long fasting window. A key question that remains is how long of a fasting window is necessary to see the protective benefits of TRF in humans. Is it necessary to fast as long as 18 h (108) or are 11–12 h sufficient (68)? The answer almost certainly depends on baseline metabolic health and the extent of the desired change in weight and metabolic parameters.

## CONCLUSION

The circadian expression of the core clock and the genes under its regulation is found not only in the master clock (i.e., SCN) but also throughout other tissues. Peripheral clocks respond not only to the synchronizing cues emanating from the light-entrained master clock but also to rhythms in feeding and fasting. Because peripheral clocks such as that of the liver are more sensitive to food as a zeitgeber, they are not necessarily coupled with the SCN when feeding and fasting rhythms are not aligned with the light/dark cycle. Furthermore, different peripheral tissues have varying degrees of responses to food intake during the inactive phase, thus potentiating peripheral misalignment. Obesogenic diets also disrupt feeding rhythms and thereby circadian metabolism. In modern humans, the discordance between behavioral and endogenous clock rhythms is prevalent, and this temporal misalignment leads to systemic metabolic dysregulation. While evening light exposure will likely continue to be a reality, food intake is a powerful zeitgeber around which behaviors are more plastic. Active phase TRF may have remarkable potential to prevent the deleterious metabolic effects of both obesogenic diets and night shift work.

## FUTURE DIRECTIONS

The relative importance of the core molecular clock as a mediator of food intake signals remains to be delineated. Does TRF affect the rhythm-generating clock itself, or does it instead affect the "hands of the clock" (outputs of the core oscillator)? The answers to these questions could be important in developing potential pharmacological interventions (e.g., fasting mimetic drugs and direct clock gene agonists) for use when lifestyle changes are not feasible, such as in the case of shift work.

Other lifestyle interventions that could influence circadian metabolism and prevent its misalignment are also being examined. Exercise in particular may influence peripheral clocks in skeletal muscles and adipose tissue (179) as it activates many of the same pathways as fasting does, and these feed back into the core clock (180). Indeed, acute exercise was found to alter the human subcutaneous adipose tissue transcriptome (181). Recently, a human phase response curve for exercise was created (182). Phase response curves illustrate the relationship between the time of zeitgeber exposure and the resultant phase shifts (advances or delays) of the clock. A phase response curve for food intake will likewise be an essential tool for understanding how to prevent or alleviate circadian misalignment by TRF.

Accessing the nutrient-responsive peripheral circadian system in humans remains challenging, and innovative methods are required to translate the large body of mechanistic work in animals. Serial sampling is necessary to observe 24-h rhythms, which presents the particular challenge for invasive studies (e.g., muscle or adipose biopsies). Nevertheless, because

animal studies have shown that the circadian metabolome and its response to nutrition are highly tissue-specific (144, 145), this work is highly important. As Roenneberg and Merrow (4) indicated, in order to combat misalignment, it is necessary to understand tissue-specific variations in response to "eigen-zeitgebers" (particular/own zeitgebers, e.g., nutrients and sympathetic innervation).

Circadian transcriptomics and metabolomics studies in humans have been carried out using serial samples of plasma, muscle biopsies, and subcutaneous adipose tissue. However, further studies are needed to synthesize these large datasets toward identification of biomarkers of circadian metabolic function. Indeed, preliminary efforts are underway to identify human circadian biomarkers (183). If they are sufficiently well-defined, these biomarkers could be screened at their peaks and nadirs to determine interpretable signatures of circadian alignment and identify early markers of circadian disruption in metabolic pathophysiology. Alternatives to plasma for non-invasive serial sampling include urine (184) and breath. The latter was used in circadian metabolomics in a proof-of-principle study (185), and it has recently been highlighted as an ideal method for continuous sampling and rapid untargeted metabolomic analysis (186), making this an exciting avenue for translational work.

A key challenge in the translational application of circadian biology is the large interindividual variations in metabolite rhythms (92, 183, 187). What causes these variations, and how are they related to health outcomes? In some cases, variability may be associated with chronotype (93). The interindividual differences in the circadian metabolic response to misaligned zeitgebers, especially in the context of shift work, warrant further attention. Moreover, the response of the human circadian metabolome under various diet conditions deserves further exploration given the largescale alterations observed in mice (145). At the same time, future research in animal models may elucidate the circadian effects of specific macronutrient ratios, micronutrients and supplements [e.g., fish oil: (188)]. Together this will inform novel therapeutic approaches to combat metabolic disease in the modern environment.

## AUTHOR CONTRIBUTIONS

LP and H-KS conceived, designed, and wrote the manuscript.

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# Metabolomic Profiling Revealed Potential Biomarkers in Patients With Moyamoya Disease

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Metabolomics is increasingly used to observe metabolic patterns and disease-specific metabolic biomarkers. However, serum metabolite analysis of moyamoya disease (MMD) is rarely reported. We investigated serum metabolites in MMD and compared them with those of healthy controls (HCs) using a non-targeted gas chromatography–mass spectrometry (GC–MS) approach to identify metabolic biomarkers associated with MMD. Forty-one patients with MMD diagnosed by cerebral angiography and 58 HCs were recruited for our study. Comparative analyses (univariate, multivariate, correlation, heatmaps, receiver operating characteristi curves) were performed between MMD patients and HCs. Twenty-five discriminating serum metabolic biomarkers between MMD patients and HCs were identified. Compared with HCs, MMD patients had higher levels of phenol, 2-hydroxybutyric acid, L-isoleucine, L-serine, glycerol, pelargonic acid, L-methionine, myristic acid, pyroglutamic acid, palmitic acid, palmitoleic acid, stearic acid, octadecanamide, monoglyceride (MG) (16:0/0:0/0:0), and MG (0:0/18:0/0:0), and lower levels of L-alanine, L-valine, urea, succinic acid, L-phenylalanine, L-threonine, L-tyrosine, edetic acid, and oleamide. These metabolic biomarkers are involved in several pathways and are closely associated with the metabolism of amino acids, lipids, carbohydrates, and carbohydrate translation. A GC–MS-based metabolomics approach could be useful in the clinical diagnosis of MMD. The identified biomarkers may be helpful to develop an objective diagnostic method for MMD and improve our understanding of MMD pathogenesis.

**Keywords: moyamoya disease, gas chromatography–mass spectrometry, biomarkers, serum, metabolomics**

## INTRODUCTION

Moyamoya disease (MMD) is a chronic, rare cerebrovascular-occlusive disease with a poor prognosis and considerable regional and racial differences (Kuroda and Houkin, 2008). The occurrence and development of MMD is multifactorial, and the underlying etiological and pathogenic mechanisms remain largely unclear (Huang et al., 2017). Most studies have focused on geographical distributions, sex predispositions, clinical manifestations, and age-specific

characteristics (Okazaki et al., 2019; Sato et al., 2019). Basic research, including genomic and proteomic approaches, has been extensively conducted in the past 60 years. However, the physiological and metabolic mechanisms remain unclear.

China, especially southwest Shandong, has a large number of affected patients. MMD incidence peaks in children aged 5–9 years and adults aged 40–50 years with a female/male ratio of ∼2 and familial occurrence of 15% (Scott and Smith, 2009). The most common symptoms of MMD are headache, transient ischemic attack, infarction, and intracerebral hemorrhage. MMD often results in death, and early diagnosis is important. Cerebral angiography is the "gold standard" for diagnosis (Lee et al., 2013). However, cerebral angiography has flaws such as an inherent risk of anaphylaxis and nephropathy due to the use of contrast medium. The identification of new biomarkers to assist physicians in timely MMD diagnosis is needed to decrease morbidity and mortality.

Metabolomics is a promising approach that can identify metabolic patterns and disease-specific metabolic markers. This pivotal tool for biomarker discovery has improved the diagnosis of psychiatric illnesses (Cai et al., 2012), atherosclerosis (Chen et al., 2015), coronary artery disease (Turer et al., 2009), cerebral infarctions (Jung et al., 2011), and intracranial tumors (Pandey et al., 2017). However, information on metabolite profiles for MMD is limited. Only one metabolomic study on MMD patients has been conducted, and it focused on identifying metabolites in cerebrospinal fluid (CSF) using nuclear magnetic resonance (NMR) spectroscopy (Jeon et al., 2015). Therefore, more metabolomics studies are urgently needed to better understand the physiological and metabolic mechanisms involved in MMD.

We sought to elucidate the metabolic mechanisms underlying the occurrence and development of MMD. A GC–MS-based metabolomics approach coupled with uni- and multivariate analyses was employed to identify metabolic biomarkers associated with MMD. These results could help guide the development of an objective diagnostic method for MMD and provide insights into MMD pathogenesis.

# MATERIALS AND METHODS

## Participants

Forty-one patients diagnosed by cerebral angiography as MMD and 58 HCs treated at the Jining First People's Hospital, Jining Medical University between January 2018 and June 2019 were recruited for our study. Patients with MMD are often accompanied by basic diseases, such as hypertension, diabetes, and coronary artery disease. In order to make our research more rigorous, MMD patients with other types of diseases were not included in our study. In addition, the patients with MMD that we recruited were hospitalized patients who had a light diet 3 days before the operation. Similarly, 3 days' diet of healthy people was recorded before we recruited to minimize the errors caused by these factors. Blood samples from all MMD patients were collected and subjected to centrifugation at 5,000 rpm for 10 min at room temperature and then stored at −80°C until analyses.

## Materials and Instruments

Heptadecanoic acid (purity: ≥98%; lot no. SLBX4162), as an internal standard (IS), was from Sigma-Aldrich (St Louis, MO, United States). Methanol was of chromatographic grade and purchased from Thermo Fisher Scientific (Waltham, MA, United States). Water was purchased from Hangzhou Wahaha Company (Hangzhou, China). Pyridine (lot no. C10486013) was from Shanghai Macklin Biochemical (Shanghai, China). *O*-Methyl hydroxylamine hydrochloride (purity, 98.0%; lot no. 542171) was obtained from J&K Scientific Industries (Ambala, India). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA + 1% TMCS) (*v*/*v*; lot no. BCBZ4865) was purchased from Sigma-Aldrich.

## Preparation and Derivatization of Samples for GC–MS

Serum samples were processed according to the following procedure. First, 350 μl of methanol (containing 100 μg/ml of IS) was added to 100 μl of serum, vortexed, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a 2-ml tube and evaporated to dryness at 37°C under the gentle flow of nitrogen gas. After the extracts had been dried, 80 μl of *O*-methyl hydroxylamine hydrochloride (15 mg/ml in pyridine) was added and mixed. The solution was incubated for 90 min at 70°C. Subsequently, 100 μl of BSTFA + 1% TMCS was added to each sample, followed by incubation for 60 min at 70°C. Samples were then detected by GC–MS.

## GC–MS

GC–MS was done on a 7890B GC system equipped with a 7000 C mass spectrometer. Separation was conducted on an HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm) with high-purity helium as the carrier gas at a constant flowrate of 1.0 ml/min. Each 1-μl aliquot of derivatized solution was run in split mode (50:1), with helium as the carrier gas and a front inlet purge flow of 3 ml/min; the gas flowrate was 1 ml/min. The GC temperature program was set to begin at 60°C for 4 min, increased to 300°C at 8°C/min, with a final 5-min maintenance at 300°C. The temperatures of the injection, transfer line, and ion source were 280, 250, and 230°C, respectively. Electron impact ionization (−70 eV) was used, with an acquisition rate of 20 spectra/s in the MS setting. MS detection was conducted by electrospray ionization (ESI) in full-scan mode from mass/charge (*m*/*z*) values of 50–800.

In general, 20 μl of each serum sample from MMD and HCs was obtained and then vortexed and mixed. Thus, this is the so-called quality control (QC) sample. Fifteen samples and five QCs were performed for 1 day (one batch), and 15 samples run randomized while the five QCs were distributed evenly among the 15 samples. At last, nine samples and five QCs were done. The peak area and retention time (RT) of the IS (heptadecanoic acid) were applied to evaluate the stability of the sample injection.

## Multivariate Statistical Analyses

GC–MS equipped with Unknowns Analysis and Agilent MassHunter Quantitative Analysis (for GC–MS) was used to

process the GC data. This process enabled deconvolution, alignment, and data reduction to produce a list of m/z and RT pairs, with the corresponding intensities for all detected peaks from each data file in the dataset. The resulting table was exported into Excel™ (Microsoft, Redmond, WA, United States), and the normalized peak area percentages were used as the percentage of corresponding intensities of each peak/total peak area. The resulting three-dimensional dataset including peak index (RT–m/z pairs), sample names (observations), and normalized peak area percentages was imported into SIMCA-P 14.0 (Umetrics, Umeå, Sweden) for statistical analyses. Center scaling, unit variance scaling, and pareto scaling are commonly used to perform the normalization data. In our paper, we adopted the pareto scaling. Too many missing values will cause difficulties for downstream analysis. There are several different methods for this purpose, such as replace by a small values, mean/median, k-nearest neighbor (KNN), probabilistic principal components analysis (PPCA), Bayesian PCA (BPCA) method, and singular value decomposition (SVD) method to impute the missing values (Kumar et al., 2017; Do et al., 2018). In our work, the default method replaces all the missing values with small values (the half of the minimum positive values in the original data) assuming to be the detection limit, and the data were not transformed.

A modified multicriteria assessment strategy was used to select variables. The assessment was used to reduce the number of variables and explore those that were most sensitive to interventions. The statistically significant threshold of variable importance in projection (VIP) values from the orthogonal partial least squares discriminant analysis (OPLS-DA) model was $>1.0$, and two-tailed Student's $t$ test differences of $p < 0.05$ were considered significant. "Fold change" was defined as the average mass response (area) ratio between two groups. Analyses of correlations, heatmaps, receiver operating characteristic (ROC) curves, and pathways were done using MetaboAnalyst 4.0.[1] All results were shown in the "metabolome" view.

# RESULTS

## Basic Participant Characteristics

Overall, 41 patients with MMD and 58 HCs were included in the analyses. Detailed information on the demographical and clinical characteristics of these participants is summarized in **Table 1**.

MMD patients were not significantly different from HCs with regard to age, sex, body mass index (BMI), tobacco smoking, or alcohol consumption (all $p > 0.05$). The types of MMD onset were cerebral infarction (39 cases) and hemorrhage (2 cases). In addition, there were 34 cases of bilateral MMD and 7 cases of unilateral MMD.

**TABLE 1 |** Clinical characteristics of the participants.

| | | No. of patients(%) | | |
|---|---|---|---|---|
| | **Variables** | **MMD** ***n* = 41** | **HCs** ***n* = 58** | ***p* value** |
| Age | Mean ± SD, age (years) | 42.94 ± 14.18 | 41.76 ± 11.06 | 0.6430 |
| Sex, n(%) | | | | 0.9605 |
| | Male | 21(51.2) | 30(51.7) | |
| | Female | 20(48.8) | 28(48.3) | |
| BMI | Mean ± SD, BMI (kg/m$^2$) | 23.48 ± 2.81 | 24.59 ± 3.91 | 0.1030 |
| Smoking, *n*(%) | Smoking (%) | 11(26.8) | 15(25.9) | 0.9142 |
| Drinking, *n* (%) | Drinking (%) | 8(19.5) | 11(19.0) | 0.9458 |
| Type of onset, *n* (%) | | | | NA |
| | Infarction | 39(95.1) | | |
| | Hemorrhage | 2(4.9) | | |
| Pathogenic site, *n*(%) | | | | NA |
| | Bilateral | 34(82.9) | | |
| | Unilateral | 7(17.1) | | |

## Metabolomics Analyses

Representative GC–MS total ion current (TIC) chromatograms of the QC serum sample showed strong signals (**Figure 1**). In addition, the relative standard deviation (RSD) in intra- and interday of the peak area and retention time (RT) of the IS were <15%, indicating that the analytical instrument operated within acceptable standard variations.

After analyses of unknown compounds and quantitative analyses, 114 metabolites were identified in each serum sample and then used in the subsequent multivariate analysis. The PCA scores plot for HCs and MMD patients were $R^2X = 0.816$, $Q^2 = 0.301$. The pairwise PLS-DA score plots also suggested that the MMD patients were statistically different from the HCs: $R^2X = 0.579$, $R^2Y = 0.836$, and $Q^2 = 0.603$. OPLS-DA analyses were carried out to maximize discrimination. The results suggested that this model was efficient and clearly separated the MMD patients and HCs ($R^2X = 0.823$, $R^2Y = 0.913$, and $Q^2 = 0.783$). Values approaching 1.0 indicate a stable model with predictive reliability. Additionally, a permutation test with 200 iterations verified that the constructed OPLS-DA model was valid and not overfitted, as the original $R^2$ and $Q^2$ values to the right were significantly higher than the corresponding permutated values to the left: $R^2 = 0.289$, $Q^2 = -0.556$. They are shown in **Figure 2**.

## Identification of Potential Biomarkers

Twenty-four metabolites to distinguish between the HCs and MMD patients were identified (VIP $> 1$, $p < 0.05$) (**Table 2**). Compared with HCs, patients with MMD were characterized by higher levels of phenol, 2-hydroxybutyric acid, L-isoleucine, L-serine, glycerol, pelargonic acid, L-methionine, pyroglutamic acid, myristic acid, palmitoleic acid, palmitic acid, stearic acid, octadecanamide, monoglyceride (MG) (16:0/0:0/0:0), and MG (0:0/18:0/0:0), as well as lower levels of L-alanine, L-valine, urea, succinic acid, L-threonine, L-phenylalanine, L-tyrosine, edetic acid, and oleamide. Their relationships were revealed by correlation analyses (**Figure 3A**). To better

[1] www.metaboanalyst.ca/

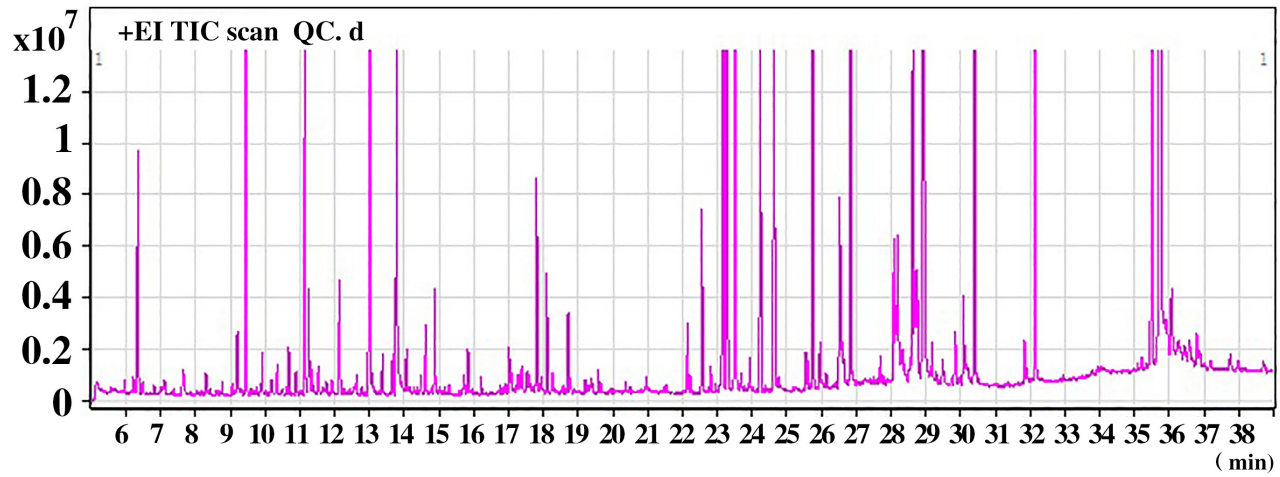


**FIGURE 1 |** A representative gas chromatography–mass spectrometry (GC–MS) total ion chromatogram (TIC) of the quality control (QC) serum sample.

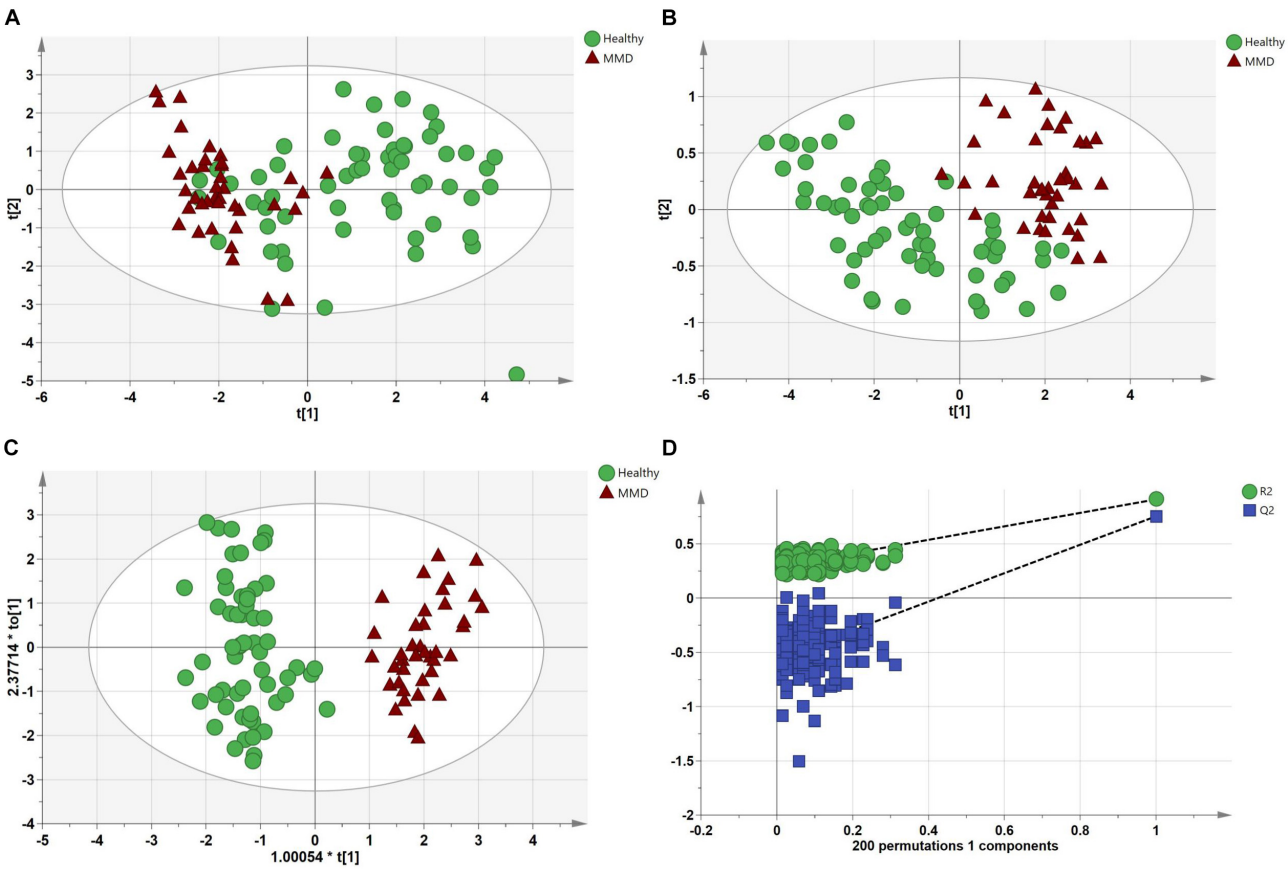


**FIGURE 2 |** Multivariate statistical analysis between the control group and moyamoya disease (MMD) group. **(A)** Principal components analysis (PCA) scores plot; **(B)** partial least squares discriminant analysis (PLS-DA) scores plot; **(C)** orthogonal PLS-DA (OPLS-DA) scores plot; and **(D)** statistical validation of the OPLS-DA model through 200 × permutation testing.

understand the metabolic differences between patients with MMD and HCs, data on identified metabolites were analyzed using clustering heatmaps. Even though sample clusters overlapped slightly, as shown in **Figure 3B**, most samples clearly grouped into two differentiated clusters, in agreement with OPLS analyses.

**TABLE 2 |** List of assigned statistically significant metabolites between moyamoya disease (MMD) and healthy control (HC) group.

| Metabolites | HMDB | RT (min) | VIP | *p* value | FDR | Fold change |
|---|---|---|---|---|---|---|
| Phenol | HMDB0000228 | 9.144 | 1.144 | 8.00E-07 | 2.04E-06 | 1.601 |
| L-alanine | HMDB0000161 | 10.344 | 1.414 | 2.27E-10 | 1.27E-09 | 0.235 |
| 2-hydroxybutyric acid | HMDB0000008 | 10.882 | 1.374 | 9.39E-10 | 4.78E-09 | 1.885 |
| L-isoleucine | HMDB0000172 | 11.765 | 1.221 | 1.05E-07 | 3.45E-07 | 2.162 |
| L-valine | HMDB0000883 | 12.632 | 1.470 | 2.80E-11 | 2.24E-10 | 0.127 |
| Urea | HMDB0000294 | 13.030 | 1.432 | 1.17E-10 | 7.31E-10 | 0.382 |
| L-serine | HMDB0000187 | 13.372 | 1.118 | 1.54E-06 | 3.76E-06 | 1.775 |
| Glycerol | HMDB0000131 | 13.779 | 1.658 | 5.71E-15 | 1.60E-13 | 1.811 |
| Succinic acid | HMDB0000254 | 14.359 | 1.158 | 5.63E-07 | 1.50E-06 | 0.666 |
| Pelargonic acid | HMDB0000847 | 15.090 | 1.092 | 2.85E-06 | 6.64E-06 | 1.991 |
| L-threonine | HMDB0000167 | 14.059 | 1.303 | 9.39E-09 | 4.04E-08 | 0.086 |
| L-methionine | HMDB0000696 | 15.945 | 1.207 | 1.56E-07 | 4.61E-07 | 1.845 |
| Pyroglutamic acid | HMDB0000267 | 17.317 | 1.678 | 1.96E-15 | 1.10E-13 | 4.578 |
| L-phenylalanine | HMDB0000159 | 19.372 | 1.465 | 3.45E-11 | 2.41E-10 | 0.141 |
| Myristic acid | HMDB0000806 | 22.216 | 1.041 | 9.07E-06 | 2.03E-05 | 1.314 |
| L-tyrosine | HMDB0000158 | 22.811 | 1.476 | 2.25E-11 | 2.10E-10 | 0.228 |
| Palmitoleic acid | HMDB0003229 | 26.512 | 1.165 | 4.67E-07 | 1.31E-06 | 1.336 |
| Palmitic acid | HMDB0000220 | 24.630 | 1.507 | 6.21E-12 | 6.96E-11 | 1.437 |
| Stearic acid | HMDB0000827 | 26.837 | 1.606 | 7.63E-14 | 1.42E-12 | 1.510 |
| Edetic Acid | HMDB0015109 | 28.943 | 1.207 | 1.55E-07 | 4.61E-07 | 0.413 |
| Octadecanamide | HMDB0034146 | 28.331 | 1.523 | 3.22E-12 | 4.50E-11 | 3.034 |
| Oleamide | HMDB0002117 | 28.624 | 1.240 | 6.11E-08 | 2.28E-07 | 0.333 |
| MG(16:0/0:0/0:0) | HMDB0011564 | 30.402 | 1.257 | 3.73E-08 | 1.49E-07 | 1.424 |
| MG(0:0/18:0/0:0) | HMDB0011535 | 32.140 | 1.307 | 8.37E-09 | 3.91E-08 | 1.457 |

*FDR, false discovery rate; fold change, MDD/healthy; RT, retention time; VIP, variable influence on projection.*

## ROC Curve Analyses

Further selection of potential indicator was performed by ROC analysis. Using different models, the value of the sensitivity and the area under the ROC curve (AUC) of these biomarker panels were both ≥0.8 (**Figure 4A**). An area of 1 represents a "perfect" test, so we obtained "good" efficiency for a clinical diagnosis for this set of metabolite biomarkers.

## Analyses of Metabolic Pathways

We identified several pathways that may be significant (raw $p < 0.5$, impact >0) (**Table 3**). Nine pathways had the greatest significance: aminoacyl-tRNA biosynthesis: valine, leucine, and isoleucine biosynthesis; propanoate metabolism; phenylalanine metabolism; cysteine and methionine metabolism; alanine, aspartate, and glutamate metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; tyrosine metabolism; and glycerolipid metabolism (**Figure 4B**). The detailed results of the pathway analyses are shown in **Table 3**, with a summary shown in **Figure 5**.

# DISCUSSION

Moyamoya disease is a multifactorial disorder that likely presents unique pathophysiological profiles in each individual. Genetics, proteomics, and imaging have been used to discover markers for MMD (Araki et al., 2010; Maruwaka et al., 2015). However, there is no existing marker that could aid the diagnosis of MMD.

GC–MS-based metabolomics was applied to profile metabolic biomarkers in the serum of 41 MMD patients and 58 HCs. Our study is the first to identify serum biomarkers in MMD patients. Novel biomarkers may assist researchers in understanding MMD pathogenesis and provide new therapeutic strategies.

Owing to high separation efficiency, sensitivity, specificity, and throughput, as well as the development of various derivatization technologies, GC–MS has become important for the application of metabolomics. However, a single technology that can cover all metabolites in biological systems is lacking, and each technology has its own technical advantages and disadvantages. We discovered more metabolites than that in an NMR study of CSF samples of MMD patients (Jeon et al., 2015). There are several reasons for this diffidence, such as different matrix, different controls, and different instrument analysis. Twenty-four discriminating metabolites between HCs and MMD patients were identified using GC–MS analysis and used to establish a biomarker panel using logistic regression. The AUC was ≥0.8 for all samples (**Figure 4A**), indicating a "good" classifier of MMD patients and HCs. Furthermore, the mechanism of MMD could be obtained by assessing the pathways underlying these biomarkers.

As seen in **Figure 3B**, these discriminating metabolites were involved in nine significantly different pathways related to the metabolism of amino acids, lipids, carbohydrates, and translation of carbohydrates.

Overall, 20% of the human body is composed of amino acids and their metabolites that are basic substrates and regulators in

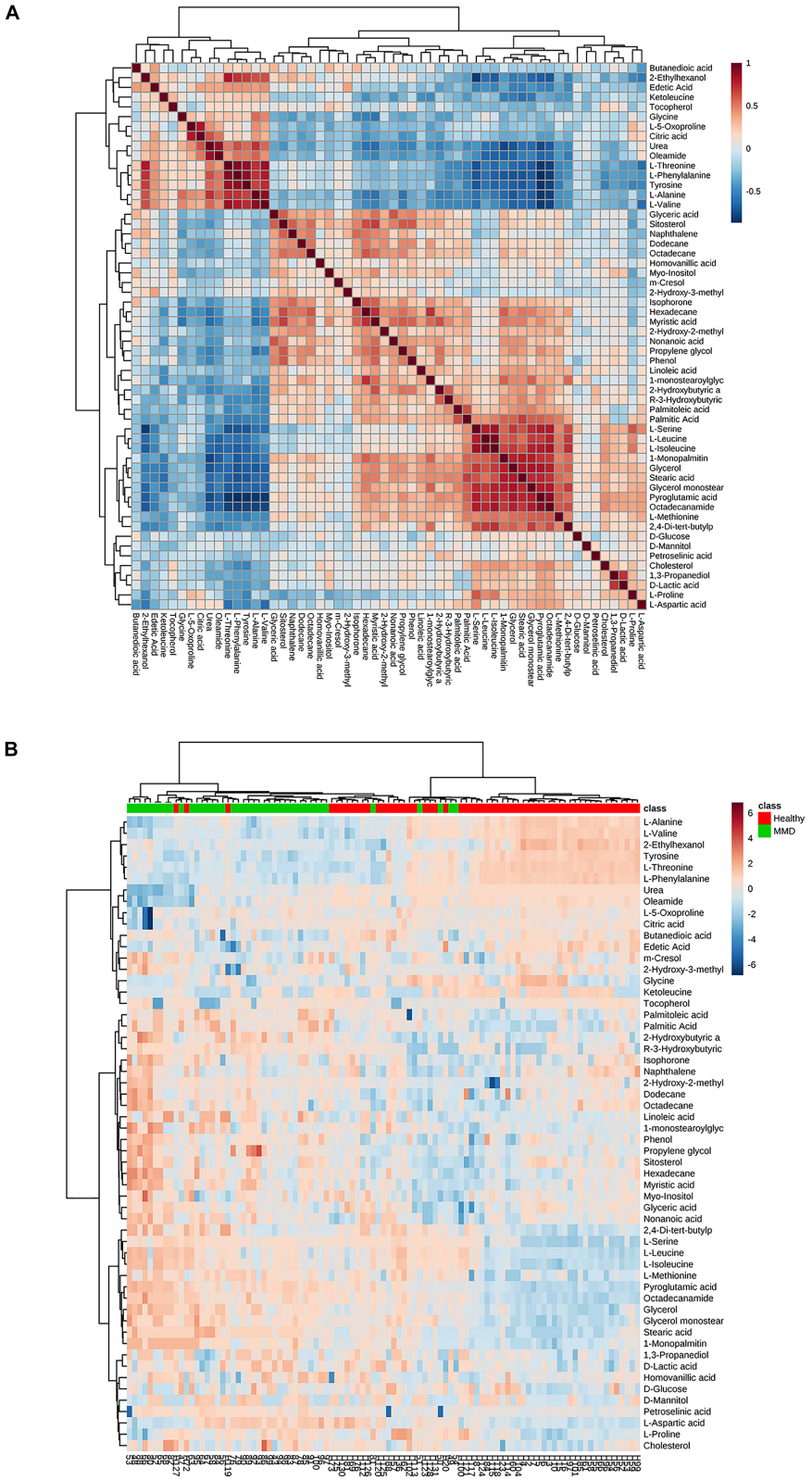


**FIGURE 3 | (A)** Correlation analysis of the differential metabolites in moyamoya disease (MMD) patients and healthy controls (HCs). **(B)** Heat map for identified metabolites in MMD patients and HCs. The color of each section is proportional to the significance of change of metabolites (red, upregulated; blue, downregulated). Rows, samples; columns, metabolites.

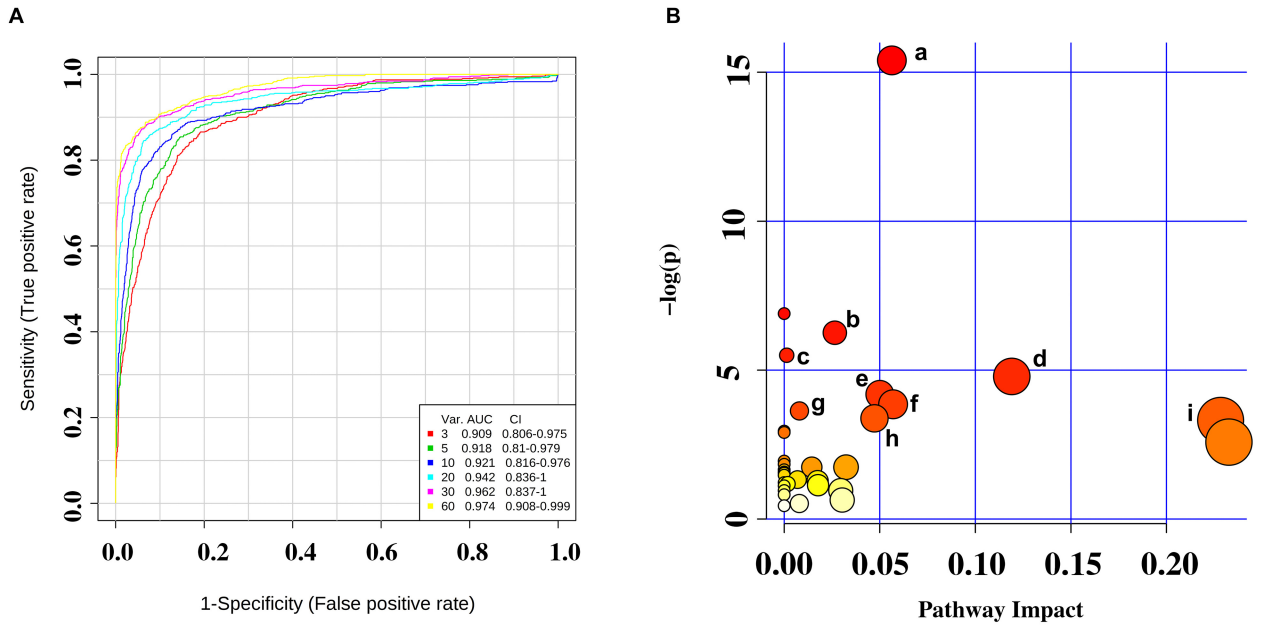


**FIGURE 4 | (A)** Receiver operating characteristic (ROC) curves were for the sets of biomarker metabolites. **(B)** Summary of pathway analysis with MetaboAnalyst 4.0. (a) Aminoacyl-tRNA biosynthesis; (b) valine, leucine, and isoleucine biosynthesis; (c) propanoate metabolism; (d) phenylalanine metabolism; (e) cysteine and methionine metabolism; (f) alanine, aspartate, and glutamate metabolism; (g) phenylalanine, tyrosine, and tryptophan biosynthesis; (h) tyrosine metabolism; and (i) glycerolipid metabolism.

**TABLE 3 |** Results from pathway analysis from MetaboAnalyst 4.0.

| Pathway name | Total | Expected | Hits | Raw *p* | Holm adjust | FDR | Impact |
|---|---|---|---|---|---|---|---|
| Aminoacyl-tRNA biosynthesis | 75 | 0.72 | 8 | 2.05E-07 | 1.64E-05 | 1.64E-05 | 5.63E-02 |
| Valine, leucine, and isoleucine biosynthesis | 27 | 0.26 | 3 | 1.92E-03 | 1.50E-01 | 5.12E-02 | 2.65E-02 |
| Propanoate metabolism | 35 | 0.33 | 3 | 4.09E-03 | 3.15E-01 | 8.18E-02 | 1.34E-03 |
| Phenylalanine metabolism | 45 | 0.43 | 3 | 8.33E-03 | 6.33E-01 | 1.33E-01 | 1.19E-01 |
| Cysteine and methionine metabolism | 56 | 0.53 | 3 | 1.52E-02 | 1.00E + 00 | 2.03E-01 | 5.00E-02 |
| Alanine, aspartate, and glutamate metabolism | 24 | 0.23 | 2 | 2.12E-02 | 1.00E + 00 | 2.43E-01 | 5.70E-02 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 27 | 0.26 | 2 | 2.65E-02 | 1.00E + 00 | 2.65E-01 | 8.00E-03 |
| Tyrosine metabolism | 76 | 0.73 | 3 | 3.40E-02 | 1.00E + 00 | 2.91E-01 | 4.72E-02 |
| Glycerolipid metabolism | 32 | 0.31 | 2 | 3.64E-02 | 1.00E + 00 | 2.91E-01 | 2.28E-01 |

*The Raw p is the original p value calculated from the enrichment analysis; the Holm p is the p value adjusted by Holm–Bonferroni method; the FDR p is the p value adjusted using false discovery rate.*

many metabolic pathways. Amino acid levels in patients with various diseases often differ from those of healthy individuals. Alterations in plasma concentrations of amino acids have been reported in liver fibrosis, non-small-cell lung cancer, aortic dissection, first-episode psychosis, and type-2 diabetes mellitus in patients with coronary artery disease (Yang et al., 2013; Leppik et al., 2018).

We found that levels of a panel of amino acids (L-alanine, L-isoleucine, L-valine, L-serine, L-threonine, L-methionine, L-phenylalanine, and L-tyrosine) were significantly different in MMD patients relative to HCs. Levels of L-alanine, L-valine, L-threonine, L-phenylalanine, and L-tyrosine were decreased in MMD patients, whereas L-isoleucine, L-serine, and L-methionine levels were increased. Such alterations of several amino acids suggest that amino acid metabolism is disturbed in MMD.

The synthesis of L-alanine is derived from pyruvate by alanine aminotransferase directly involving in gluconeogenesis and the alanine–glucose cycle and regulates glucose metabolism (Chen et al., 2017). Like gamma-aminobutyric acid, taurine, and glycine, L-alanine is an inhibitory neurotransmitter in the brain and is involved in lymphocyte reproduction and immunity. L-Alanine is disturbed in serum of MMD patients, but the association between L-alanine and MMD has not been investigated. Our study did not elucidate how altered levels of L-alanine affect MMD development; more studies are needed. L-Isoleucine and L-valine are branched chain amino acids (BCCAs). They are critical to human life and are particularly involved in stress, energy generation, and muscle metabolism (Zheng et al., 2017; Zhenyukh et al., 2017). The biosynthesis of L-isoleucine is initiated by the L-threonine deaminase reaction, whereas the

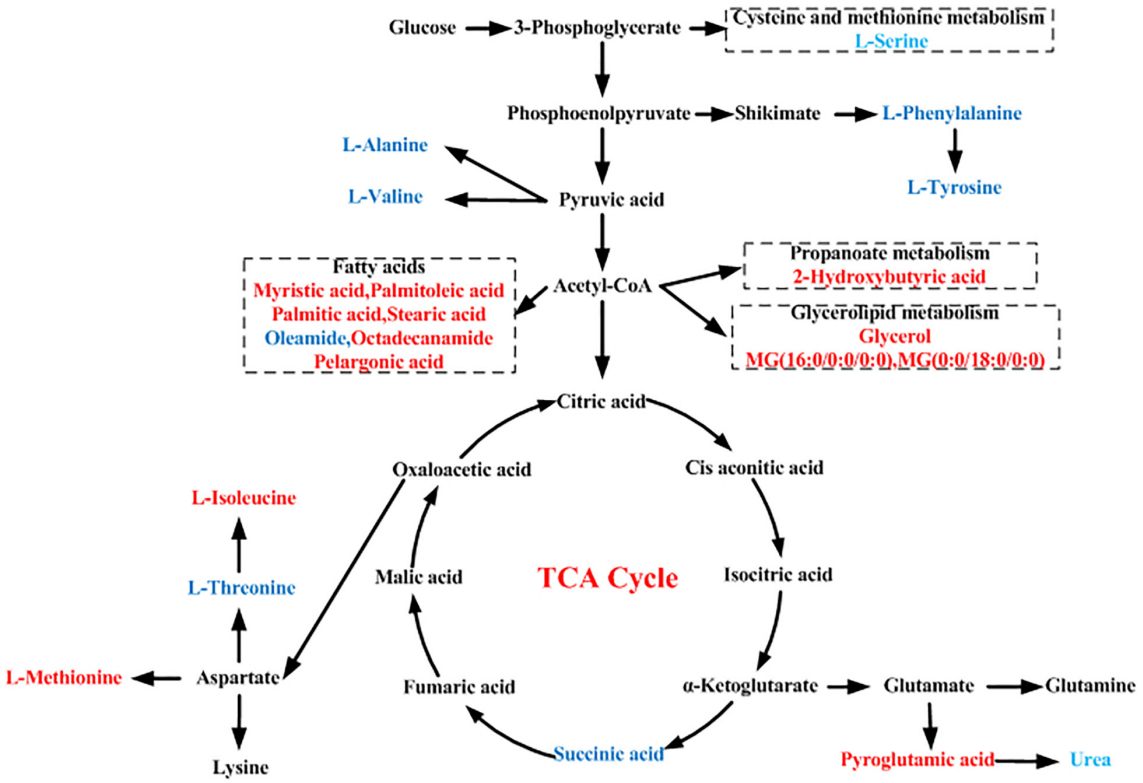


**FIGURE 5 |** Schematic diagram of the proposed metabolic pathways in moyamoya disease (MMD) compared to healthy controls (HCs). Red and blue represent up- and downregulated metabolites, respectively.

pathway toward L-valine starts from pyruvate. Acetohydroxyacid synthase, ketol acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), and branched chain amino transferase (BCAT) were the four enzymes operating in L-isoleucine and L-valine biosynthesis (Galili et al., 2016). Abnormal changes in L-isoleucine and L-valine levels have been documented in first-episode psychosis (Leppik et al., 2018). Our study suggested that abnormal changes in L-isoleucine and L-valine levels are also associated with MMD.

Like glutamate, aspartate, and glycine, L-serine can act as an excitatory and inhibitory neurotransmitter (Yu et al., 2017; Martin et al., 2018). As seen in **Figure 5**, phosphoenolpyruvate (PEP) is one of the precursors to the shikimate pathway; in addition, PEP is also derived from oxaloacetate by phosphoenolpyruvate carboxykinase (PEPCK) in glycolysis; thus, they are linked to each other. L-Serine may be derived from the biosynthesis of the glycolytic intermediate 3-phosphoglycerate, which participates in cell proliferation and is necessary for specific functions in the central nervous system. Altered levels of L-serine in patients with psychiatric disorders underscore the amino acid's importance in brain development and function. Thus, L-serine level alterations may be involved in MMD pathogenesis by affecting brain development and function. L-Threonine is an essential amino acid in humans, and severe deficiency causes neurological dysfunction (Liu et al., 2010). In our study, L-threonine level was lower in serum of MMD patients compared to HCs, indicating that L-threonine was altered in serum of MMD patients. Alterations in the ratio of L-tyrosine and L-phenylalanine levels could be a sign of compromised function of the dopaminergic system, which may be partly associated with MMD pathogenesis.

Collectively, our findings indicate that altered levels of amino acids could be linked to MMD. The precise mechanism by which amino acid levels influence the genesis and development of MMD should be investigated further. Pyroglutamate is converted to urea over glutamate and allantoin reactions; additionally, L-arginine could be converted to urea via urea cycle. Altered levels of pyroglutamate and urea were found in MMD. A panel of fatty acids (succinic acid, myristic acid, palmitoleic acid, palmitic acid, stearic acid, oleamide, octadecanamide, and pelargonic acid) could be used to distinguish between MMD patients and HCs. Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle (Bechthold et al., 2010; Lauritzen et al., 2014). Levels of succinic acid were significantly decreased in MMD patients compared with HCs. Lower levels of TCA-cycle intermediates may indicate alterations in the cycle. The TCA cycle is the core of cellular respiratory machinery and produces energy to power manufacture of compounds needed to defend against oxidative stress. Oxidation of fatty acids in mitochondria is responsible for approximately half of the total amount of adenosine triphosphate generated. Fatty acids also serve as the "building blocks" of cellular membranes after their esterification into phospholipids

and are involved in signal transduction. Altered levels of fatty acids would lead to decreased energy production. However, whether such lower energy levels are associated with an increased risk of MMD is unknown.

The OPLS-DA method was used to analyze metabolic features and maximize discrimination between classes of compounds (Westerhuis et al., 2010). This approach reduced the effects of variability of non-relevant metabolites and helped identify serum metabolites contributing to differences between MMD patients and HCs. The reliability of OPLS-DA results was confirmed by correlation analyses and heatmaps.

Our study had two main limitations. First, MMD is an uncommon disease. We recruited 41 MMD patients, which may have introduced selection bias because of interindividual differences. Second, we only used a metabolomics approach, and the possibility that single-omic data restricted interpretation of our results cannot be excluded. Proteomics and genomics analyses are needed to confirm our findings.

In summary, we used a GC–MS platform to characterize the metabolic profiles of serum from MMD patients. Our analysis revealed important candidate metabolic biomarkers for MMD. Verification and validation studies with larger independent samples are necessary to demonstrate the utility of these metabolites as potential disease markers and to elucidate the pathophysiological mechanisms underlying MMD.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

Our work was carried out according to the Declaration of Helsinki. All participants provided written informed consent to allow use of their samples in these analyses, and our study protocol was reviewed and approved by the Medical Ethics Committee of the Jining First People's Hospital (Jining, China) (No. 20170021).

## AUTHOR CONTRIBUTIONS

PJ and CC conception and design. CW, JZ, and WH acquisition of the data. CG and PJ analysis and interpretation of the data. CG drafting the manuscript. PJ and FJ critically revising the manuscript. CG, PJ, CC, and FJ reviewed submitted version of manuscript. PJ approved the final version of the manuscript on behalf of all authors. CG, DC, and YG statistical analysis. PJ study supervision.

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# A Novel 7-Days Prolonged Dietary Deprivation Regimen Improves ALT and UA After 3–6 Months Refeeding, Indicating Therapeutic Potential

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**Objectives:** The aim of this study was to evaluate a total fasting regimen assisted by a novel prebiotic, Flexible Abrosia (FA), in more than 7 days of continual dietary deprivation (7D-CDD). Our analysis included basic physical examinations, bioelectrical impedance analysis, and clinical lab and ELISA analysis in normal volunteers.

**Methods:** Seven healthy subjects with normal body weight participated in 7D-CDD with the assistance of a specially designed probiotic. Individuals were assigned to take FA (113.4 KJ/10 g) at each mealtime to avoid possible injuries to intestinal flora and smooth the hunger sensation. During 7D-CDD, the subjects were advised to avoid any food intake, especially carbohydrates, except for drinking plentiful amounts of water. The examination samples were collected before CDD as self-control, at 7 days fasting, and after 7∼14 days of refeeding. Three subjects were also tested after 6-m refeeding.

**Results:** The FA-CDD regimen significantly decreased suffering from starvation, with tolerable hunger sensations during the treatment. With the addition of daily mineral electrolytes, the subjects not only passed through the entire 7D-CDD regimen but also succeed in 12∼13 days total fasting in two subjects. There was a significant reduction in blood glucose, insulin, and high-density lipoprotein levels during fasting, and the blood concentrations of uric acid (UA), alanine aminotransferase (ALT), and creatine kinase (CK) were increased. However, after more than 2 months of refeeding, the disease markers ALT, GOT, and CK either remained stable or were slightly downregulated compared to their initial D0 control level.

**Conclusion:** Our experiment has supplied the first positive evidence that, with the assistance of a daily nutritional supply of around 100 kcal total calories to their intestinal flora, human subjects were able to tolerate hunger sensations. We have found that, although 7D-CDD induced increases in UA, CK, and transferases during fasting, refeeding led the markers to become either down-regulated or unchanged compared to their initial levels. This phenomenon was further confirmed in longer-term (6 m) recovery.

Our results failed to support the hypothesis that fasting induced liver damage, since ALT, GOT, and CK remained low after longer-term refeeding. Our findings indicate that the 7D-CDD regimen might be practical and that it might be valuable to design larger clinical fasting trials for improvement of health strategy-targeting in metabolic disorders.

**Keywords: flexible abrosia (FA), continual dietary deprivation (CDD), alanine aminotransferase (ALT), glutamic oxalacetic transaminase (GOT), creatine kinase (CK)**

## BACKGROUND, MOTIVATION, AND OBJECTIVES

Fasting paradigms have been proved to be more effective and efficient at achieving health benefits than are other types of dietary paradigms, including dietary or calorie restriction (DR/CR) (1). Fasting in higher species has only been studied extensively as short-term or intermittent fasting schedules (IF) (2). Among various forms of IF, a recent report considered alternate day fasting (ADF), defined as strict 36-h periods without caloric intake ("fast days") followed by 12-h intervals with *ad libitum* food consumption ("feast days"), to be one of the most extreme dietary interventions (3). This study demonstrated an improvement in markers of general health in healthy, middle-aged humans without adverse effects, even after >6 months' continuous application. Eventually, the treatment caused a 37% calorie reduction on average plus improved cardiovascular markers, reduced fat mass, improved fat-to-lean ratio, and increased beta-hydroxybutyrate, even on non-fasting days. Other applications of alternative fasting schedules have been reported that used a very low-calorie diet (200∼500 kcal/day) to substitute for complete fasting for 1 to 3 weeks or a year for weight management, disease prevention, and chemotherapy facilitation (2, 4). The health improvement benefit of those paradigms was obvious. However, an individual must face months or even years of low-calorie dieting or fasting, which seemed to be less efficient and could be quite a long, uncomfortable, and inconvenient practice in human society.

It has been reported that some individuals could survive continual no-food fasting from 40 days (5, 6) or even more than a year (7, 8). Historical books from China and India also record some examples of months to years of absolutely no food status (bigu) as a way to attain longevity and spiritual distillation (9, 10). However, there was a lack of detailed records of any practical methodology and reports on measurable health-related impacts. To date, there have been three types of systematic studies related to prolonged fasting: (1) those initiated during 1950 and 1970 that created Guinness World Records of total fasting for more than a year (2, 7, 8) a series of metabolism studies led by G.F. Cahill Jr. involving prolonged starvation that led to the establishment of ketone-body metabolism during fasting (3, 11) a therapeutic fasting protocol established by O. Buchinger Jr. in 1952 that used fresh fruit or vegetable juice servings as alternative energy supplies, which led to an average total calorie intake of 200–250 kcal per day to assist an up to 21-days incomplete fasting status (4). Most other prolonged fasting studies also focused on metabolic studies and found ketone body metabolism, which eventually led to alternative fasting protocols (12, 13). There has been insufficient attempts to build up a proper, more tolerable prolonged total fasting regime as a health-improvement practice for society since the dawn of the new century due to the following concerns: (1) safety issues—whether prolonged fasting is safe for practice in human society; (2) tolerance and discomfort during the entire total-fasting procedure. In fact, during the 60∼80 s of the last century, some safety concerns were reported regarding prolonged total fasting. They were usually due to extensive complete dietary deprivation. Usually, if the total fasting persisted for more than 40 days, negative consequences might start to happen (14, 15).

Based on the above historical realities, we have designed a special natural form of prebiotic intervention, Flexible Abrosia (FA, with 113.4 KJ in each 10-g pack), which consists of food-grade polysaccharose, which can be mostly absorbed by bacteria rather than the human host (16, 17). It was hypothesized that long-term DD leads gut microbiota to resort to host-secreted mucus glycoproteins as a nutrient source, which causes erosion of the colonic mucus barrier and hunger pangs (18). We have applied a daily intake of 10 g FA at three mealtimes (3 × 113.4 KJ, which is <100 kcal per day) under a fasting paradigm. The calorie supply was not targeted at maintaining basic energy supply in human; rather, it was designed to reduce the starvation of intestinal flora, which might cause injury to the human digestive system. Compared with previously reported fasting-mimicking diet (FMD, 3,000–4,600 kJ per day) facilitated-fasting paradigms (12) and Buchinger's Periodic Fasting intervention, which contained fruit juice to maintain a minimum calorie intake of 200 kcal (4), our FA-facilitated continual dietary deprivation paradigm (FA-CDD, with <100 kcal non-human-absorbable daily calorie intake) would be close to a no-calorie intake protocol. Considering the life-threatening negative consequences that usually occur after more than 40 days' complete fasting (14, 15, 19), we have suggested a practical fasting period of 1 week for the entire FA-CDD treatment. We also found that maintaining the real no food status would make the subject more efficient in overcoming the hunger sensation. Since then, the FA functional food supplement has assisted up to 1,000 volunteers to achieve the FA-CDD paradigm with tolerable sensations of hunger for a continual 7 days for the purpose of weight control or chronic disease treatment (20). Some of the volunteers voluntarily chose to extend to 14-d total fasting and experienced tolerable and favorable consequences (21).

Here, we collect seven individual subjects with typical 7D-CDD experience and focus on reporting special phenomena of and scientific evidence on the 7-D FA-CDD. Our report

will supply some preliminary results of this paradigm prior to applying more sophisticated clinical trials to further evaluate the potential health improvement value of its application in human society.

# MATERIALS AND METHODS

## Study Design and Participants

A complete clinical trial registration has been deposited with the Chinese clinical trial registration organization (http://www.chictr.org.cn with registration # ChiCTR-OOC-17010377). Approval of the study protocol was given by the University of Henan Human Research Protection Program under the guidance of the China Association for Ethical Studies. The protocol was also recorded with the Medical Ethics Committee of Henan Medical Association of Henan Province, and the entire clinical study was under the supervision of the ethics board of The First Affiliated Hospital of Henan University. Before initiating the program, signed informed consent was obtained before everyone participated, and history, physical, electrocardiogram, laboratory, physical, and ultrasonic exams were also performed as pre-med checks. Inclusion criteria were as follows: (i) volunteers from the hospital staff, including doctors, nurses, lab and medical technicians, etc., and their relatives, (ii) age 21–65 years, (iii) absence of any exclusionary factor among the individuals participating, such as active medical or psychiatric problems, history of heart disease and potential heart problems such as heart failure, myocardial infarction, and cardiac arrhythmia, renal dysfunction, serious blood clots, intestinal obstruction or ulcer, or type-1 diabetes patients with islet dysfunction (18).

## Procedures

Volunteers were recruited and introduced to the FA-CDD program. Before the trial started, the individuals received medical and laboratory examinations, including collections of serum, plasma, urine, and feces samples. FA-CDD involved daily oral application of a solid beverage of Flexible Abrosia (FA, Beijing Cloud Medical International Technology, Inc. China) 10 g/bag/person per treatment at three mealtimes every day on *an outpatient basis* during the fasting period. *The ingredients of FA were designed to include dietary fiber and cordyceps polysaccharide, ganoderma lucidum polysaccharide, and hericium erinaceus polysaccharide (18), which were regarded as bacteria- but not human-consumed saccharides (22). The National Food Inspection Center of China has reported the analyzed energy of 10 g FA as 113.4 KJ (27 kcal), which indicated that even if the calories from each treatment were completely absorbed by the human being, it would be <100 kcal daily in total, significantly less than recently reported low-calorie (500 kcal per day) intake in the treatment of cancer (1).*

During the 7D-CDD period, individuals were advised to avoid any food intake, especially carbohydrates, and only to drink plenty of water and keep mineral electrolyte intake and vitamin supply constant. During the first 3∼5 days, to overcome extreme hunger sensations and cravings for food, some individuals might consume a few pieces of fruits such as cucumber or tomato. Further, participants could also consume either 1 bag of 375 mg potassium/400 mg magnesium (DAS Gesunde Plus, Deutschland) or 1 pellet of 1.08 g (K = 10 mEq) potassium citrate extended-release tablets (Dawnrays China) every day during extreme hunger periods in the day time, which could further ameliorate fasting-induced mineral loss and reduce peristaltic pangs of the smooth muscles of the gut. According to the written informed consent forms, the individuals could quit the ongoing program at any time and at any step of the experiment without giving an explanation.

During the entire experimental fasting period, concomitant hunger sensations and tolerance limits categorized into different levels were recorded in a questionnaire supplied by the project administration. Physical and neurological examination, weight, blood and urine chemistries, electrocardiogram, bioelectrical impedance-directed body composition analysis, and ultrasound exam were checked at three time-points by the medical authorities of the hospital: (1) before the regimen was initiated (control baseline—*ad libitum*), (2) 7th day of fasting (7D-CDD), and (3) after recovery with food intake for 7–14 days or (4) after recovery with food intake for more than 3 months to as much as 6 months (**Figure 1**). The medical and basic laboratory exams were performed in The First Affiliated Hospital of Henan University. The extra serum and plasma samples were collected and stored under −80°C for future molecular and mechanistic analyses. The ELISA and metagenomics tests were performed at Beijing Institute of Radiation Medicine.

Bioelectrical impedance analysis (BEIA) was performed using the InBody 720 Body Composition Analyzer at the Nutritional Department of the First Affiliated Hospital of Henan University. InBody 720 has received the approval of the FDA to analyze impedance, reactance, and resistance. The instrument expresses the relationships of water, protein, muscle, mineral, and fat content, and more, rather than just measuring Body Mass Index (BMI). The device can determine the weight of lean muscle tissue in each limb, water content, percentage body fat, mineral content, protein content, and visceral fat levels. Measurements at five time points during the CDD experiment data were supplied via a full-color print-out and were analyzed by a professional analyst at the hospital.

## Biological Sample Analysis

In addition to the routine clinical biochemical and blood/urine tests, we also performed molecular and biochemical tests on biological samples collected during the CDD experiment:

1) Plasma or serum preparations. Plasma or serum was centrifuged at 3,000 rpm and analyzed according the protocol of the hospital.
2) Serum factor measurements. TNF-α (E-EL-H1205c) and Insulin-like growth factor 1 (E-EL-H0086c) levels in serum were detected with ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China http://www.elabscience.cn/). The optical density was read at 450 nm using a microtiter plate reader.

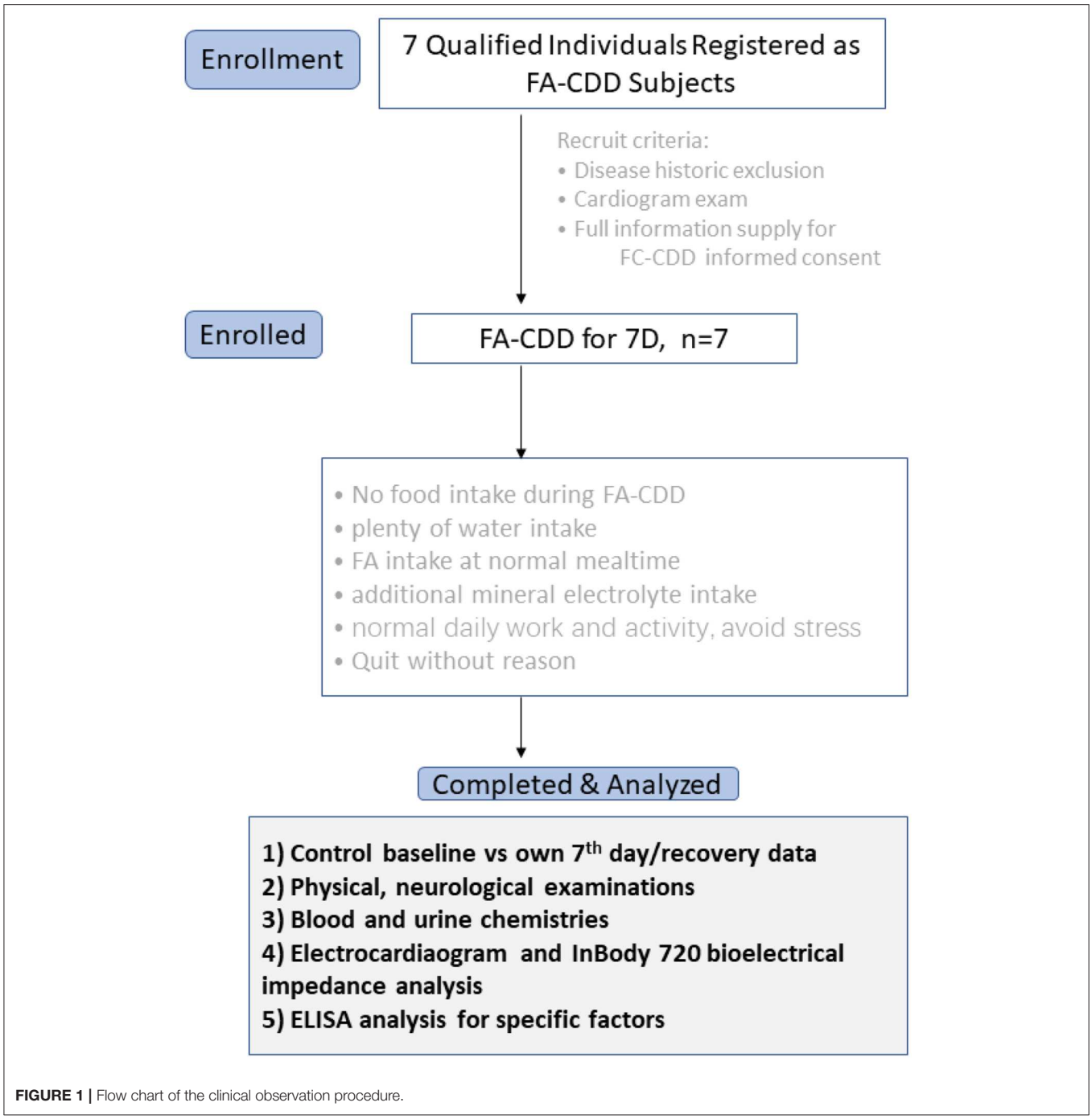


**FIGURE 1 |** Flow chart of the clinical observation procedure.

## Statistical Analysis

One-way ANOVA with repeated measures was applied to data from the individuals and plotted in GraphPad Prism 8.0.1 software (GraphPad Software, Inc.). Maintaining prolonged fasting was not an easy task for all participants. To accurately reveal the effect of 7D-CDD and restrict the variation in individuals' control baselines, we used Dunnett's multiple comparisons test to evaluate the fasting and refeeding parameters by comparing them with the individual's own pre-fasting (0D-CDD) point as control. We also reported multiple comparisons between fasting and refeeding procedures. Among the seven subjects, there were two males and one female subject for whom the refeeding sample collections were achieved for more than 6 months. The individual parameters of different treatments are reported in **Table 1**.

**TABLE 1 |** Physical, bioelectrical impedance (BEIA) and biochemical results under Flexible Abrosia- facilitated 7D Continual Dietary Deprivation (FA-CDD).

| Female Subjects | Ctrl 0D | Fast 7D | Refeed 7D | Ctrl 0D | Fast 7D | Refeed 7D | Ctrl 0D | Fast 7D | Refeed 6M | Control 0D | Fasting 7D | Refeed 20D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight (kg) | 67.20 | 62.10 | 65.50 | 65.10 | 60.10 | 63.40 | 76.40 | 71.40 | 74.10 | 70.20 | 71.40 | 74.10 |
| Basal Metabolic Rate (%) | 1245.62 | 1160.42 | 1251.43 | 1355.50 | 1266.93 | 1341.54 | 1351.02 | 1253.05 | 1334.68 | 1351.02 | 1253.05 | 1334.68 |
| Skeletal muscle mass (kg) | 21.82 | 19.65 | 21.89 | 25.46 | 23.20 | 24.83 | 24.93 | 22.53 | 24.71 | 24.93 | 22.53 | 24.71 |
| Body fats (kg) | 26.70 | 25.50 | 24.70 | 19.50 | 18.60 | 18.40 | 31.00 | 30.50 | 29.40 | 31.00 | 30.50 | 29.40 |
| Trunk muscle mass (kg) | 18.26 | 16.88 | 18.10 | 20.98 | 19.23 | 20.23 | 20.12 | 18.83 | 20.03 | 20.12 | 18.83 | 20.03 |
| Body mass parameters (kg $m^{-2}$) | 26.92 | 24.88 | 26.24 | 26.41 | 24.38 | 25.72 | 29.84 | 27.89 | 28.95 | 29.84 | 27.89 | 28.95 |
| **Uric Acid (μmol/L)** | **255.80** | **688.00** | **61.00** | **296.20** | **339.40** | **335.10** | **302.00** | **689.20** | **238.50** | **258.00** | **494.80** | **188.50** |
| **Creatine kinase U/L** | **68.00** | **121** | **92.00** | **84.00** | **96.00** | **57.00** | **87.00** | **97.00** | **96.00** | **125.00** | **192.00** | **121.00** |
| **Alanine aminotransferase (U/L)** | **13.30** | **17.20** | **14.00** | **70.40** | **21.80** | **45.80** | **21.70** | **143.90** | **22.10** | **25.00** | **30.50** | **18.10** |
| **Glutamic Oxalacetic Transaminase (U/L)** | **24.00** | **32.00** | **36.60** | **50.40** | **27.70** | **33.80** | **39.20** | **130.90** | **32.40** | **23.10** | **15.00** | **28.30** |
| | **3DBPS008, Male, Age 30** | | | **DBX009, Male, Age 45, fast 13D in total** | | | **3DBPS003, Male,Age 34, fast 12D in total** | | | | | |
| **Male Subjects** | **Ctrl 0D** | **Fast 7D** | **Refeed 14D** | **Ctrl 0D** | **Fast13D** | **Refeed 6M** | **Ctrl 0D** | **Fast 7D** | **Fast 12D** | **Refeed 16D** | **Refeed 6M** | |
| Weight (kg) | 87.20 | 83.00 | 82.10 | 85.00 | 78.00 | | 104.40 | 98.10 | 96.00 | 97.10 | | |
| Basal Metabolic Rate (%) | 1690.52 | 1654.12 | 1655.27 | 1414.2 | 1351.6 | | 1912.16 | 1832.55 | 1784.27 | 1870.56 | | |
| Skeletal muscle mass (kg) | 35.35 | 34.23 | 34.13 | 36.7 | 34.9 | | 41.05 | 39.14 | 37.44 | 39.57 | | |
| Body fats (kg) | 26.10 | 23.60 | 22.60 | 38.3 | 33.8 | | 33.00 | 30.40 | 30.50 | 27.60 | | |
| Trunk muscle mass (kg) | 27.85 | 27.05 | 26.72 | 31.83 | 30.71 | | 32.36 | 30.81 | 29.44 | 30.81 | | |
| Body mass parameters (kg $m^{-2}$) | 29.48 | 28.06 | 27.75 | 32.6 | 29.8 | | 32.22 | 30.28 | 29.63 | 29.97 | | |
| **Uric Acid (μmol/L)** | **523.10** | **900.80** | **429.70** | **325.4** | **297.4** | **385.80** | **369.00** | **901.40** | **952.00** | **289.60** | **378.30** | |
| **Creatine kinase U/L** | **201.00** | **196** | **157** | **145** | **165** | **120** | **259.00** | **297** | **267** | **191** | **203.00** | |
| **Alanine aminotransferase (U/L)** | **145.40** | **176.90** | **85.60** | **19.00** | **28.50** | **18.40** | **57.40** | **48.90** | **43.60** | **65.10** | **52.70** | |
| **Glutamic Oxalacetic Transaminase (U/L)** | **74.00** | **97.00** | **34.70** | **36.10** | **32.10** | **28.90** | **33.50** | **32.30** | **33.30** | **35.60** | **25.10** | |

*Blood sample collection and BEIA tests were performed under different timing schedules for subjects DBB1020 and 3DBPS003.*
*Therefore, the timing points of biochemical examination (in bold font) and BEIA were not the same.*
*Subjects 3DBPS003 and DBX009 automatically experienced 12D and 13D total fasting and accepted biochemical examination after 54 d and 6 m refeeding recovery, respectively.*

# RESULTS

## Physiological and Bioelectrical Impedance Analysis

Seven subjects successfully accomplished a 7D-CDD trial. Among them, both DBX009 and 3DBPS003 voluntarily experienced 13D and 12D total fasting under the assistance of FA and strict medical monitoring. During the experiment, the subject's physical experience was recorded in a diary table supplied by the project organizers, and their medical conditions were strictly monitored by hospital medical experts. Bioelectrical impedance analysis (BEIA) indicated that the body weight (BW) and body mass parameters reduced moderately during 7D-CDD (1∼2 lbs. per day) and that the refeeding recovered these parameters at a reasonable rate (**Table 1**). Meanwhile, the basal metabolic rate (BMR) had reduced to a lower speed at 7D fasting and recovered after refeeding (**Table 1**). However, it seems that muscle-related parameters (skeletal muscle mass and trunk muscle mass) recovered faster than fat-related parameters (Body fats and Visceral fat area) during refeeding (**Table 1**). These results might imply that the human under fasting may utilize more fat than muscle after longer-term fasting, which might explain the slow drop in BW during total fasting (**Table 1**).

## The Different Metabolic Patterns of Lipid and Protein During and After Fasting

Based on the different patterns of fat and protein indicated by BEIA, we further tested and analyzed blood biochemical lab results. At 7D fasting, there were increases in both total cholesterol and low-density lipoprotein (LDL-"bad") levels and decreases in triglyceride and high-density lipoprotein (HDL-"good"), which confirmed previous reports in cleansing cholesterol during fasting (**Figures 2A–D**, Total Cholesterol $F = 10.67$, $p = 0.0054$; LDL $F = 9.621$) (23). These results indicated the possibility of increased consumption of lipid-related energy supply (triglyceride and HDL) to support ketone bodies while enhancing side product clearance during autophagy of unhealthy tissue during starvation.

Meanwhile, although the protein metabolism levels tested showed a relative increase at 7D fasting, they were either slightly decreased or stabilized after refeeding (**Figures 2E–H**, Creatine Kinase $F = 6.27$, $p = 0.021$). The pattern of action in albumin (**Figure 2F**, $p > 0.05$), which free fatty acids attach and transport throughout the body for the alternative energy supply, might indicate activation of alternative energy supply from ketone metabolism. Accordingly, blood triglyceride levels, which involve fatty acid delivery via albumin, were decreased during fasting and quickly returned to control (**Figure 2A**, $P < 0.5$, limited sample size). Regarding carbohydrate metabolism, both glucose and insulin levels were down-regulated during 7D fasting as expected (**Figures 2I–L**). Prolonged fasting decreased insulin resistance (HOMA-IR), which was related to an increase in insulin sensitivity (**Figures 2I–K**, Glucose $F = 8.015$, $p = 0.0243$). Glycated hemoglobin (HbA1C), a form of hemoglobin used in clinic to identify the 3-month average plasma glucose concentration, remained unaffected during fasting and even after more than 2 months of recovery (**Figure 2L**, $p > 0.5$).

## Tissues With Unhealthy Status Seem to Be Preferentially Eliminated During Prolonged Fasting

While 7D-CDD induced a reduction in blood urea nitrogen (BUN), this prolonged fasting effectively increased both creatinine (Cr) and uric acid (UA) at 7D CDD (**Figures 3A–C**). Except for the markers of kidney function indicators, increase in daily Cr excretion, a breakdown byproduct in muscle metabolism, is usually related to high-protein diets, and a decrease in BUN may indicate the operation of protein recycling procedures (24). Besides, long-term fasting efficiently enhanced both UA and Cr blood levels, even under limited sample size, and these returned to normal levels, as reported previously (1) (**Figures 3A–C**, BUN $p = 0.085$; Creatinine $F = 7.14$, $p = 0.0093$; UA $F = 28.62$, $p = 0.0016$). In addition, both Alanine Aminotransferase (ALT) and Glutamic Oxalacetic Transaminase (GOT) showed a tendency to increase during fasting, as previously reported, but we found that the recovery from prolonged fasting led to a decrease in the tendency, though without statistical significance due to the limited sample size (**Figures 3G,H**). Correspondently, the results from creatine kinase (CK) also showed an increase during 7D-CDD (**Figure 2H**). CK is an enzyme found in the heart, liver, brain, and skeletal muscle. A higher level of CK usually indicates tissue damage, which releases it into circulation, and is regarded as a biomarker of heart failure, myocardial injury, or liver damage in clinic. In the unhealthy subjects whose markers showed abnormal liver or heart function, 7D prolonged fasting caused a higher increase in concentration. Longer-term recovery levels of ALT, GOT and CK from 7D-CDD have shown lower than control levels in most of the subjects. The results indicated that, after the subject recovered from 7D fasting, the values of each unhealthy subject tended to return at a level that was lower than prefasting control (**Table 1**).

Additionally, results from Insulin-like growth factor type I (IGF-I), tumor necrosis factor (TNF-α), and C-reactive protein (HS-CRP) showed significant changes during 7D fasting but returned to normal after refeeding. However, there was a lack of significance due to the limited sample size (**Figures 3D–F**, $p > 0.5$).

# DISCUSSION

Our 7D FA-CDD paradigm has been demonstrated to be a more tolerable and efficient regimen and is the most practical for long-term total fasting practice. The subjects' personal experience records indicated that, under the assistance of FA with proper mineral supply at every mealtime, subjects were more able to tolerate hunger sensations, with fewer pangs. Under the recommendation of drinking plenty of water to speed up the cleansing of metabolic wastes and sufficient mineral supply such as potassium and magnesium to release spasms of the smooth muscle of the digestive system, subjects experienced reasonable body weight decrease (about 1∼2 lb per day) plus moderate hunger sensations. In addition, the speed of body-weight reduction was moderate, which might be safely buffered

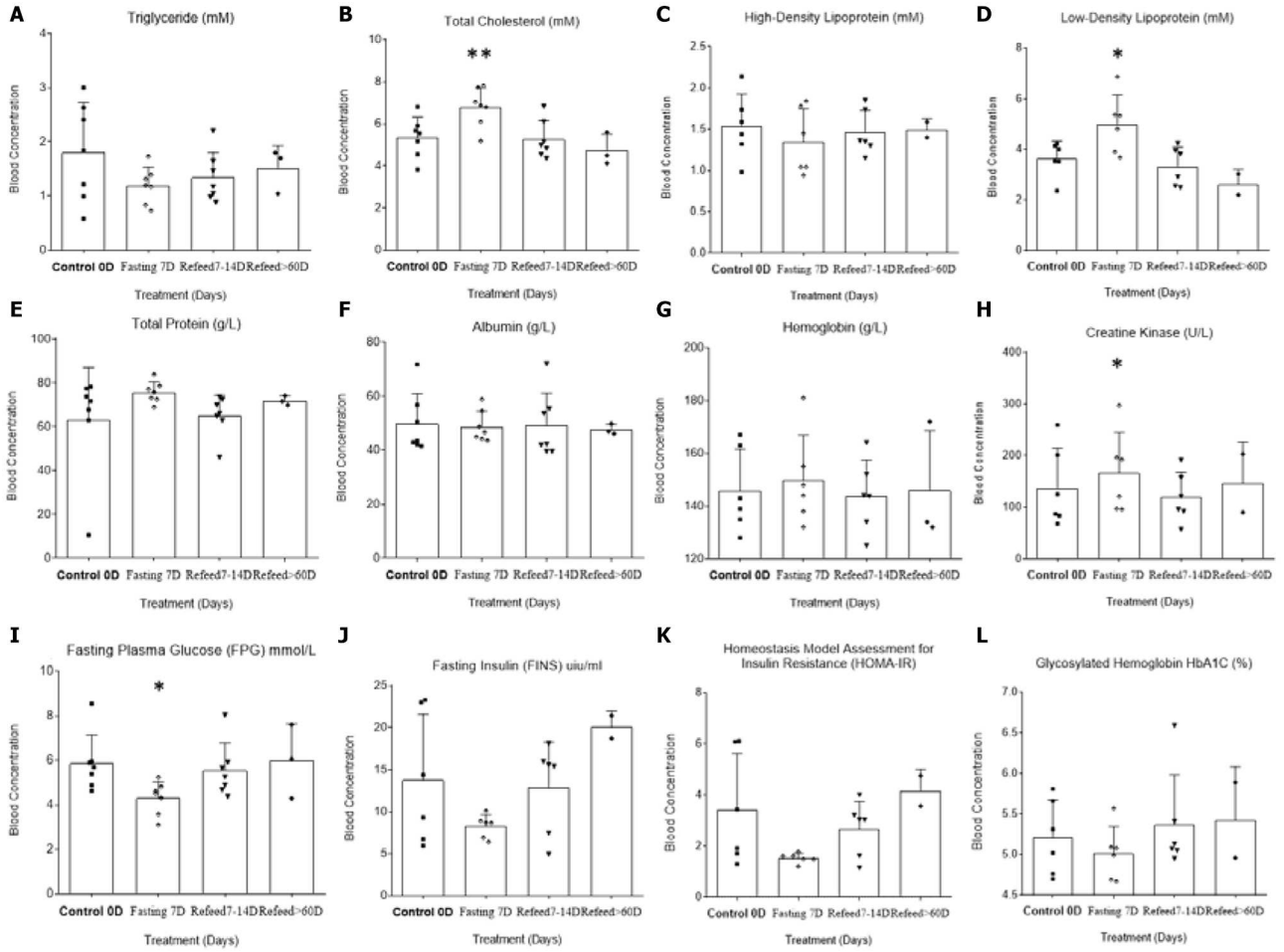


**FIGURE 2 |** Differential analysis among 0D, 7D fasting, and refeeding results from clinical laboratory tests. All differences in 0D vs. 7D fasting and either 15D or more than 2∼6 months' refeeding (three subjects donated biological samples over a longer recovery period) were analyzed. X-axes refer to the control 0D, fasting 7D, and refeeding 7–14D and >60D groups, respectively. Graph were created with GraphPad Prism 8.0.1 software using one-way ANOVA with repeated measures, and the dots around each column represent the actual values of group subjects on the specific treatments. The larger * symbol above the Fasting 7thD column indicates statistical significance (*$p < 0.05$, **$p < 0.01$; the actual values are represented in the manuscript).

and protected without showing dramatic dehydration, as in some current commercial body-weight reduction programs.

Of the factors tested, we found that triglyceride, HDL, glucose, insulin, BUN, and IGF-1 showed tendencies to decrease. On the other hand, cholesterol, LDL, total protein, hemoglobin, lactate dehydrogenase, creatinine, uric acid, TNF-α, Cr, CK, ALT, and GOT all showed tendencies to increase (**Figures 2**, **3**). The factors that were up-regulated during the long-term fasting seemed to be related to life-critical and beneficial survival nutrition, which the system would conserve sparingly. Those factors that were down-regulated during fasting were usually related with either alternative energy supply (such as total protein and hemoglobin), reserved system consumption (such as BUN, triglyceride, and IGF-1), or negative factors such as cholesterol, LDL, UA, TNF-α, ALT, GOT, and CK. It seems that the system automatically chose the beneficial and critical factors to reserve and selected the harmful elements to eliminate during total fasting, which might indicate that prolonged fasting-related autophagy is preferentially targeted to damaged or unhealthy tissue.

Fatty acid oxidation disorders (FAODs) has been reported to lead to deficient energy production and intermittent symptoms through increased β-oxidation, which may occur after 48 h of fasting in adults (25). Therefore, previous evidence assumed that 48-h long-term fasting may cause injury to liver function. Choline-deficient diets were reported to cause hepatic dysfunction and steatosis. However, most of the previous so-called prolonged fasting studies were related only with short-term animal models (either alternate day fasting or a shorter fasting period of <3 days). There was a lack of evidence for liver injury being induced by real prolonged fasting for more than 5 days in human. In fact, other clinical studies indicated that prolonged fasting only modestly diminished plasma choline but was not associated with signs of choline deficiency, such as perturbed lipoprotein secretion and liver

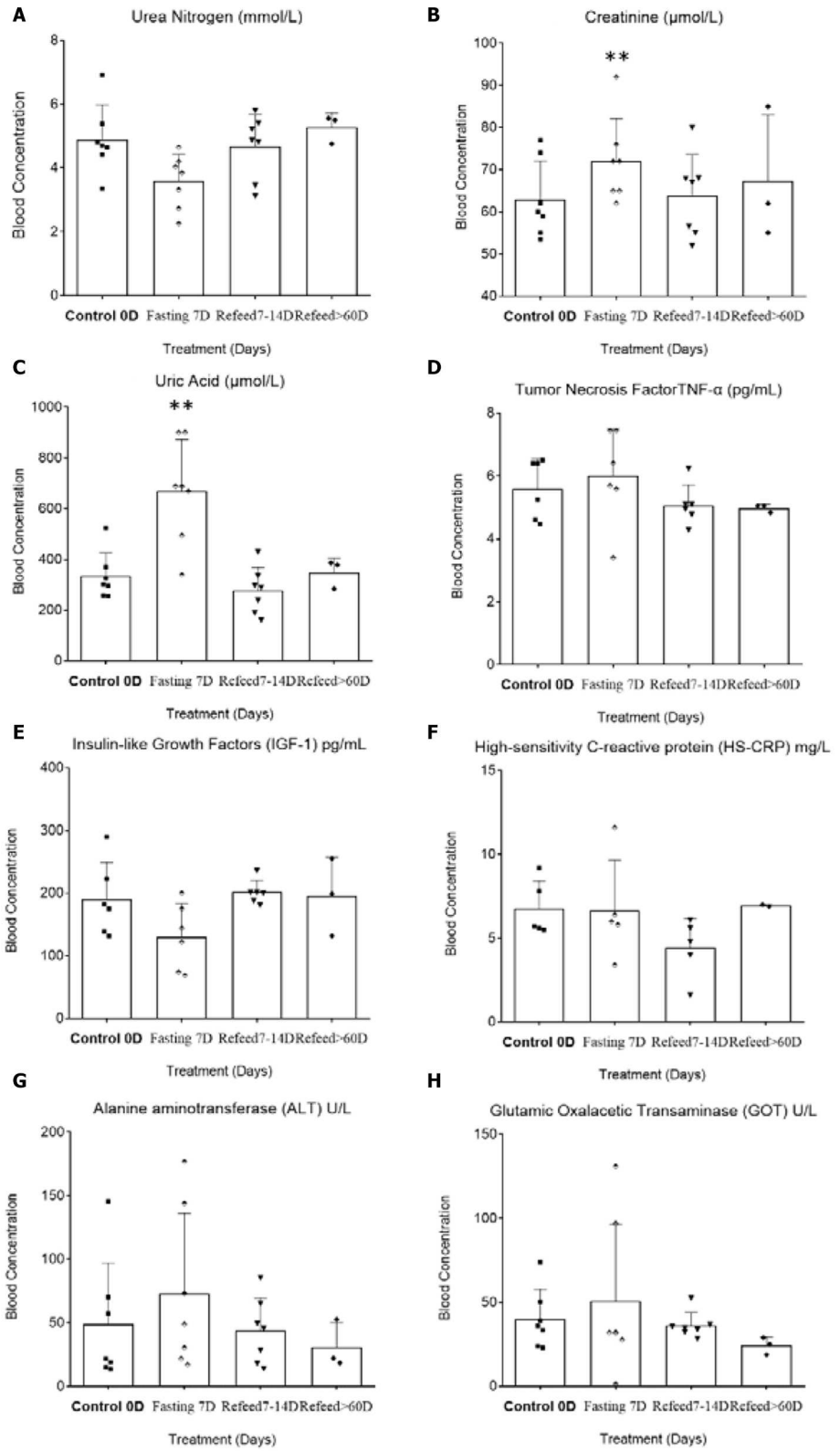


**FIGURE 3 |** Differential analysis among 0D, 7D fasting, and refeeding results from either clinical laboratory tests or ELISA detection. All differences in 0D vs. 7D fasting and either 15D or more than 2∼6-months refeeding (three subjects donated biological samples after 6 months) were analyzed. For the specific ELISA analysis, there

*(Continued)*

**FIGURE 3 |** was a lack of results from further refeeding experience. X-axes refer to control 0D, fasting 7D, and refeeding 7-14D and >60D groups, respectively. The graph was created with GraphPad Prism 8.0.1 software using one-way ANOVA with repeated measures, and the dots around each column represent the actual values of group subjects on the specific treatments. The larger * symbol above the Fasting 7thD column of **(B)** and **(C)** indicates statistical significance (*$p < 0.05$, **$p < 0.01$; and the actual values are represented in the manuscript).

damage (26). Our results indicated that the changes in ALT, GOT, and CK after 7D or longer CDD might be quite beneficial, especially in our three subjects providing 6-months refeeding recovery data (**Table 1**). It seems that, along with the fully metabolized fat tissues to ketone body metabolism, it might start to clean up any potential harmful metabolize, which created a healthier environment to the liver or myocardial function (unpublished results).

## CONCLUSION

Ketogenic or very-low-carbohydrate diets favor mitochondrial respiration for energy metabolism by imitating the fasting process (27). Wei tested the effects of a fasting-mimicking diet (FMD) associated with aging and age-related diseases and demonstrated that three FMD cycles could reduce BW and trunk and total body fat (12). If we could apply some specific period of ketone-generating food such as a low-carbon diet in the refeeding session after our long-term CDD regimen, we could expect a significant reduction in gain of body weight in fat and resolve the critical concern of such a fasting regimen in longer-term practice. In that way, we would be able to apply such a paradigm for treating metabolic syndrome. Either the prolonged fasting could be more efficient in treating the chronic disease, or metabolic syndrome needs to be confirmed in more detail by larger, more strict clinical trials with longer-term analysis.

## DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article, and further inquiries can be directed to the corresponding author Garrick D. Lee at Garricklee@biomed-sci.ac.cn.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Henan Human Research Protection Program was under the guidance of China Association for Ethical Studies. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

GL and CgZ have designed the study and the trial. GL, XW, HX, ZZ, SC, and CN fully managed and performed the trials. YZ, ZL, YX, and QW managed clinical laboratory analysis. YY and ClZ performed InBody 720 body composition analysis. ZL, QW, and YX performed ELISA and other blood tests. GL wrote and revised the paper.

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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.

frontiers in Nutrition

REVIEW
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# Effects of Physical Exercise on Autophagy and Apoptosis in Aged Brain: Human and Animal Studies

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The aging process is characterized by a series of molecular and cellular changes over the years that could culminate in the deterioration of physiological parameters important to keeping an organism alive and healthy. Physical exercise, defined as planned, structured and repetitive physical activity, has been an important force to alter physiology and brain development during the process of human beings' evolution. Among several aspects of aging, the aim of this review is to discuss the balance between two vital cellular processes such as autophagy and apoptosis, based on the fact that physical exercise as a non-pharmacological strategy seems to rescue the imbalance between autophagy and apoptosis during aging. Therefore, the effects of different types or modalities of physical exercise in humans and animals, and the benefits of each of them on aging, will be discussed as a possible preventive strategy against neuronal death.

**Keywords: aging, exercise, autophagy, apoptosis, brain**

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## AGING

The aging process is characterized by a series of molecular and cellular changes over the years that could culminate in the deterioration of physiological parameters important to keeping an organism alive and healthy. This widespread loss of body function, or loss of fitness, is extremely variable and can result in increased individual vulnerability, the onset of various illnesses, and death (1, 2). At the cellular level, aging is characterized by aggregation and accumulation of misfolded proteins and disabled organelles in a progressive way that may lead to cell homeostasis interruptions. Therefore, progressive degeneration may occur, increasing the risk of cell death [reviewed by (1, 3)].

In the Central Nervous System (CNS) normal aging is accompanied by alterations in brain structure such as white matter atrophy (4, 5), and functional and cognitive decline. It is still unclear what relationship the cognitive and functional dysfunctions have with the decrease in neurogenesis observed during aging (6). Age-related cognitive decline can reduce the quality of an individual's life and is related to an increased risk of neurodegenerative diseases (7, 8).

Neurogenesis consists in the generation of new neurons from neural stem cells and progenitor cells that reside in germinal niches in the subgranular zone (SGZ) of the hippocampal dentate gyrus and in the subventricular zone (SVZ) of the lateral ventricle (9). In relation to humans, neurogenesis is still a highly controversial topic, because of the inherent difficulty in marking neurogenic niches and newborn cells *in vivo* (10). Notwithstanding, a recent study raised the debate about the existence of neurogenesis in the human brain and its meaning. Analyzing 37 postmortem and 22 intraoperative tissue samples from human hippocampus, Sorrells et al. concluded that, different to

other species, dentate gyrus (DG) proliferating progenitor cells and newborn neurons in humans decline during childhood without being detected in adult brain samples, suggesting a decline in neurogenesis during life (11). Besides, some studies suggest that neurogenesis happens daily in human DG (12–14), whereas others find a decrease in neurogenesis with just a few neurons being generated in adults (15, 16). Indeed, in 2019 new studies supported the evidence of hippocampal neurogenesis in adult humans, with great individual variability (17) up to the ninth decade of life in healthy subjects (18) and also in patients with mild cognitive decline and Alzheimer's Disease (17).

In animal models, physical exercise has been related to increased hippocampal neurogenesis (19, 20) which is reduced in old rodents (21), whereas in humans it is still speculative (22). However, in humans of all ages, physical exercise is related to improved memory function (23–25), as well as reduction in brain atrophy observed during aging in humans (26) and rodents (27). In the face of such an interesting approach against age-related cognitive decline, the focus of this review is to present and discuss some cellular mechanisms by which different kinds of physical exercise can unleash possible benefits in the CNS in human and animal models.

## PHYSICAL EXERCISE AND AGING

Physical exercise was defined by Caspersen as a planned, structured and repetitive physical activity done with the objective of improving or promoting physical fitness (28). Exercise has been an important force of evolution for the human species in order to hunt for food sources, adapt to the environment and, in consequence, to alter physiology and development of the brain, displaying co-evolution of neuroplasticity signaling pathways (29–32).

The benefits of physical exercise can be observed throughout different stages of life. During pregnancy, supervised moderate exercise attenuates prenatal depression (33). It is also associated with a shorter first stage of labor (34), and newborns whose mothers exercised during pregnancy presented a better auditory memory response related to sound differentiation (35). Thus, an active lifestyle during pregnancy should be encouraged and promoted by public health policies (36). Among adolescents, physical activity may have beneficial effects on attention capacity and cognitive functions (37, 38), and is likely to be effective in reducing depression symptoms amongst both adolescents and young adults (39–42). Meanwhile, different modes of exercise are investigated for the older population, including stretching exercise, such as Pilates (43, 44) and Tai Chi Chuan (45, 46), resistance exercise (26, 47), multimodal exercise (48–50) and aerobic exercise (51). These modes of exercising seem to be similarly effective regarding cognitive improvement (23), but such improvement may not be seen in a short period of time (52).

Some studies and public organs recommend 150 min of moderate physical exercise per week to be sufficient for beneficial outcomes (53, 54). However, few elderly people accomplish the recommendation, especially of moderate to vigorous physical exercise; some believe physical exercise may be potentially harmful or even unnecessary [reviewed by (55)]. A review of nine cohort studies indicated that lower doses of the recommended physical exercise may reduce mortality risk by 22% (56), indicating that, even though it is believed that it is necessary to reach the suggested amount of activity, there is also the need to investigate whether light intensity exercise could ameliorate health or function and motivate the practice of more intense exercise (57).

Among the various interventions that affect aging, physical exercise seems to be the main ally in the prevention of aging-related diseases (58).

Studies regarding the effects of physical activity on elderly people also extend to several types of aging-related disorders, comprising dementia (59), late-life depression (60, 61), frailty syndrome (62, 63), Parkinson's Disease (64) and Alzheimer's Disease (65), through evaluation of epigenetic changes [see (66, 67) for review]. However, studies about the effects of physical exercise on elderly people's epigenetics are still emerging. Lavratti et al. conducted one of the first human studies to demonstrate the relationship between physical exercise and levels of global histone acetylation in schizophrenic patients (68). A meta-analysis on elderly people supports the protective effects of physical activity, a healthy diet and higher educational levels (69); however, in 2018, Gale et al. investigated the effect of physical activity on the epigenetic clock [reviewed in (69)] and found no correlation between them, indicating that exercise alone might not be enough to exert a protective effect in this specific regard.

It has already been demonstrated in the literature that physical exercise can promote neuroprotection. For example, treadmill physical exercise carried out in a mouse model of Alzheimer's disease (69) and voluntary running physical exercise in elderly mice (70) demonstrated, among other effects, that running physical exercise decreased glia activation and amyloid-beta (Aβ) peptide levels, suggesting possible mechanisms for exercise-induced neuroprotection.

The literature brings some ways in which physical exercise promotes its neuroprotective effects. In mice, it has been shown that neuroprotection induced by resistance physical exercise occurs in combination with multiple synergistic neuroprotective pathways: increased neurogenesis, decreased loss of dopaminergic neurons, increased antioxidant capacity, and improved autophagy (70). A study carried out in rats suggested that aerobic physical exercise reverted the synaptic loss in the cortex and hippocampus in old rats, which may be related to the up-regulation of Rho-GTPases (a G protein family, which plays a fundamental role in synaptic morpho-functional changes) (71). In mouse model of Parkinson's disease induced by MPTP, endurance physical exercise promoted neuroprotection possibly due to its contribution to the improvement of mitochondria biogenesis and reduction of apoptosis (72), decrease of pro-inflammatory cytokines and α-synuclein protein (73). In ischemic brain injury rat model, aerobic physical exercise can contribute to neuroprotection by blocking glia activation and preventing neuronal death (74).

Given the data presented here, it is likely that physical exercise is able to promote neuroprotective effects, which seem to depend on the type of physical exercise performed.

In addition to that, in a more general view about the effects of physical exercise in aging, there are many studies regarding cerebrovascular function, gut microbiota, hormone release, sleep quality, and neurotrophic factors production. These topics are quite large and complex and are beyond the scope of this review, however we will briefly mention the main data to contextualize the reader.

Cerebral blood flow (CBF) is the main marker of cerebrovascular function and its decrease, mainly due to detriment of energy depletion or brain ischemia [reviewed by (75)], seems to be related to the generation of cognitive impairment and dementia (72). Disruption of neuronal environmental homeostasis through impaired CBF can be highly related to the decline of the cerebrovascular system during aging (73, 74). In patients with Alzheimer's disease, in addition to decreased brain volume, there is also a decrease in CBF, and it is a potential marker of the severity of the disease (76).

In humans, physical exercise can prevent cognitive impairment by enhancing cerebral vasomotor reactivity, increasing CBF, and consequently increasing cerebrovascular function in older adults (77, 78). Such an increase seems to be dependent on exercise intensity (79). In sedentary older men, aerobic physical exercise was able to increase CBF in the region responsible for regulating cognitive functions, part of this mediated by improvements in glucose metabolism (78). Moreover, another study involving sedentary older men verified the increase in brain function mediated by the regional CBF, increased cognition assessed by memory and executive functions, and the increase in cardiovascular fitness measured by VO2 max, after a protocol of 12-week exercise (80). Otherwise, a study with master athletes ranged in age between 50 and 80 demonstrated that the exercise cessation for a short period of time reduced CBF levels in hippocampus and gray matter regions (81).

In animals, physical exercise through treadmills or running wheels was able to improve endothelium-dependent vasorelaxation as well as increase CBF, reducing functional deficits and protecting the brain from cerebral ischemia and reperfusion (82).

Regarding the gut-brain axis, it is known that gut microbiota plays important roles on metabolic and immunological activities in humans (83). Infrequent bowel movements can decrease gut microbiota, which increases the risk of individuals developing colorectal cancer (84, 85). A combination of moderate physical exercise [$\geq$ 7000 steps/day or 15 min/day at >3 METS (metabolic equivalents)] and lactobacillus ingestion has been shown to decrease infrequent bowel movements in elderly people aged 65–92 (86). In elderly humans, it was shown that 5-week endurance physical exercise was not able to significantly change gut microbiota diversity and composition (87). In the animal model, it has been shown that 11-month-old mice submitted to treadmill physical exercise for 7 months had their gut microbiota diversity augmented, suggesting that physical exercise is able to increase microbiota diversity during the aging process (88). However, Fielding et al. found that mice presented no changes either in their entire lean body mass or in treadmill endurance capacity when treated with human feces coming from elderly people who exercised. Therefore, data regarding physical exercise influence on gut microbiota along aging seems contradictory both in animals and humans, likely depending on duration, intensity and type of physical exercise (89).

It is well-established that growth hormone (GH) secretion decreases during aging process (90–93), which seems to be associated with changes in the organism such as loss of lean mass, gain of fat tissue, diminution in muscle strength, decline in cognitive function, among others [reviewed in (94)]. Physical exercise in the elderly has been described to influence GH level/activity; studies in humans, independently of gender, have demonstrated that regular physical exercise can increase GH levels in plasma (95, 96) or serum (97–103).

However, other studies have not shown an increase of GH levels in elderly marathon runners and sedentary controls (104), in middle-age men (40–50 years old) (105), in old men (47), in old women (106) and in old men and women. The comparison was conducted after subjects had been submitted to heavy resistance training (107), low volume resistance exercise (108), and low intensity physical exercise (109).

Furthermore, experiments done in 21-month-old rats showed that mild physical exercise in treadmill (8 m/min, 1 h/day) in combination with GH administration for 73 days, increased both muscle mass and strength compared with GH by itself (110). However, Marzetti et al. showed that short-term treadmill training attenuates age-related skeletal muscle apoptosis and the same effect was not observed with short-term administration of GH in older rats (111). Studies performed in old rats showed that exercise and GH reduced age-related decay in myocardial relaxation, avoiding diastolic dysfunction (112) and increasing bone strength (113, 114). It is known that GH can augment muscle mass in humans (92). In humans, administration of GH and testosterone together in elderly males produced a gain in lean mass and increased muscle strength, and consequently aerobic endurance (115). Another study about GH supplementation in elderly men did not observe increased muscle strength, and consequently no changes in resistance exercise (116).

Sleep disturbances are common features in older adults, such as sleepiness at daytime, fractionated sleep at night (117–119), among others. It has been shown in the elderly, both men and women, that low to moderate physical activity improved sleep quality (120–133). In animals, sleep derangement related to the aging process also happens (134, 135). It seems that regular moderate physical exercise ameliorates sleep architecture in old rats (136).

A great number of signaling pathways seem to be involved in physical exercise benefits, and one of them is brain-derived neurotrophic factor (BDNF) which is positively induced by physical exercise (137). BDNF is a protein that participates in neuronal proliferation and differentiation, synaptogenesis, synaptic function, plasticity, and neuroendocrine actions [see Review (138)]. In 1995, Neeper et al. measured BDNF mRNA in different brain regions of adult rats with different levels of physical activity, and found a positive correlation between the distance run per night and the BDNF produced in the hippocampus and caudal neocortex of these animals (139). Moreover, BDNF acts as a regulator of the ubiquitin-proteasome system (UPS) as it increases ubiquitin conjugation in synaptic

proteins during synaptic remodeling. In addition, the use of a proteasome pharmacological inhibitor prevented BDNF-mediated action and had the same profile as the BDNF signaling block (140).

In summary, the benefits of physical exercise can be observed throughout different stages of life. Especially during aging, neuroprotection is promoted according to type and intensity of physical exercise. In general, it improves cerebrovascular health and gut microbiota diversity, which seems to be related to healthy aging. Furthermore, physical exercise improves quality of sleep and increases BDNF production, and can decrease neuronal death and improve cognitive performance, due to better functioning of the proteostasis system, among many other effects. Besides, data regarding physical exercise influence on gut microbiota, GH, CBF during aging seems contradictory both in animals and humans, likely depending on duration, intensity and type of physical exercise.

## PROTEOSTASIS AND AGING

The mammalian protein pool is subject to a constant quality control system that integrates the pathways related to protein synthesis, folding, unfolding, secretion, trafficking and degradation (**Figure 1**). This quality control system is known as proteostasis and its failures rely on increased levels of protein aggregates, which contribute to the development of

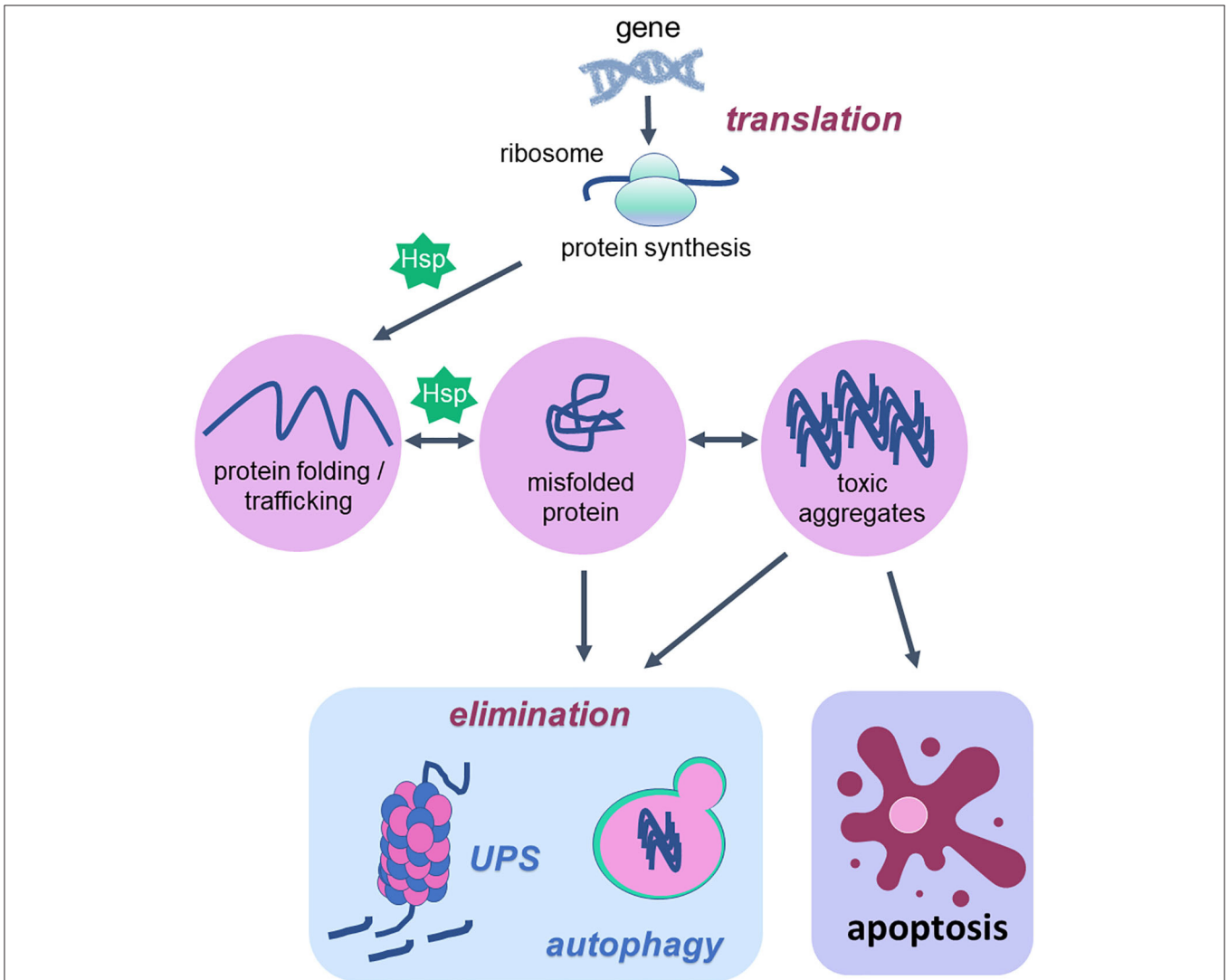


**FIGURE 1 |** Cellular proteostasis and apoptotic cell death. Proteostasis network is the protein pool quality control system that integrates the pathways related to protein synthesis since translation, protein folding, protein unfolding, secretion, trafficking and degradation or elimination. To succeed in protein folding, unfolding, refolding and trafficking, chaperone proteins (heat shock proteins—Hsp) are of fundamental importance. In the elimination phase that usually happens when misfolded proteins turn into toxic aggregates, degradation of damaged proteins occurs through proteolytic systems: autophagy and ubiquitin-proteasome. The activity of both systems avoids cell death. However, when the proteostatic network cannot avoid protein aggregates accumulation, the cell undergoes apoptosis. Thus, degradation system and apoptosis are both important mechanisms for organism homeostasis due to the elimination of damaged proteins and damaged cells, respectively.

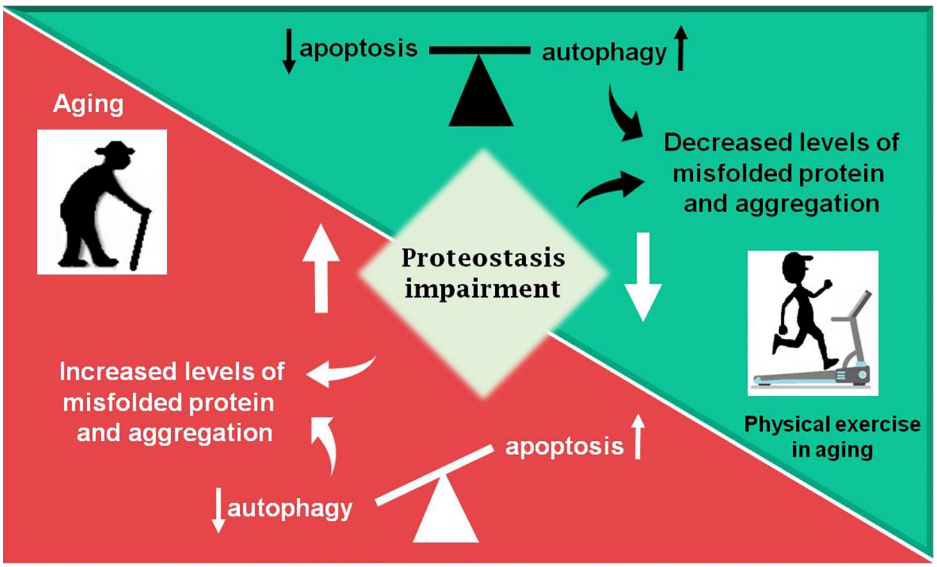


**FIGURE 2 |** Summary of the review. During aging (red box) an increase in proteostasis impairment is observed in the Central Nervous System, which may lead to increased levels of misfolded proteins, in part due to decreased autophagic process. The balance between apoptosis and autophagy is lost and an increase in programmed cell death is observed. Otherwise, physical exercise in aging (green box) can partially revert the disbalance observed in aging, decreasing proteostasis impairment and improving autophagic process, as well as decreasing levels of misfolded proteins and toxic protein aggregates, which lead to less apoptosis activation.

proteinopathies and thus neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (141, 142). The proteostasis decline is one of the hallmarks of aging (1, 143) and this decline can be explained by increased generation of oxidative damage within the cells (144).

According to a vast literature in the field, the proteostasis network is mediated by the degradation of damaged proteins by proteolytic systems (autophagy and ubiquitin-proteasome) and correction or sequestration by chaperones (141, 145, 146). Although proteostasis involves all of these processes, this review will focus on the balance between two vital cellular processes such as autophagy and programmed cell death, apoptosis, based on the fact that there are several studies done with physical exercise as a non-pharmacological strategy that rescues the lost balance between them during aging (**Figure 2**).

The ubiquitin-proteasome system (UPS) is extremely important for the maintenance of protein homeostasis in the cytosolic and nuclear compartments. Ubiquitination occurs through catalytic enzymes, E ligases, which activate ubiquitin and covalently bind this polypeptide to the substrate, a tag for proteasome degradation. The proteasome, or 26S, is a multicatalytic complex with a proteolytic core, 20S, flanked by regulatory units that recognize the ubiquitinated substrates, misfolded and damaged proteins or healthy proteins, and lead them to degradation (147, 148). During aging, this system may be compromised due to defective proteasome activity, proteasome damage, proteasome assembly changes and ubiquitination defects (141). Studies with *Drosophila melanogaster* demonstrated a change from the 26S "activated" proteasome (1–32 days old) to the weakly active 20S form (43–47 days old) during aging, together with decline in ATP levels, highly necessary for the 26S proteasome activity (149). Therefore, UPS is one of the proteolytic systems that degrades damaged proteins regulating the proteostasis network (150, 151).

## AUTOPHAGY IN AGED BRAIN

During the aging process, organelles and proteins are prone to damage affecting their normal functionality, besides that these dysfunctional proteins, and organelles accumulate in the body progressively, thereby increasing the rate of cell death (1, 3). Studies indicate that the loss of autophagic activity in cell aging contributes to a progressive reduction in cell function and may precipitate cell death by restricting the ability of cells to support a healthy population of proteome and organelles (152, 153). Aging is associated with reduced autophagy potential and it has been shown in the literature that autophagic inhibition may result in premature aging (153).

Autophagy (divided into macroautophagy, microautophagy, and chaperone-mediated autophagy) is an important process of cell renewal in maintaining homeostasis and perfect cellular functionality, characterized by the elimination of non-functional proteins, damaged/defective organelles, and intracellular pathogens. Autophagy is an important cell survival mechanism with an important role in cell maintenance and homeostasis and with a positive influence on useful life and longevity (153–159).

The macroautophagy, here referred to as autophagy, has been the most studied. In summary, when damaged proteins and/or organelles are free in the cytoplasm, a nascent membrane originated from Golgi Complex, endoplasmic reticulum (ER),

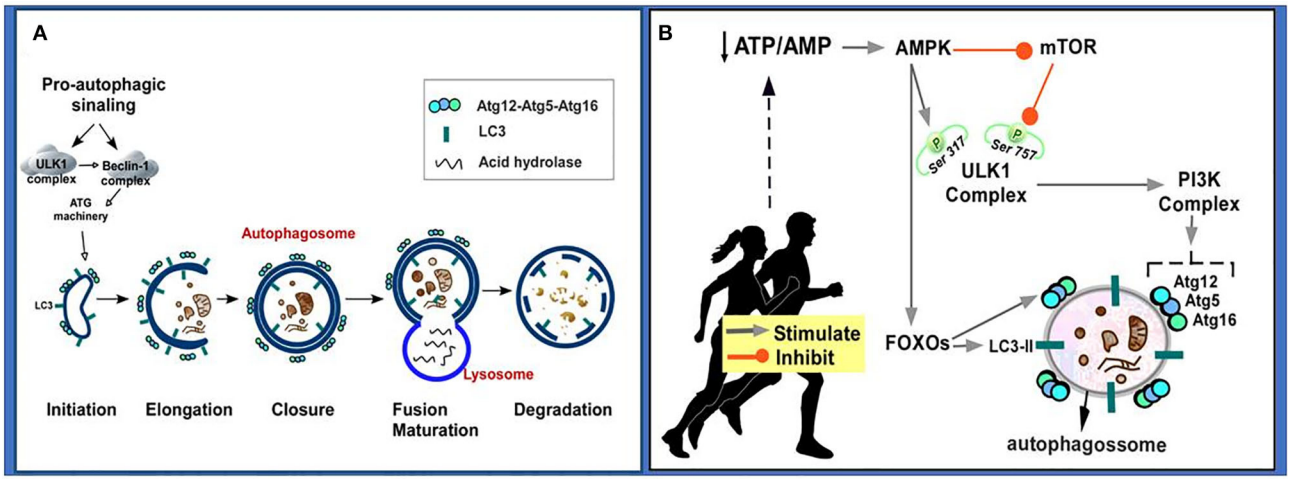


**FIGURE 3 |** Autophageal mechanism and stages. **(A)** Pro-autophagic signaling induces ULK1 activation and Beclin-1 complex, activating the ATG machinery, thereby initiating the autophagy stages consisting of initiation, elongation, closure and autophagosome formation, autophagosome fusion with lysosome and degradation. **(B)** Physical exercise causes a decrease in the ATP/AMP ratio by activating AMPK. AMPK inhibits the mTOR pathway, so ULK $Ser^{757}$ phosphorylation will be decreased and it may interact with AMPK and be activated by phosphorylation on $Ser^{317}$. AMPK phosphorylated ULK1 becomes active and may initiate autophagy. PI3K activation occurs culminating in increased expression of autophagy-related proteins (Atg12, Atg5, Atg16). AMPK also promotes signaling of autophagy-related transcription factors (FOXOs), thereby increasing the expression of autophagy-related proteins (Atg12, Atg5, Atg16, LC3-II). Atg, autophagy-related protein; ATP/AMP, adenosine triphosphate/adenosine monophosphate; AMPK, AMP-activated protein kinase; Beclin-1, autophagy-related protein; FOXO, Forkhead box O; LC3II, microtubule-associated protein 1 light chain 3–form II; mTOR, mammalian target of rapamycin; PI3K, Phosphatidylinositol 3-kinase; UKL1, Unc-51-like kinase 1.

mitochondria, plasma membrane or endosomes is formed to engulf and sequester these damaged elements. This primordial membrane is called phagophore, which will, in a second step, fuse at its edges forming a double-membrane vesicle called autophagosome. The autophagosomes will undergo a maturation step in which they fuse with acidified lysosomal or endosomal vesicles to finally degrade a damaged element and recycle it (**Figure 3A**) (160, 161).

Among the main proteins that control the autophagic process, we can mention: autophagy-related (Atg) protein, which is associated with cytosolic component sequestration and autophagosome formation and is crucial for normal autophagic function (162, 163), for example Atg5, Atg12, Atg16; LC3, which participates in the phagophore and autophagosome expansions (164, 165); and Beclin-1, protein which participates in the initiation of the autophagic process by interacting directly with the phosphatidylinositol 3-kinase (PI3K) complex (166, 167). Autophagy is a very tightly controlled process that can adapt cellular metabolism to a stressful situation, such as starvation and growing factors deprivation, and can also be involved in turnover of organelles and long-lived proteins. Thus, autophagy and apoptosis are both important mechanisms for organism homeostasis due to the elimination of damaged or superfluous cellular components and damaged cells, respectively (160).

Autophagy dysfunctions may contribute to neurotoxicity associated with neurodegeneration and aging (168). Decreased age-related autophagy disrupts neuronal homeostasis and may thus promote the process of neurodegenerative disorders (169–171). However, data in the literature indicates that exercise can activate autophagy, thus preventing age-related diseases as well as retarding neurodegenerative processes [see reviews for more information (161, 172)].

Atgs knockout experiments have shown defects associated with aging, such as high accumulation of non-functional organelles (173–175), endoplasmic stress (173) and mitochondrial disorder (174–176). However, it remains unclear whether these Atgs reductions are, in fact, the main reason for age-related autophagic malfunction. Literature data suggests that basal autophagy decay may be mediated by excessive activity of rapamycin complex 1 (TORC1), a protein kinase that negatively regulates autophagy. The literature has shown that inhibition of TORC1 may increase longevity (177–179).

## INFLUENCE OF PHYSICAL EXERCISE IN THE AUTOPHAGY PROCESS IN AGED BRAIN

It has been postulated that regular physical exercise can promote a beneficial effect on the health of individuals and is considered an important autophagic inducer (180–183). It was observed that treadmill exercise (8 weeks) in mice modulated the levels of autophagy-associated proteins, including Beclin1, and improved autophagy (184). Based on literature data, it is suggested that physical exercise can induce autophagy through the following mechanism: exercise induces decreased adenosine triphosphate/adenosine monophosphate (ATP/AMP) in the cell, and this induces AMP-activated protein kinase (AMPK) activation; AMPK activation promotes inhibition of mammalian target of rapamycin (mTOR), leading

to Unc-51 kinase 1 (ULK1) disinhibition, which is also phosphorylated and activated by AMPK; the ULK1 complex induces activation of the Phosphatidylinositol 3-kinase (PI3K) complex culminating in increased expression of autophagy-related proteins (Atgs); AMPK also promotes activation of autophagy-related transcription factors such as forkhead box O (FOXO), thereby increasing the expression of LC3-II and Atgs [for more detailed information on these signaling pathways, see (172, 185, 186) (**Figure 3B**)].

Based on data published in the literature, it is possible to suggest that the induction of autophagy by stimulating physical exercise is regulated according to exercise type, duration and/or intensity-dependent manner (182).

Kou et al. noted that swimming can delay the aging process, rescuing the impaired functional state of autophagy and abnormal mitochondrial dynamics. In addition, Luo et al. observed that 10-week swimming exercise in rats promoted adjustments in lysosomal degradation, activation of autophagy and mitochondrial quality control in the hippocampus, preventing age-associated cognitive decline. These findings indicate that the conservation of cognitive function in older rats by exercise is associated with mitochondrial improvement in the hippocampus, and lysosomal degradation is required in this process, suggesting that exercise and lysosomal degradation may be effective in decreasing age-related cognitive decline (187).

Huang et al. show that 8-week running exercise in mice can activate the autophagy pathway and improve lysosomal biogenesis, suggesting improvement in brain function of mice; besides that, they also observed that prolonged physical exercise promoted nuclear translocation of transcription factor EB (TFEB–main regulator of autophagic and lysosomal biogenesis) in the cortex, positively regulating the transcription of genes associated with autophagy and lysosome (lysosomal degradation is a fundamental step to completing the autophagy process) (188). It has also been observed that moderate exercise contributes to the prevention of early neurodegeneration in the substantia nigra region in aged rats by improving autophagy and mitophagy (189).

Given the data mentioned here, we can conclude that during aging there are dysfunctions in autophagy leading to CNS damage. Physical exercise could attenuate or prevent such autophagic dysfunctions. However, further studies are still necessary to indicate which modality, duration and intensity of physical exercise induce the greatest positive effects on CNS autophagy.

## APOPTOSIS IN AGED BRAIN

Apoptosis is a process of programmed cell death modulated by the B cell leukemia/lymphoma 2 (Bcl-2)/Bcl2 associated X protein (Bax) family and upregulated during the aging process (190, 191), which is important for tissue homeostasis (192). Apoptosis basically occurs in two different pathways: extrinsic and intrinsic. The extrinsic pathway is induced by death receptors and their ligands (Fas/ FasL complex) or via pro-inflammatory marker (tumor necrosis factor (TNF)α), and the intrinsic pathway is regulated by mitochondrial stress which activates caspase 9 and cleaves caspase 3 (**Figure 4**) (193, 194).

The Bcl-2 family is related to apoptotic intrinsic pathway and has a range of 20 different proteins. Each protein of this family has homology domains: BH1 to BH4. Pro-apoptotic proteins are related to BH3 domains called BCL2 antagonist killer 1 (BAK) and BAX. The other domains are related to anti-apoptotic proteins, known as BCL2, BCLXL, BCL2L2, myeloid cell leukemia 1 (MCL1) and BCL2A1 (194–196). In the intrinsic pathway an increased expression of anti-apoptotic Bcl-2 proteins modulates the expression of cell cycle inhibitors and induces cellular senescence, while the expression of pro-apoptotic factors such as Bax and Bak proteins results in macropores formation in the mitochondrial surface, called mitochondrial outer membrane permeabilization (MOMP). This pore allows the exit of cytochrome c from mitochondria, which culminates in activation of caspase cascade causing cell death. Bcl-2 acts as an anti-apoptotic factor that can inhibit the activation of Bax or Bak and inhibits autophagy by beclin-1, which modulates cellular senescence (194–197). Caspase-3 is one of the key proteins of apoptosis and could be responsible for the proteolytic cleavage of many proteins such as poly (ADP-ribose) polymerase (PARP) responsible for DNA repair observed by Cechella et al. in aged rat brains (27).

In the aged brain, there is a reduction in the availability of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), especially in the hippocampus; therefore, these changes may be linked with the large reduction in cell proliferation and increase in apoptosis in the dentate gyrus (8, 198). Besides, TNF-α, a pro-inflammatory protein, could increase the process of apoptosis via intrinsic and extrinsic pathways, which is more significantly visible in aged brain and could be implicated in neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (**Figure 4**) (199–201).

## INFLUENCE OF PHYSICAL EXERCISE IN THE APOPTOSIS PROCESS IN AGED BRAIN

Sedentary lifestyle could be a risk factor for cognitive dysfunction and neurodegenerative process, and regular exercise has anti-aging effects, more specifically in the CNS, whose benefits include an increase in hippocampus neurogenesis, which improves learning and memory in aged rodents. Exercise can upregulate BDNF in hippocampal and cortical neurons promoting synaptic remodeling and improving cell survival (202, 203). Physical exercise inhibits the production of the pro-inflammatory cytokine TNF-α, which in low concentration decreases the process of apoptosis via the extrinsic pathway (200).

Aerobic Physical Exercise (APE) is the most common type of training for rodents, such as running and swimming, and they must be conducted repeatedly with an established frequency; however, few studies have compared the effects of many different modes of exercise on cognition (7). APE is able to positively affect the dynamic adaptations of the neuronal terminal zones in aging.

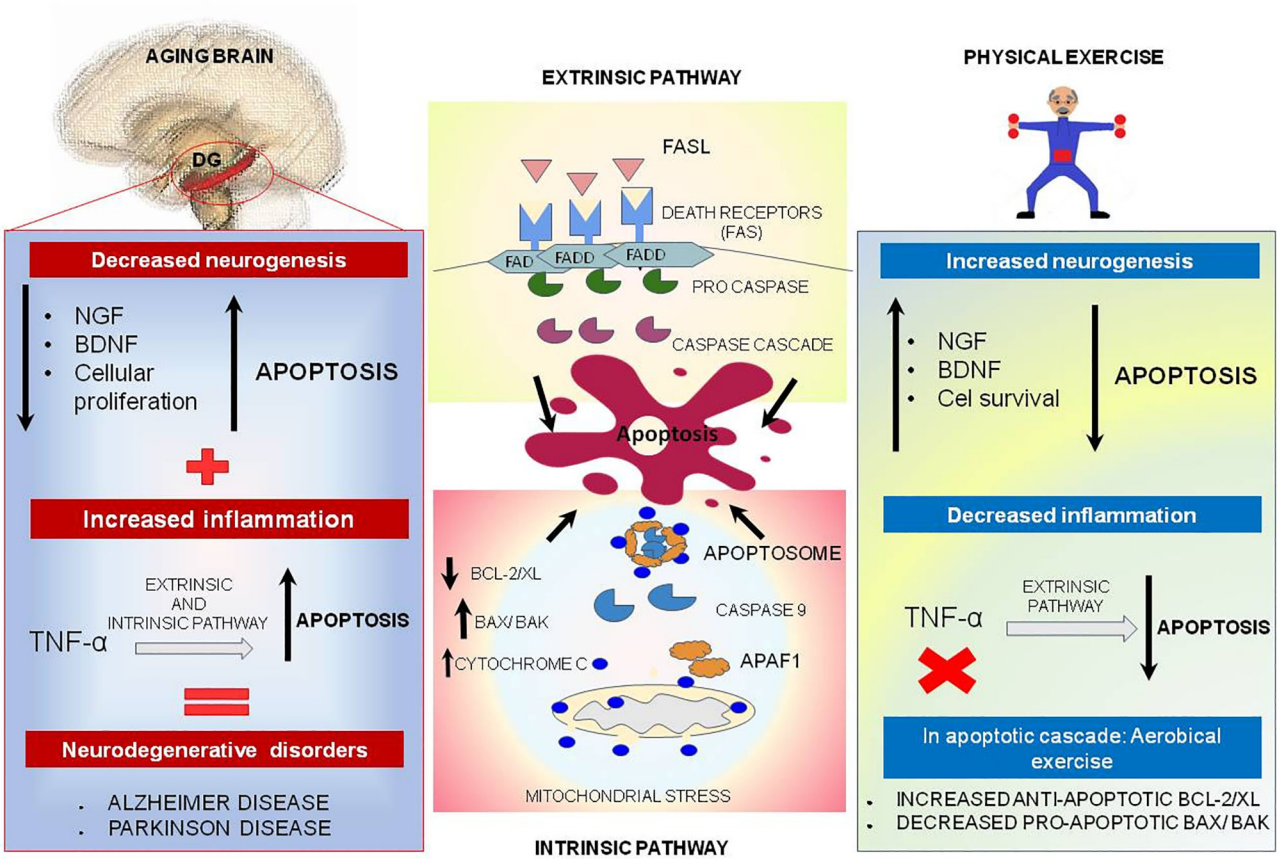


**FIGURE 4 |** Physical exercise actions against aging brain and the apoptotic process. In the central boxes: Apoptosis can be divided into extrinsic and intrinsic signaling pathways. The extrinsic pathway illustrated in the upper central box is induced by death receptors such as FASL, and their ligands, FAS. On the other hand, the intrinsic pathway illustrated in the central inferior box is induced by signs that cause changes in the mitochondrial membrane, called mitochondrial outer membrane permeabilization (MOMP). This process generates macropores on the mitochondrial surface and a release of Cytochrome C, which once released by mitochondria binds to and induces APAF-1 and pro-caspase 9, forming the Apoptosome complex, which activates caspase 9 causing cell death. This pathway is mediated by anti-apoptotic proteins such as BCL-2 and BCL-XL, and pro-apoptotic proteins such as BAX and BAK. In the left box, brain aging is illustrated, and a decrease of neurotrophic factors such as nerve growth factor (NGF) is observed, and brain-derived neurotrophic factor (BDNF), especially in dentate gyrus located on hippocampus, may be related to decreased cell proliferation and increased apoptosis. In addition, TNF-alpha, a pro-inflammatory cytokine, can increase the apoptosis process by intrinsic and extrinsic pathways and lead to the emergence of neurodegenerative diseases. Physical exercise effects, illustrated in the right box, act in reverse, increasing the amount of NGF and BDNF, increasing cell survival and culminating in decreased apoptosis. In addition, physical exercise inhibits the production of pro-inflammatory cytokine TNF-alpha which, in low quantities, decreases the extrinsic apoptosis process.

Fattoretti et al. observed effects of APE in the hippocampus of old mice (27 months) submitted to a 4-week aerobic training in a treadmill apparatus, five times per week, and found that hippocampal regions are not uniformly influenced by physical training: they show an increase in the number of synapses and synaptic area per $\mu m^3$ of tissue in CA1 caused by physical exercise protocol in aged animals, while in DG they could only see an increased number in the synaptic area (204).

Moderate intensity APE protocol for 28 days in 18-month-old mice induced an upregulation of hippocalcin, α-spectrin and ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) in the hippocampus (6). Hippocalcin, which is a calcium ligand, has been shown to protect neurons against apoptosis by regulating inhibitory proteins (205). Xie et al. using an intracerebral hemorrhage method in rats, verified that OUTB1 colocalized with active caspase 3 and attenuated neuronal apoptosis (206). Besides, hippocalcin and spectrin-α in the hippocampus of old mice could be related to an increase in neurogenesis (7).

Fang et al. tested a 12-week protocol of treadmill aerobic exercise in aged rats, and observed by TUNEL staining and immunohistochemistry that the number of TUNEL positive cells (dead cells) had diminished in cortex and hippocampus after the exercise protocol. Moreover, anti-apoptotic protein Bcl-2 levels were augmented. On the other hand, pro-apoptotic proteins, Bax and cleaved caspase-3 levels were decreased in hippocampus and cortex of these rodents submitted to the APE protocol. Another important evidence for the benefits of APE is the decrease of the cytoskeletal protein tau hyperphosphorylation in the brain of old rodents (197, 207–209).

APE is not restricted only to the treadmill apparatus. Cechella et al. submitted rats to a forced swimming protocol for 4 weeks and one experimental group of rodents were supplemented with diphenyl diselenide ($(PhSe)_2$), a potential antioxidant compound. Both groups, exercise and exercise plus $(PhSe)_2$, could decrease the level of pro-apoptotic proteins in 27-month-old rats. They observed an increase in BDNF levels that has led to a downregulation of pJNK/JNK ratio which induced caspase 3 cleavage. The cleaved caspase 3/caspase 3 ratio was also decreased, while Bcl2 expression was increased in animals submitted to both protocols (27). Conversely, Liu et al. affirmed that, after 10 weeks of treadmill exercise, apoptotic striatum cells in old-aged rats increased by 68.24% in comparison to sedentary animals, showing that intense physical activity might not be beneficial for the organism (210).

Do et al. tested voluntary running in triple transgenic AD mice for 4 and 8 weeks. At the end of this period, they showed a reduction of apoptotic cells in the hypothalamus (211). In addition, 4-week voluntary exercise training reduced pro-inflammatory cytokines TNF-α and IL-6 to the levels compared to the control, which supports previous studies in the literature (212, 213).

There is a great amount of research related to aerobic exercise and apoptosis, but little is known about the effects of resistance training (RT) on the brain. Aerobic exercise may induce events such as cell proliferation, survival and death through distinct mechanisms from those of resistance exercise (214, 215). The RT consisted of bodybuilding exercises like adductor, abductor, leg presses, triceps and biceps exercises, or in a simpler way for rodents, it could be climbing a ladder repeatedly, for example (200, 216, 217). de Smolarek et al. (217) showed that RT could be very suitable for the elderly population because they were able to see an improvement in cognition in elderly women (between 65 and 69 years old) submitted to resistance training for 12 weeks. Another study that used RT protocol in elderly women (between 65 and 75 years old) for 52 weeks showed, after this period, an increase in these women's cognition, mainly in learning, measured by Mini-Mental State Examination and another verbal quiz (26).

There are few studies in the literature showing that RT could be used for hospitalized elderly people who spend most of the time lying on a bed relying exclusively on physiotherapy exercises (218–221). Martínez-Velilla et al. used an RT protocol that includes physical exercises such as line walking, stepping practice, proprioceptive exercises, among others (219). The results showed that RT protocol was efficient in preventing functional decline caused by hospitalization (218). Other clinical trials with RT protocol applied in 65-year-old healthy men and women, consisting of a 3-month body-mass-based exercise program, revealed that only working memory was improved (222).

Frailty is a syndrome characterized by dysregulation of several physiological systems (223, 224). Few studies with physical exercise (not only RT exercise, but also APE) were conducted to evaluate its efficiency in ameliorating frailty phenotype (218, 225). Yoon et al. tested an RT high speed program in elderly people (74 years old) with frailty syndrome for 16 weeks and they observed that RT improved cognitive and physical functions (218). Based on clinical trials data, we can conclude there is a good outcome in using RT protocol in the elderly to improve their cognition (218, 219, 222).

In another perspective, regarding animal studies, Henrique et al. (200) compared two protocols, APE and RT. APE protocol used treadmill running, and RT protocol consisted of a series of eight climbs with a progressively heavier load, both for 7 weeks in 21-month-old rats. They observed that the RT group had a reduction in hippocampal levels of macrophage inflammatory peptide (MIP)-2 protein, a pro-inflammatory mediator which seems to be related to inducing apoptosis (226), so the effects of RT on MIP-2 levels deserve to be more explored.

Vilela et al. (215) also compared APE and RT in 24-month-old rats and observed, in both protocols, an increase of the hippocampal neurotrophin receptor P75 ($P75^{NTR}$), a transmembrane receptor involved in many cellular functions including apoptosis, cell survival, neurite outgrowth, migration, and cell cycle arrest (227, 228). In this case, the researchers believed that $P75^{NTR}$ was involved in neuroprotection through activation of the apoptosis pathway to induce death process on damaged neurons and to provide a conducive environment for insertion of new cells (215, 229).

In summary, both APE and RT could improve spatial memory or activate different mechanisms that lead to cell survival and induce a decrease of apoptotic cells. RT can be a strategy to protect the brain and maintain healthy cognition during the aging process, while APE could alter intracellular pathways, even though the related mechanisms that explain these effects still remain unknown.

## CONCLUSION AND PERSPECTIVES

Based on what we cited in this review, we can conclude that during the aging process dysfunctions can occur in several cellular events, such as autophagy and apoptosis, which can culminate in CNS damage. Physical exercise could attenuate or prevent such autophagic or apoptotic dysfunctions. At the same there is controversial data from the literature both in human and animal studies. Therefore, further studies are still necessary to clarify the effects of physical exercise during the aging process and also to demystify underlying mechanisms of physical exercise effects and indicate which modality, duration and intensity of exercise is able to induce the greatest positive effects in the CNS, thus preventing neuronal death.

## AUTHOR CONTRIBUTIONS

EK conceived the review idea and edited the final version of the text and figures. JS, AO, AM, and DA contributed equally by writing the text as well as drawing the figures, and participated in the discussion of the review idea. PM contributed by drafting the review and drawing the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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# The Future of Shift Work: Circadian Biology Meets Personalised Medicine and Behavioural Science

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Shift work is commonplace in modern societies, and shift workers are predisposed to the development of numerous chronic diseases. Disruptions to the circadian systems of shift workers are considered important contributors to the biological dysfunction these people frequently experience. Because of this, understanding how to alter shift work and zeitgeber (time cue) schedules to enhance circadian system function is likely to be key to improving the health of shift workers. While light exposure is the most important zeitgeber for the central clock in the circadian system, diet and exercise are plausible zeitgebers for circadian clocks in many tissues. We know little about how different zeitgebers interact and how to tailor zeitgeber schedules to the needs of individuals; however, in this review we share some guidelines to help shift workers adapt to their work schedules based on our current understanding of circadian biology. We focus in particular on the importance of diet timing and composition. Going forward, developments in phenotyping and "envirotyping" methods may be important to understanding how to optimise shift work. Non-invasive, multimodal, comprehensive phenotyping using multiple sources of time-stamped data may yield insights that are critical to the care of shift workers. Finally, the impact of these advances will be reduced without modifications to work environments to make it easier for shift workers to engage in behaviours conducive to their health. Integrating findings from behavioural science and ergonomics may help shift workers make healthier choices, thereby amplifying the beneficial effects of improved lifestyle prescriptions for these people.

**Keywords: chronomedicine, chrononutrition, chronotherapy, circadian disruption, light exposure, physical activity, shift work, time-restricted eating**

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## INTRODUCTION

Simplistically, our species once lived by two "clocks." One of these clocks is the environmental clock, which generates roughly 24-h changes in the light/dark (LD) cycle. The other clock is the endogenous biological clock, which among other rhythms generates roughly 24-h (circadian) rhythms in biological outputs such as the sleep/wake cycle. Prior to the introduction of artificial light at night, these two clocks were probably tightly synchronised (1, 2). Following industrialisation, however, people can more easily work outside of conventional daytime hours, and 15–20% of the working population now work shifts (3). The burden of shift work is striking: Shift workers are not only at increased risk of accidents (4), they are also disposed

to developing numerous diseases, including certain cancers, coronary heart disease, stroke, and type-two diabetes (5). Few studies have explored whether shift work makes individuals prone to neurodegenerative diseases (6, 7), but shift work frequently disrupts biological rhythms and sleep, and such disturbances propagate a slew of pathobiological changes that contribute to neurodegeneration (8). While at the time of writing little is known about the effects of the 2019 novel coronavirus disease (COVID-19) pandemic on the lives of many shift workers, we would be remiss not to mention that many healthcare professionals at the frontlines of the outbreak are currently working long shifts in conditions that dispose them to developing COVID-19 (9). Many of the chronic conditions associated with shift work are also associated with greater risk of poor outcomes in those with COVID-19 as well as other coronavirus and influenza infections (10–17). As shift workers often work jobs considered essential during the COVID-19 pandemic, improving the health of shift workers should become a key part of current and future pandemic preparedness. Importantly, however, at present there is no strong evidence that people fully adapt to shift work (18). And considering that the unconventional schedules of shift workers also interrupt the lives of cohabiting non-shift workers, the burden of shift work is greater still. The purpose of this manuscript is therefore to summarise some ways by which we might be able to reduce this burden.

## OPTIMISING SHIFT WORK SCHEDULES

Optimising shift work schedules is fundamental to the health and productivity of shift workers. In general, it appears that most shift workers tolerate rapid, forward (clockwise) rotation schedules best (5). To support worker wellbeing, these shifts should each last no longer than 10 h, have at least 11 h of recovery between them, and amount to no more than 60 h of work per week (5). To hone shift work schedules for individual workers, workers may benefit from having some control of their schedules. This autonomy helps account for differences between people in non-work responsibilities, tolerance to shift work, and commuting to and from work.

Chronotype is another tailoring variable that is particularly germane to optimising shift work schedules. Chronotype is defined as interindividual differences in the phenotypic expression of behavioural outputs regulated by the circadian system (19), the most conspicuous of which is the timing of the sleep/wake cycle, and in industrialised societies there exist large differences between individuals in their chronotypes (20). Chronotype appears to modify the association between shift work schedules and risk of health problems (21), such that the health of early chronotypes may be especially negatively affected by working night shifts (22), whereas late chronotypes find working morning shifts particularly problematic (23). While it is not clear precisely why this interaction exists, shift workers who have closer alignment between their chronotypes and their work schedules appear to have more robust melatonin rhythms than their fellow shift workers, suggesting that they have better circadian system function (24). Shift workers who have chronotypes that are better matched to their work schedules may also sleep better (23), and it is increasingly clear that circadian system and sleep health are essential to perhaps all facets of human health (25, 26).

## OPTIMISING ZEITGEBER SCHEDULES

The period of each individual's circadian system is one determinant of his or her chronotype. In the absence of time cues (zeitgebers), the free-running period of the human circadian system is slightly longer than 24 h, on average (27). The circadian system therefore needs to be synchronised (entrained) each day with the 24-h day, and shift work complicates this process.

### Exposure to Light

Retinal light exposure is generally regarded as the most important stimulus in entraining the human circadian system (28), and changes in patterns of light exposure can rapidly and substantially shift circadian system timing (phase). This is especially true of short-wavelength light, which most potently suppresses melatonin synthesis (29). Exposure to such light in the biological morning tends to advance circadian phase, whereas exposure to such light in the late biological evening tends to delay circadian phase. The implication of this is that it is possible to bolster how well-shift workers adapt to work schedules through timely use of means to increase exposure to high-intensity, short-wavelength light at specific times of day (e.g., by using light-therapy lamps) and means to reduce exposure to such light at specific times of day (e.g., "blue-blocking" glasses and blue-light filtering apps on electronic devices). While not all studies that have used interventions to modify exposure to light in shift workers have proven beneficial, this inconsistency likely reflects marked heterogeneity in the methods used by researchers (30), as well as large variation between people in how they respond to light (31).

### Melatonin

During darkness, retinal photoreceptors no longer register exposure to light, relaying this to the central clock in the circadian system (the suprachiasmatic nucleus), which in turn signals the pineal gland to synthesise melatonin. Melatonin therefore acts as an endogenous marker of darkness, agonising its receptors in cells in numerous tissues to signal them to fulfil time-of-day-specific functions. Simplistically, when the concentration of melatonin in the blood surpasses a certain threshold in humans who are melatonin-proficient, it is the biological night-time. Conversely, when the concentration of melatonin is below this threshold, it is the biological daytime.

Melatonin supplementation can shift the phase of the circadian system (32). Melatonin ingestion in the late biological afternoon tends to advance circadian phase, while ingestion in the early biological morning tends to delay it. Melatonin is therefore a chronobiotic – an agent that can modify circadian phase. When timed appropriately, light exposure and melatonin ingestion additively shift circadian phase (33).

## Exercise

A growing body of evidence also shows that exercise can shift circadian phase. Early research demonstrated that 15 min of cycling exercise each hour of night shifts helped workers adjust their circadian systems to a 9-h delay in bedtime (34). More recent work has begun to clarify the precise nature of the relationship between exercise and circadian phase, showing that treadmill exercise done in the early biological morning or early biological afternoon advances circadian phase, whereas the same exercise done in the biological evening delays it (35). This relationship is therefore similar to how timing of exposure to light affects circadian phase, and timely exposure to both light and exercise can also additively shift circadian phase (36).

## Nutrition

The influence of timing of food availability on patterns of activity in rats was documented as early as a century ago (37), and numerous studies of such "food anticipatory activity" have since implicated nutrition as an influence on circadian system timing. Whereas the LD cycle is the primary zeitgeber for the suprachiasmatic nucleus, some scientists have hypothesised that the eating/fasting cycle may be the primary time cue for some peripheral clocks in the circadian system. We now know that changing the timing of food consumption rapidly alters the timing of gene transcription in peripheral clocks in mice, for example (38). Recent work has shown that this may be true of humans too, for changing meal timing independently shifts the expression of some genes in peripheral tissues as well as the timing of the blood glucose rhythm, without changing the phase of the melatonin rhythm (39). We acknowledge, however, that lack of control of variables such as LD cycles in most studies of the effects of nutrition on the human circadian system mean that this is arguably the only study of people that fulfils at least one of the criteria for diet to be classified as a zeitgeber (40). It could be that entrainment to LD cycles largely nullifies any zeitgeber effects of nutrition (41).

Summarising the above, it is plausible that carefully timed exposure to light, melatonin ingestion, and exercise may result in additive shifts in the phase of the suprachiasmatic nucleus. As eating/fasting cycles appear to affect the phases of some peripheral circadian clocks, we anticipate that coordinated changes in all of these variables could be used to expedite adaptation to new shift work schedules. If one could estimate shift workers' circadian phases in real time and model how subsequent changes in zeitgeber schedules would influence their circadian systems, one could develop tools that use this information to expedite adaptation to shift schedule changes by providing personalised guidance and perhaps even individual-level changes in exposure to light. This may be a particularly fruitful topic for further study.

# CHRONONUTRITION: THE IMPORTANCE OF DIET TIMING

While it is plausible that one could change nutrient timing to accelerate adaptation to new shift work schedules, in many instances shift workers do not seek to fully adjust to their new shifts. This raises the question of whether workers undergoing transient changes in work schedules should adjust their diets accordingly. However, it is also crucial to consider contextual factors that influence when shift workers eat and drink. Work schedules, time constraints, timing of breaks within shifts, family commitments, and prioritising behaviours such as sleep over meals all influence diet timing in shift workers, leading to erratic diet timing patterns in these people (42). Diet timing irregularities are also affected by cultural factors (e.g., Ramadan) and the nature of some jobs (e.g., many on-call workers have especially unpredictable work schedules). Temporarily putting these complexities to one side, controlled experiments have begun to explore the effects of diet timing during pre-clinical and clinical simulations of shift work. **Table 1** summarises our dietary and supplementation suggestions for shift workers based on our interpretation of the current literature.

## Diet Timing in Shift Workers

Beginning with preclinical research, studies of mice have shown that restricting food access to the dark period (the active phase for these nocturnal animals) may protect against the obesogenic effects of repeated 6-h advances in the LD cycle (48). In addition, restricting food access to the active phase may also accelerate adaptation of circadian rhythms in core body temperature and locomotor activity to repeated 12-h changes in LD cycles (49). These findings imply that people would better cope with rotating shift work if they fixed their eating to the daytime, which is somewhat counterintuitive given that fixing eating time during shifting LD cycles might be expected to uncouple circadian rhythms between the suprachiasmatic nucleus and peripheral clocks. It is, however, intuitive that restricting food access to the active phase may be preferable to restricting it to the rest phase, and findings from initial research on humans support this contention.

Among healthy young men undergoing simulated night shift work for 4 days, those who confined their consumption of calorie-containing foods and drinks (i.e., the caloric period) to between breakfast at 07:00 and dinner at 19:00 had superior post-breakfast glucose tolerance after the intervention compared to men who had dinner at 19:00, a meal at 01:30, and breakfast at 07:00 (50). The group that restricted food intake to the daytime also had superior overnight cognitive function (51). This is especially salient given that many shift workers redistribute their energy intakes into the night when working shifts (52). Additional studies using larger sample sizes and investigating the effects of diet composition on a range of round-the-clock postprandial responses will be instructive.

## Time-Restricted Eating: Findings From Non-shift Workers

Studies of adults undergoing time-restricted eating (TRE) also indicate that optimising nutrient timing is likely to be important to cardiometabolic health, although the participants in these studies have generally not been shift workers. We arbitrarily define TRE as consumption of all calorie-containing items within a period of 12 h or less each day. Conversely, we define

**TABLE 1 |** Dietary and supplementation suggestions for shift workers.

| | |
|---|---|
| The caloric period | Workers should restrict consumption of all items containing > 5 calories to a 6- to 12-h period each day, when possible. They should keep the timing of this period as regular as is feasible from day to day. Workers should self-select the timing of this period, and the ideal time for this period may be relatively early in each worker's biological daytime. We therefore recommend that workers select a caloric period that finishes at least 3 h before their most common bedtime. |
| Distribution of macronutrient intakes within the caloric period | Workers who have poor cardiometabolic health should aim to consume at least half of daily energy intake in the first half of the caloric period (e.g., by increasing the size of breakfast and reducing the size of dinner). This is less relevant to people who exercise in the second half of their caloric period.<br>Workers should also aim to evenly divide their protein intakes between dietary events. As a starting point, we recommend that workers aim to consume ∼ 0.4 g protein per kg bodyweight at each of 3 to 4 evenly-spaced dietary events each day (43). |
| Sequence of macronutrient intakes within dietary events | Workers who have poor glycaemic control should consume carbohydrate-rich foods last at dietary events, when practical (e.g., consuming fibre- and protein-rich salads before meals or eating meat and vegetable foods before carbohydrate-rich foods). |
| Snacking outside the caloric period | When workers feel it would be beneficial to snack outside of the caloric period (e.g., to abate hunger and/or support alertness), they may benefit from consuming relatively small (i.e., ∼10–20% daily caloric intake), minimally processed, micronutrient-dense, satiating, easy to digest, convenient snacks. We hypothesise that relatively high-protein, low carbohydrate snacks are ideal at these times (e.g., snack items may include boiled eggs, dairy products, minimally-processed fish jerky or meat jerky, high-protein drinks, nuts, whole vegetables, and/or low-sugar whole fruits such as berries). |
| Caffeine | If their goal is to support cognitive function during shifts, workers may benefit from individual doses of 1–4 mg caffeine per kg bodyweight, favouring the upper end of this range if short on sleep (44). Repeated doses of caffeine every 2 h or so may maximally support cognitive function during extended wakefulness (45). As consuming caffeine as gum leads to faster absorption than consuming caffeine as capsules (46), caffeinated gum may be particularly helpful if the goal is to affect cognition as quickly as possible. Since mistimed caffeine intake impairs sleep, workers should also stop consuming caffeine at least 7 h before the main sleep period, if possible (47). Individuals differ remarkably in their responses to caffeine ingestion, so they should moderate their intakes according to their individual responses. As a starting point, we recommend consuming no more than 6 mg caffeine per kg bodyweight per 24 h. |
| Creatine | Creatine monohydrate consumption may help shift workers cope with sleep loss. During periods of insufficient sleep, we tentatively recommend that shift workers consume 0.1 g creatine monohydrate per kg bodyweight per day. Because of its potential alertness-boosting properties, we speculate that the ideal time to consume creatine is with the first meal of each day. |
| Melatonin | Well-timed melatonin use may help some shift workers adapt to new work schedules and sleep better during these transitions. We tentatively recommend that workers consume a dose of 0.3–5 mg melatonin at these times, beginning with a dose at the low end of this range and adjusting the dose according to responses. Because of its potent chronobiotic properties, the optimal timing of melatonin ingestion depends on variables such as the individual's circadian phenotype and work schedule. We therefore do not offer guidance related to melatonin ingestion timing. |

intermittent fasting as periodic abstinence from consumption of *any calories for at least 24 h*.

Skipping breakfast is one way to implement TRE, and doing so leads to *late* TRE. While breakfast-skipping is a controversial topic, epidemiologic studies have tended to associate breakfast consumption with lower risk of developing cardiometabolic diseases such as heart disease and type-two diabetes (53, 54). However, controlled studies have not shown large effects of skipping breakfast on cardiometabolic health (55). For example, lean adults who skipped breakfast for 6 weeks inadvertently decreased their daily energy intakes, but this change was compensated by reductions in physical activity energy expenditure, resulting in no changes in energy balance or body composition (56). Skipping breakfast did not affect most measures of cardiometabolic health - the only noteworthy difference between groups was that afternoon glycaemic variability was higher in adults who skipped breakfast. A subsequent study implemented the same intervention but only included obese adults (57). In this study, participants in the breakfast-skipping group expended less energy in the morning, but they did not burn fewer calories over the entire day. Daily energy intake was similar in breakfast-skippers and breakfast eaters, and both groups gained weight during the study. People who skipped breakfast did have higher insulinaemic responses to an oral glucose tolerance test, however. These two rigorous studies show that skipping breakfast minimally affects energy balance but may negatively affect glycaemic regulation and some of its determinants. As sleep timing did not differ between groups, breakfast skipping led to a form of late TRE, so these studies imply that late TRE may not be optimal for some aspects of cardiometabolic health.

Skipping breakfast imposes a relatively late caloric period, and an alternative is to shorten the caloric period by way of skipping dinner or having an early dinner. Several recent carefully controlled experiments have shown that such *early* TRE may exert numerous positive effects on health. The first of these experiments reported that compared with a ∼ 12-h daily caloric period for 5 weeks, 5 weeks of early TRE (∼ 6-h daily caloric period, finished by 15:00) improved insulin sensitivity, blood pressure, appetite regulation, and a marker of oxidative stress in men who have prediabetes (58). The same group of scientists recently reported that in overweight adults, just 4 days of early TRE reduced mean 24-h blood glucose levels and improved metabolic flexibility, among other benefits (59, 60).

These experiments did not compare early TRE to later TRE while keeping the caloric period fixed, however, and to our knowledge, only one study has done this to date (61). The study in question showed that 7 days of both early (08:00 to

17:00) and late (12:00 to 21:00) TRE improved oral glucose tolerance in men at high risk of developing type-two diabetes, although only early TRE lowered fasting glucose, suggest a small advantage of early TRE (61). While this hypothesis needs careful testing, we believe that early TRE may also enhance diet *composition* by reducing intakes of foods and drinks commonly consumed in the evening, such as processed snacks and alcohol.

Together, these studies support the superiority of relatively *early* TRE in adults who have poor cardiometabolic health. However, non-self-selected TRE schedules may interfere with some social activities and be difficult to adhere to in the context of work schedules and family commitments (62, 63). Letting people self-select their TRE periods helps mitigate these undesirable consequences. Indeed, 12 weeks of self-selected TRE minimised these issues in adults with metabolic syndrome, also reducing daily energy intake and potently improving numerous aspects of cardiometabolic health including bodyweight, waist circumference, and blood pressure (64). Moreover, TRE led to more regular diet timing, which may independently be beneficial for cardiometabolic health (65). Interestingly, TRE also improved sleep timing regularity and increased how often participants self-reported restorative sleep. However, this study was an unblinded, single-arm study with only 19 participants included in the data analysis (64).

Based on existing studies, TRE appears to be a safe strategy that is likely to reduce energy intake, which would be especially beneficial for people who have unavoidably sedentary lifestyles. We hypothesise that fixing the timing of each worker's caloric period within regular hours each day supports metabolic health, and it is plausible that this may be especially important in workers who are subject to unpredictable changes in zeitgebers such as LD cycles (e.g., emergency service workers). We further speculate that each worker's biological daytime is the optimal time at which to fix the individual's caloric period, but self-selection of TRE schedules will help people adhere to TRE and avoid undesirable effects on social and family life. This said, scheduling TRE as early as is practical may maximise the beneficial cardiometabolic effects of TRE.

## Distribution of Energy and Macronutrient Intakes Within the Caloric Period: Findings From Non-shift Workers

While a detailed discussion of this subject is beyond the scope of this review, several recent controlled studies have shown that when daily energy intake is fixed, the distributions of energy and macronutrient intakes *within the caloric period* strongly influence cardiometabolic health. For example, one study divided overweight and obese women into two groups that consumed isocaloric weight loss diets for 12 weeks (66). One group consumed half of their daily energy intakes at breakfast, the other group consumed half at dinner. The group that consumed half at breakfast lost more than twice as much bodyweight, more than twice as many centimetres off their waists, and had greater improvements in oral glucose tolerance. Subsequent work by the same scientists demonstrated that when energy intake is controlled, concentrating energy and carbohydrate intakes early in the day leads to enhanced appetite regulation, weight loss, and dramatic improvements in glycaemic control in adults with type-2 diabetes (67). This builds on research demonstrating that having carbohydrate-rich meals early in the day reduces 24-h glycaemia in adults with impaired fasting glucose and/or impaired glucose tolerance (68).

While these studies highlight the advantages of concentrating energy and carbohydrate intakes relatively early in the caloric period, we note that that intelligent inclusion of physical activity leads to acute improvements in postprandial responses to dietary events such that relatively high energy and carbohydrate intakes late in the biological day may not be so problematic if they bookend exercise (69). And staying on the subject of exercise, there is tentative evidence that distribution of daily protein intake affects skeletal muscle protein synthetic responses to resistance training (70). As muscle protein synthesis is the main determinant of muscle protein balance, it is reasonable to assume that evenly dividing and spacing protein intakes between 3 and 4 daily dietary events may help maximise fat-free mass, a key determinant of cardiometabolic health (43).

## Sequence of Macronutrient Intakes Within Dietary Events: Findings From Non-shift Workers

We would be negligent to not mention that the sequence of macronutrient intakes *within* dietary events may also meaningfully affect postprandial responses. Several studies by one research group have shown that consuming carbohydrate last at a given dietary event (e.g., a full meal) dramatically reduces postprandial glycaemia and insulinaemia in adults who have prediabetes or type-two diabetes (71–73). Shift workers who have poor glycaemic control may hence benefit from consuming carbohydrate-rich foods last at dietary events, when practical.

## Snacking in Shift Workers

Most shift workers snack during night shifts. The problem is that night shifts often occur during the workers' biological night-times, and digestive and metabolic responses to dietary events are impaired during the biological night (74). As highlighted earlier, eating and/or drinking during the biological night-time may disrupt peripheral clocks. If workers snack during night shifts, it is therefore important to minimise energy intake and select dietary choices that lead to favourable postprandial responses. These snacks should also be convenient, minimally processed, micronutrient-dense, satiating, easy to digest, and minimally perishable, when applicable.

Preliminary research has shown that when 24-h energy and macronutrient intakes are controlled during simulated night shifts, a small snack (containing 10% of daily energy intake) may support cognitive function and performance in simulated driving compared with no snacking or a larger meal containing 30% of daily energy intake (75). In this instance, the small snack also reduced hunger to a comparable extent to the meal, without leading to significant digestive discomfort (76). Compared to large night-time snacks, small night-time snacks may also be

better for metabolic health. Glycaemic control is relatively easy to measure and predictive of many health outcomes, and some researchers have therefore focused on the effects of nocturnal snacking on glycaemic control. Compared with a small midnight snack (∼200 calories), a large midnight snack (∼500 calories) impaired postprandial glycaemic responses at a subsequent breakfast at 08:30 during simulated shift work (77). Research such as this is informative, but we again need additional studies of workers in which the effects of dietary changes on metabolic parameters are measured around the clock.

## CHRONONUTRITION: THE IMPORTANCE OF DIET COMPOSITION

Shift workers are not only apt to consume foods and drinks at suboptimal circadian phases, the quality of shift workers' diets is often worse than that of day workers too. Many shift workers report consuming few fruits and vegetables while also consuming a variety of processed foods at work, such as biscuits, cakes, chocolates, pastries, sandwiches, and fried foods (42). As diet composition affects metabolic health and cognitive function, it is important to help these people make better dietary choices. One way by which diet composition influences health is via effects on the circadian clockwork, and the ketogenic diet (KD) exemplifies this. There has been a resurgence in interest in the KD of late, and while some believe that the restrictive nature of the KD is a barrier to its widespread implementation, certain properties of the KD make it an appealing option for some shift workers who are able to adhere to it.

### The Ketogenic Diet

Studies of mice have shown that the KD has chronobiotic actions on the clocks in multiple peripheral tissues, including the brain, gut, and liver (78–80). Interestingly, Tognini and colleagues found that a KD induced distinct changes in the liver and gut clocks in mice. Compared to a control diet, consumption of a KD produced greater amplitudes of clock gene transcription and their downstream products in the liver, as well as inducing 24-h oscillations in the transcription of many genes in the gut (78). As disruption of the gut clock is associated with increased intestinal inflammation and permeability, as well as endotoxaemia (78, 81), if translatable to humans these results suggest that shift workers who follow a KD may protect themselves against some of the adverse consequences of consuming calories at suboptimal circadian phases.

More generally, both the KD and less severe carbohydrate restriction may reduce some negative effects of shift work on metabolic health. Shift workers are at an increased risk of impaired glucose tolerance and type-two diabetes, and restricting carbohydrate intake is likely to reduce fasting and postprandial glycaemia, both of which are precursory to numerous chronic diseases (e.g., some cardiovascular diseases, certain cancers, and dementia) (82–87). Preliminary evidence has shown that a multicomponent lifestyle intervention centred on the KD may also improve subjective sleep quality in adults who have poor glycaemic control (88), suggesting that sleep enhancement may mediate some of the reported benefits of the KD.

In preclinical studies, ketone bodies themselves have been found to have pleiotropic beneficial physiological effects, including modulation of inflammation, tissue-specific suppression of mTOR signalling, and increased production of brain-derived neurotrophic factor (89–91). If translatable to humans, these systemic effects of ketone bodies imply that long-term consumption of a KD could reduce risk of certain cancers and neurodegenerative diseases such as Alzheimer's in shift workers, particularly those that are already at increased risk (92, 93). Increased production of ketone bodies may also account for some benefits of fasting and TRE. For example, early TRE led to greater morning beta-hydroxybutyrate levels compared to a 12-h caloric period (59). However, there have not yet been any clinical trials of the KD in shift workers, and it will be interesting to explore how the combination of the KD and TRE and/or intermittent fasting interact to affect ketosis, metabolic regulation, and circadian biology in these people.

### Other Dietary Chronobiotics

In addition to effects of dietary patterns on the circadian system, specific dietary compounds have chronobiotic actions. A multitude of dietary compounds affects the circadian system and sleep (94, 95), and it is beyond the scope of this article to discuss them all. We therefore focus on some of those that we anticipate may be practical and beneficial for shift workers. In the future, screens for novel chronobiotics and hypnotics may yield compounds that support the health and performance of these workers (96). Identifying agents that counter decrements in health and cognitive function incited by sleep disruption would also benefit shift workers.

### Caffeine

Largely by antagonising adenosine receptors, consumption of caffeine can improve alertness, attention, reaction time, and mood, as well as physical performance in tests of endurance, strength, and power (44). Studies of caffeine consumption by shift workers have consistently shown beneficial effects on multiple aspects of cognitive function, although whether this results in improved safety is not clear (97). The trade-off is that caffeine consumption tends to prolong sleep latency, reduce slow-wave activity during sleep (which is important to numerous restorative processes), shorten sleep duration, fragment sleep, and worsen subjective sleep quality (98). Consumed late in the day as coffee, caffeine also delays circadian phase (99). Thus it is clear that while judicious caffeine intake can be used to help shift workers perform at work - especially when sleepy - mistimed caffeine intake may strongly degrade sleep, which is noteworthy given that many of the adverse consequences of shift work appear to relate to its detrimental effects on sleep (5). It therefore seems prudent to recommend that shift workers generally stop consuming caffeine several hours before their main sleep period (more specific guidance on caffeine intake is provided in **Table 1**).

## Creatine Monohydrate

Antagonising adenosine receptors is one way to reduce the accumulation of pressure to sleep (sleep homeostasis), but another is to bolster the phosphorylation of adenosine. Creatine (creatine monohydrate, specifically), a safe and inexpensive dietary supplement that increases brain phosphocreatine stores, countering the accumulation of extracellular adenosine in the brain during extended wakefulness. A study of rats showed that adding creatine to the rats' chow for 4 weeks reduced the duration and slow-wave activity of the rats' sleep (100). We do not currently know the effects of creatine supplementation on sleep in humans, however. Notably, while shorter sleep would generally be expected to impair health and performance, creatine supplementation has repeatedly been shown to *enhance* these variables in humans. Creatine supplementation routinely improves performance in - and adaptations to - many exercise tasks, and creatine has a number of therapeutic actions, including neuroprotective properties (101).

Interestingly, creatine supplementation may also acutely help protect against the deleterious consequences of sleep loss. After sleep loss, creatine supplementation seems to offset deterioration in executive function, mood, reaction time, balance, and other motor skills (102–104). Although we expect creatine supplementation to be a useful strategy to help but this people cope with shift work, we are not aware of any research on this topic. We also note that there is some evidence that concurrent consumption of caffeine may reduce some of the ergogenic effects of creatine on physical performance (105), and additional studies are needed to better identify how the two compounds interact.

## Dietary Amino Acids

Several dietary amino acids may influence circadian rhythms and sleep. For instance, L-tryptophan is a precursor to melatonin that researchers have studied with respect to circadian rhythms and sleep. As an example, there appears to be a temporal relationship between consumption of L-tryptophan in breast milk and infant urinary excretion of 6-sulfatoxymelatonin, the primary metabolite of melatonin (106). Furthermore, infants fed L-tryptophan-enriched night-time formula seem to experience more consolidated sleep/wake patterns (107). Many studies of adults have also shown that ∼ 2 g L-tryptophan each day enhances some sleep parameters, although it is not a potent hypnotic (108). To our knowledge, there are no rigorously controlled studies demonstrating that L-tryptophan affects circadian phase, however.

Overall, there has been little research on whether amino acids affect circadian system parameters. In a screen of whether amino acids affect light-induced shifts in the phase of wheel running activity in mice, L-serine increased the magnitude of phase shifts by 86%. This effect seems to translate to humans, as adults who consumed L-serine before bedtime experienced a greater advance in circadian phase in response to bright light exposure (109). Another study reported that 1 week of L-ornithine supplementation delayed the plasma melatonin rhythm by 15 min (110). However, LD cycles and meal timing were not fully controlled in these studies. Interestingly, there is also preliminary evidence that regular L-ornithine supplementation (400 mg per day) may enhance sleep quality during stressful periods (111, 112).

L-glycine may too affect sleep. Consuming 3 g L-glycine an hour before bedtime appears to shorten sleep latency, increase sleep efficiency, and reduce daytime sleepiness in healthy adults, effects that appear to be mediated via the suprachiasmatic nucleus (113, 114). Such supplementation also seems to diminish daytime fatigue and boost vigilance during sleep restriction (115), implying that L-glycine may both enhance sleep and the ability to cope with sleep loss. Given that L-glycine is safe, inexpensive, and may confer other health benefits (116), night shift workers could gain from supplementing with this amino acid. At present, however, there has been little research on effects of this amino acid on sleep.

In summary, it is plausible that supplementing with certain amino acids may help shift workers adapt more quickly to changes in their shifts and/or sleep better, but this is based on few studies that did not control zeitgeber cycles or explore whether the circadian timing of amino acid ingestion interacts with the circadian timing of light exposure. Going forward, it will be important to address these limitations. It will also be interesting to see whether concurrent consumption of different chronobiotic agents additively boosts circadian phase shifts.

# THE FUTURE

We have mentioned several ideas for future studies, and we will end by focusing on additional research avenues that may be worth exploring with respect to improving the health of shift workers.

## Using Novel Technologies to Better Personalise Guidance for Shift Workers

Rapid recent advances in the development and uptake of digital technologies such as smartphones, apps, wearables, and artificial intelligence provide scientists with an unprecedented ability to comprehensively assess people's behaviours and health in free-living contexts. The myCircadianClock app is a salient example of such technology. This app has already been used in multiple studies to monitor the circadian phenotypes of study participants, unveiling interesting insights into the effects of interventions such as TRE on human health (64, 117).

As data collected from digital devices are time-stamped, it is easier than ever to temporally map behavioural patterns and their biological sequelae, which could provide novel insights into the causes of changes in the health trajectories of shift workers. One could identify the hours of the day in which it is most frequently a shift worker's biological daytime by longitudinally assessing the timing of the individual's biological clock. As it is not currently practical to assess an individual's melatonin rhythm on a daily basis, the integration of data from surrogate markers of circadian phase such as body temperature and sleep/wake cycles could be used to approximate the timing of the biological daytime. These cycles could be monitored ambiently using data from devices such as smartphones, and the data from the devices could then be used to inform individual shift workers about how to best implement TRE. Where feasible, this process could be refined

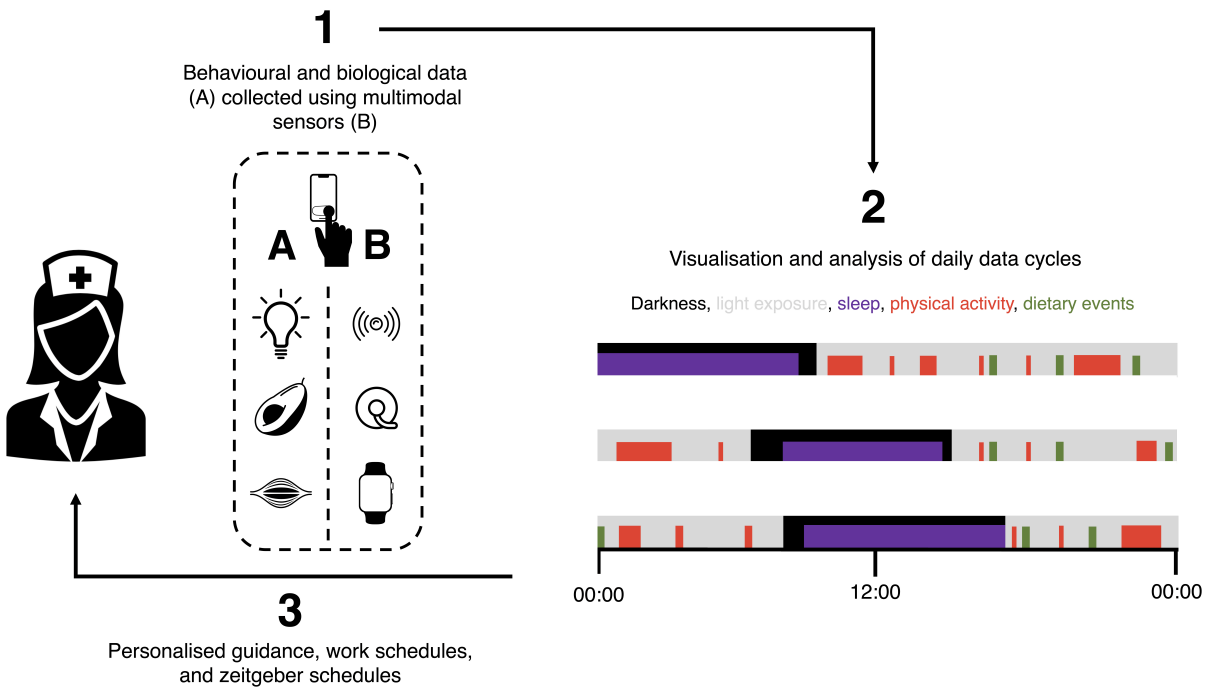


**FIGURE 1 |** Using digital devices to optimise the health and performance of shift workers by providing personalised guidance, work schedules, and zeitgeber schedules. (1) Digital devices can be used to monitor behaviours and their biological responses, including (i) exposures to variables such as light (e.g., via head-worn wearables), (ii) dietary behaviours (e.g., via food photography and continuous glucose monitoring), and (iii) physical activity (e.g., via smart watches). Digital phenotyping using patterns of smartphone use can be used to enrich this analysis. (2) These data and their interactions can then be analysed in real time and used to (3) personalise guidance, shift schedules, and zeitgeber schedules of individual workers. Guidance can be delivered digitally and in-person, and innovative technologies may eventually allow the automation of adjustments to individual workers' zeitgeber schedules.

with the addition of round-the-clock measures of metabolic regulation, such as continuous glucose monitoring.

At a small scale, the feasibility of this type of approach has already been shown (118). Ultimately, implementing such methods at a large scale and including both shift workers and non-shift-working controls may help develop models that forecast transitions in the health of shift workers, as well as how to alter these trajectories. However, the data collection process will need to be relatively frictionless (for participants, at least) to achieve this. This will be facilitated by close collaboration between scientists and workers in the technology sector. With accurate monitoring in place, digital tools could then be implemented to improve the health and productivity of shift workers by optimising variables such as zeitgeber schedules in real time (**Figure 1**).

Innovative technologies could also provide novel means of generating insightful data while minimising participant burden. For example, sensors commonly built into smartphones can now be used to monitor blood parameters such as haemoglobin that once required invasive testing (119). Smartphones can also be used to monitor some exposures that are particularly relevant to shift workers, such as patterns of locomotion and exposure to light. One problem, however, is that it would be especially useful to assess exposure to light at the level of the eye. This requires new wearable devices, for smartphones are not suited to this, and many existing wearables that measure light exposure are frequently obstructed by clothing, confounding their data. It is possible to make smart eyewear to estimate retinal light exposure, and such eyewear may be especially useful for another purpose. The utility of all of these monitoring technologies may be enhanced by the addition of the ability to digitally "envirotype" individuals, ambiently tracking information about their environments to better understand the interaction between environment and phenotype (120). Building camera technology into eyewear is one way to accomplish this.

Meanwhile, digital phenotyping – assessing changes in people's phenotypes using data from digital devices – has already been used to identify patients' disease trajectories in neurological disorders such as schizophrenia (121). Such phenotyping can proceed without active user engagement, and it can also be used to assess behaviours such as sleep (122). Ultimately, use of multimodal novel sensors that analyse biofluids including interstitial fluid (e.g., continuous glucose monitoring), saliva, sweat, and tears may prove particularly useful in monitoring variables such as dietary intakes and associated changes in metabolites (123). However, the development of these sensors poses substantial challenges related to biofouling, accuracy, power, usability, calibration, and data security.

These tools are promising approaches to forecasting changes in behaviours and health, and we hope they will help healthcare professionals intervene before individuals succumb to disease. We foresee that using sophisticated computational methods such as deep learning to concurrently analyse individuals' behavioural, health, and environmental data from multimodal sources will eventually enhance personalisation of guidance for individual shift workers (124).

## Applying Behavioural Science to Support Better Health Decisions by Shift Workers

Even if shift workers understand precisely which behaviours they should enact to improve their health, they are prone to a variety of factors that impair decision making, such as circadian system misalignment and sleep loss (125, 126). Furthermore, knowledge alone is rarely sufficient to support lasting health behaviour change (127). It is therefore imperative to support the ability of these people to make smart decisions, and this requires applying principles from behavioural science, particularly at the level of the organisations that employ shift workers.

Significantly, many new technologies are strikingly habit-forming, and this exemplifies the power of applying behavioural science principles to shape behaviour. If scientists and technologists can collaborate to effectively use behavioural science to create engaging, scalable products that deliver tailored health guidance to shift workers, all would benefit. We believe that technologies that deliver adaptive interventions to both help people avoid poor health decisions during states of vulnerability *and* support good health decisions during states of opportunity will be particularly advantageous (128).

The built environment also affects health in numerous ways (129), and given that shift workers are prone to health problems, it is particularly critical to pay attention to optimising the workplaces of these people. As shift workers commonly experience circadian system disruption and do not gain tolerance to such disruption (130), it may be valuable to create workplaces that allow close control over exposure to light, and intelligent use of "smart" lighting systems may benefit these individuals. We also anticipate the development of closed-loop devices that will personalise light exposure at the level of the individual.

The built environment influences physical activity. To support job performance and health, workplaces should have designated exercise spaces to encourage physical activity. Environmental design is relevant to nutrition too. Dietary choices depend strongly on where foods and drinks are sourced from. In work settings such as airplanes, food is provided for shift workers. However, most shift workers are left to source their own food, and when short on time many shift workers buy foods and drinks from vending machines (42). It is encouraging that many workers do select healthier dietary choices when they are available in vending machines, providing an opportunity for organisations to positively affect their employees' health decisions (131). Furthermore, workplace interventions to promote healthier diets, such as offering free fruit and labelling meals, have sometimes been shown to facilitate healthy dietary choices (132, 133). Simple changes in the placement of food in eating areas affect food selection too (134), and these changes can be leveraged to support the health of shift workers. Similarly, if workers are using products such as melatonin supplements and blue-light-blocking glasses to shift the phases of their circadian systems, it makes sense to help them acquire efficacious products.

It is also clear that social life is a strong influence on many shift workers' health behaviours, including their diets. The dietary attitudes and preferences of co-workers affect some workers' dietary choices (135), so group commitment to healthier dietary choices may aid the adoption of more nutritious diets. As stress strongly affects dietary choices in many people and shift workers often report high stress and abnormal dietary behaviours (136), interventions to nurture the resilience of shift workers and to improve workers' self-regulation skills may support their dietary choices. Such interventions include mindfulness-based approaches (137). Shift workers could also benefit from other types of social support, including provision of additional childcare, as well as groups and events designed to minimise conflicts between their work and non-work activities.

Educating shift workers about how to sleep better is likely to be pivotal to their well-being, and shift work workplaces should have spaces for sleepy workers to nap. It is of course important to identify workers who have sleep disorders too, and simple screening tools such as brief questionnaires can be used for this (138). It may too be useful to screen for people who are simply not suited to certain shift schedules, for people differ substantially in how they tolerate shift work. Certain characteristics associate with better shift work tolerance, including robust general health; young age; male sex; not having children; low languidity and neuroticism; high extraversion, flexibility in sleeping habits, and internal locus of control; and a chronotype that is neither very early nor very late (18). Promisingly, personalising shift work schedules by removing night shifts for early chronotypes and excluding morning shifts for late chronotypes has been shown to prolong self-reported sleep, improve subjective sleep quality, and enhance worker well-being (139). To estimate chronotype, a study by Vetter and colleagues used a shift work-specific version of the Munich Chronotype Questionnaire (140), and this approach may be useful to help personalise work schedules for shift workers. Nonetheless, it would be useful to develop additional questionnaires designed specifically to identify appropriate shift schedules, as well as to track how workers respond to these schedules.

Finally, it is worth noting that many workplace wellness programmes that have been tested have not yielded impressive results (141). Assessing the effects of workplace interventions is difficult for numerous reasons, not the least of which are enforcing blinding and randomisation of participants. To date, marked heterogeneity between studies has made it challenging to assess the utility of workplace interventions for shift workers (30). And as is so often the case, the participants included in many of these studies did not comprise a diversity of ages and races, nor did the scientists attempt to define determinants of which workers responded positively to the interventions. None of this means that it is not possible to implement effective programmes, however, and we hope that lacklustre

results to date do not stymie continued efforts to improve on workplace interventions by better incorporating principles from behavioural science.

### Using Alternatives to Traditional Study Designs to Better Personalise Guidance for Shift Workers

To assess the efficacy of interventions to improve shift-worker health, it may make sense to use alternatives to many of the hitherto-used study designs. Recently, studies applying "Big Data" approaches have contributed to some advances in efforts to personalise medicine. However, it may be advantageous to concurrently carry out studies that use a "Small Data" paradigm – for example, using *n*-of-1 approaches to more rapidly assess how individual workers are responding to a given intervention and to forecast which of them are at risk of health trajectory transitions towards disease (142).

## CONCLUSIONS

A large proportion of the workforce works shifts, and these individuals are integral to sustaining functional societies. However, the study of how to support the long-term health and well-being of these people has been somewhat neglected, and a relatively small proportion of relevant studies has included shift workers as participants. While the type of personalised interventions to support shift workers that we have discussed in this article are bound to produce logistical headaches for employers, the onus should be on supporting the long-term the health and performance of their employees. The acute difficulties arising from implementing customised shift schedule systems and suchlike may be more than made up for by the lasting benefits of these systems on health, safety, and productivity. We note also that as shift work increases the likelihood of adverse pregnancy outcomes and may lead to epigenetic modifications in parents that could plausibly affect the epigenetics and hence health of their children, supporting the health of shift workers could one day have critical effects on the well-being of future generations (143, 144).

Scientists now have an unprecedented ability to identify ways of helping shift workers. We hope that this ability is realised in the near future.

## AUTHOR CONTRIBUTIONS

GP conceived the idea and drafted the manuscript. TW edited the manuscript and produced the final draft. All authors approved the final version and performed the literature review.

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