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REVIEW ARTICLE

Circadian Regulation of Fatty Acid Metabolism in Humans: Is There Evidence of an Optimal Time Window for Maximizing Fat Oxidation During Exercise?

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Abstract

Exercise training performed at the intensity that elicits maximal fat oxidation improves cardiovascular function and metabolic health while simultaneously reducing visceral adipose tissue in patients with obesity and type 2 diabetes. Indeed, it is currently considered an efficient non-pharmacological approach for the prevention and treatment of cardiometabolic disorders. Over the last 5 years, several studies have reported a diurnal variation in both resting fat oxidation as well as maximal fat oxidation recorded during submaximal intensity exercise. Higher fat oxidation has been recorded during the evening in comparison with the early morning, although this has not been universally observed. If evening exercise increases fat oxidation, then this timing of exercise may be preferable for the reversal of cardiometabolic diseases. However, the physiological and molecular mechanisms behind the circadian regulation of fatty acid metabolism have not yet been fully elucidated. The present review thus aims to describe the circadian rhythmicity of several hormones, metabolites, and enzymes involved in fatty acid mobilization and oxidation. Furthermore, we discuss the relevance of circadian mitochondrial dynamics and oxidative phosphorylation to fatty acid metabolism. To conclude our discussion, we highlight those biological (e.g., age and sex) and lifestyle factors (e.g., sleep quality/disturbances or physical activity) that potentially influence the circadian regulation of fatty metabolism and which therefore should be considered for a tailored exercise prescription.

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Graphical abstract

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Circadian regulation of fatty acid metabolism in humans: is there evidence of an optimal time window for maximizing fat oxidation during exercise?

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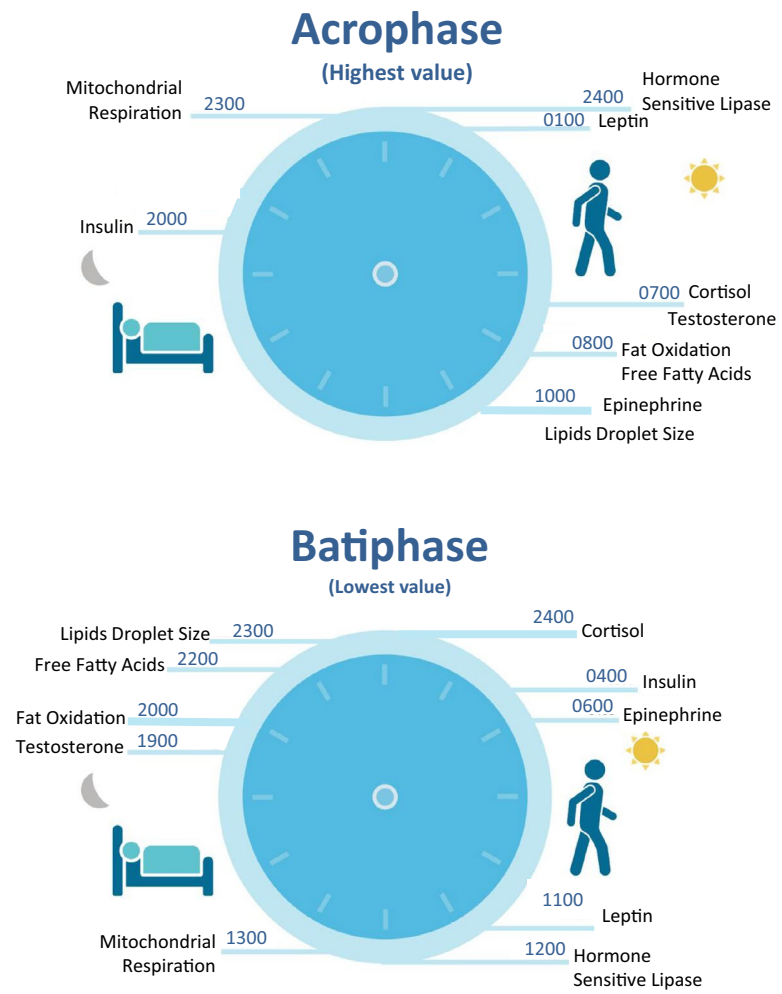
What is this study about? This review describes the circadian rhythm of key biomarkers associated with fatty acid metabolism.



Take home message: Physiological conditions in the morning stimulate a superior fat oxidation in comparison to the afternoon and the evening. Hence, morning exercise may be convenient for body weight management.

Key Points

Analysis of 24-h resting fat oxidation rhythmicity reveals higher fat utilization during the early morning in young healthy men, rebutting those findings obtained from the analysis of maximum fat oxidation rate diurnal variation in which higher fat oxidation in the early evening was observed.

Higher fat oxidation in the early morning is related to enhanced fatty acid availability and lipolytic hormones concentration but not elevated mitochondrial respiration or lipolysis in skeletal muscle.

Further studies investigating the influence of age, sex, nutritional status, sleep disorders, and chronotype on the circadian rhythmicity of fatty acid metabolism are deemed necessary to establish specific daytime recommendations for maximizing fat oxidation.

1 Introduction

Maximizing fat oxidation during exercise is a time-efficient therapeutic approach for improving physical fitness and cardiometabolic health in patients with obesity, type 2 diabetes mellitus, and metabolic syndrome [1, 2]. As evidenced by recent systematic reviews and meta-analyses, exercise training at the intensity that elicits maximal fat oxidation (FATmax) induces moderate to large reductions in body fat percentage, fasting insulin resistance, low density lipoprotein cholesterol levels, diastolic blood pressure, and waist circumference in the above-mentioned populations [1, 3]. Concurrently, FATmax training improves the leptin to adiponectin ratio, maximum oxygen uptake, ejection fraction, mitochondrial respiratory capacity, and stroke volume among subjects with obesity and metabolic syndrome [3].

In a recent systematic review, Chávez-Guevara et al. [4] provided exercise intensity guidelines for optimizing fat oxidation in subjects with obesity, establishing training volume recommendations specific to age, sex, and the type of exercise. Such recommendations, nevertheless, were limited to exercise training performed in a fasted state during the early morning (0800–1100 h), leaving it unclear if exercise at this specific time of the day would be the most effective approach for maximizing fat oxidation.

Previous investigations have reported that maximal fat oxidation (MFO) shows a diurnal variation in male athletes, young men with obesity, and adults with metabolic syndrome, observing higher MFO values during the afternoon or the evening (1700–2000 h) compared with the early morning (0800–1100 h) (Fig. 1a) [5–7]. These observations have led to the hypothesis that evening exercise would result in a higher fat oxidation in these populations. However, it should be stated that MFO commonly exhibits a poor reproducibility [8, 9]; hence, it might be possible that the MFO diurnal variation could simply reflect the day-to-day variation in exercise fat utilization, although the precision of metabolic carts used in prior studies also needs to be considered. Indeed, Amaro-Gahete et al. [5] used a metabolic cart (Ultima CPX) that showed a large measurement error for assessing fat oxidation rate during exercise (error: $39.5 \pm 20.8\%$) [10]. On the other hand, the precision of the metabolic carts used by Mohebbi and Azizi [6] (Cosmed Quark b2) and Methnani et al. [7] (Metasys TR) for assessing fat oxidation rate remains unexplored.

Adding to the above-mentioned issues, all studies instructed their participants to avoid strenuous exercise before the exercise tests; however, they did not verify if the subjects followed these recommendations by comprehensively assessing their physical activity levels (e.g., accelerometry). Regarding dietary standardization, both Amaro-Gahete et al. [5] and Methnani et al. [7] provided individualized meals 24-h before the exercise trials; however, neither blood glucose concentration nor resting metabolic rate was assessed in these studies to verify if the participants arrived at a similar metabolic condition before the exercise trials. Additionally, Methnani et al. [7] grouped the data from men and women despite a sexual dimorphism consistently reported for FATmax and MFO [1, 3]. Moreover, that same study mixed the data from young and middle-aged women, even though MFO is higher in pre-menopausal women than in post-menopausal women [11].

Contrary to the above-discussed studies, a diurnal variation in MFO was not observed in healthy young females [12], while diurnal fat oxidation during exercise has not been assessed in adolescents or elderly adults. To add further complexity to the interpretation of MFO diurnal variation, it should be mentioned that previous studies assessing the circadian rhythmicity of fat oxidation and resting metabolic rate in healthy adults did not support the notion that fat utilization is higher in the evening. In fact, the peak resting fat oxidation rate was observed in the morning (0800 h), a few hours after the nadir of energy expenditure (0400 h) [13, 14].

Given that resting fat oxidation has been positively associated with MFO in healthy adults [15–17], it seems contradictory that MFO shows higher values in the afternoon or the evening, whereas resting fat oxidation peaks in the early morning. In fact, since afternoon and evening exercise trials were performed after consuming a high carbohydrate meal (i.e., postprandial status), it does not make sense to observe a superior MFO in the late part of the day, as carbohydrate

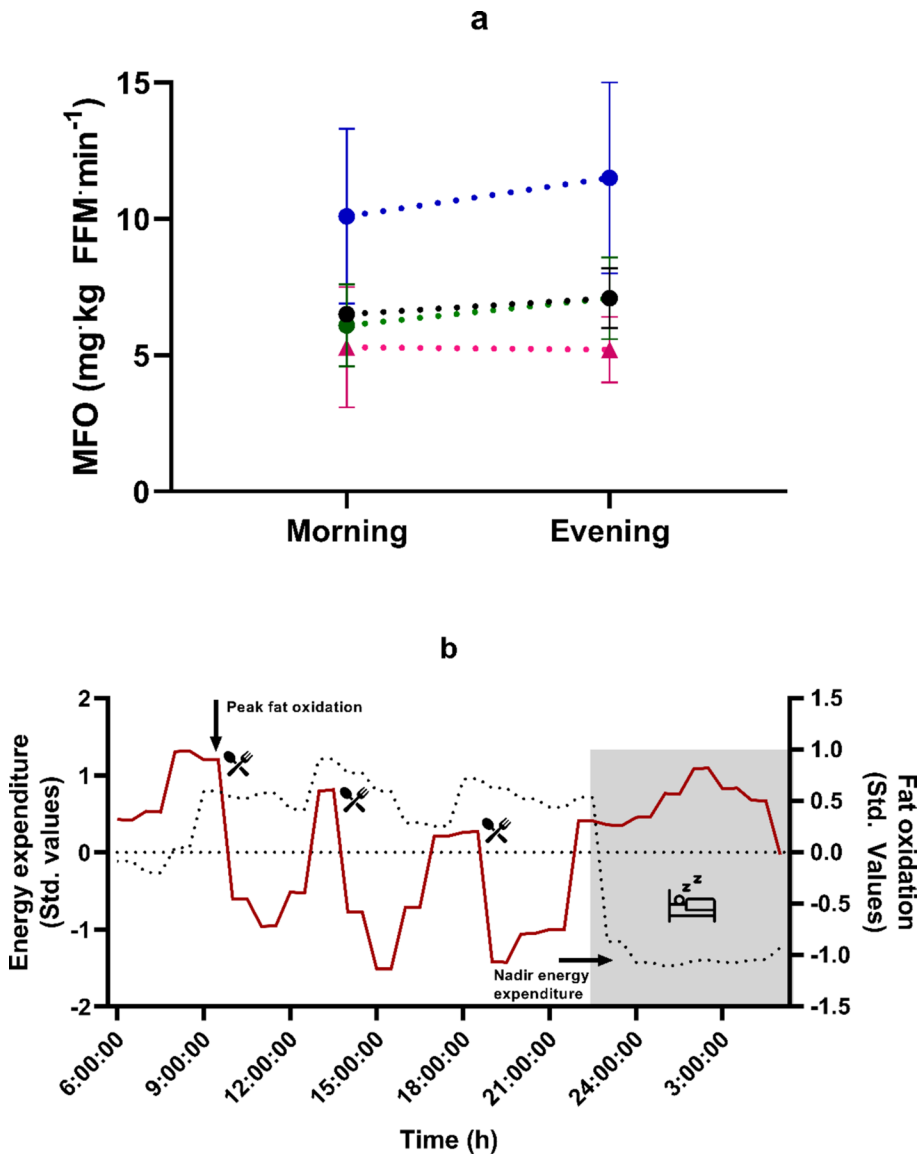


Fig. 1 **a** Diurnal variation of maximal fat oxidation rate at submaximal intensity exercise in endurance athletes (*blue*), healthy men (*black*), men with obesity (*green*) and healthy women (*pink*). Plotted values represent the mean ± SD reported by Mohebbi et al. [6], Amaro-Gahete et al. [5], and Robles-Gonzales et al. [12]. **b** Circadian rhythmicity of energy expenditure (*dotted lines*) and fat oxidation rate (*continuous lines*) in healthy men. Both kinetics were rebuilt in WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/) based on the data reported by Zhang et al. [14]. The figures were elaborated in GraphPad Prism v.8.1. *FFM* fat-free mass, *MFO* maximal fat oxidation rate

intake downregulates mitochondrial fatty acid oxidation and adipose tissue lipolysis [18].

To further explore which would be the best window of time for maximizing fat oxidation during exercise, the present review provides a detailed discussion of the molecular and physiological mechanisms behind the circadian regulation of fatty acid metabolism. In particular, we describe the circadian rhythmicity of several hormones, metabolites, and enzymes that regulate fatty acid mobilization and oxidation in humans (Supplementary file 1, see electronic supplementary material [ESM]). Furthermore, we argue the relevance of circadian mitochondrial dynamics and oxidative phosphorylation to fatty acid metabolism. Whether morning exercise close to the FATmax zone stimulates a higher body fat reduction than exercise in the evening is also discussed. Finally, we highlight several biological (e.g., age and sex) and lifestyle factors (e.g., sleep quality/disturbances, physical activity) that may influence the circadian and diurnal regulation of fatty acid metabolism. The full search strategy applied to identify all the studies discussed in this review is provided in Supplementary file 2 (see ESM).

2 Circadian Regulation of Fatty Acid Metabolism

2.1 Biological Clocks

Energy homeostasis is essential for the survival of any living organism. Therefore, complex biological clocks have evolved under selective environmental pressures to ensure an equilibrium between energy availability and energy expenditure within a 24-h period (i.e., energy balance) [19]. Human beings behave as a diurnal species and exhibit

diverse sleep–wake cycles, with three specific chronotypes: morning types (work by day, sleep by night), evening types (sleep by day, work by night) and intermediates (non-specific sleep/wake cycle) [20]. Consequently, circadian and seasonal rhythms are predominantly organized by light exposure, a stimulus that activates numerous intracellular clock genes in peripheral tissues through neuronal and hormonal signals [21]. Additional cues like temperature fluctuations, social interactions, feeding, and exercise patterns are also important modulators of the circadian rhythmicity. These environmental factors are called zeitgebers and allow the body's metabolic homeostasis to be synchronized with the environment [22].

The suprachiasmatic nucleus (SCN) of the hypothalamus acts as the central governor of circadian rhythms. This central pacemaker comprises approximately 20,000 neuronal circadian oscillators [23] and assumes a pivotal role in orchestrating the synchronization of the sleep–wake cycle, integrating metabolic, environmental, and genetic factors [24]. The SCN receives external light signals which are transmitted though the retina-hypothalamic tract, which is an anatomical structure that is responsible for communication from the retina to the SCN and preserves coherence with the 24-h day [25]. Peripheral cell tissues, nonetheless, possess an autonomous circadian clock to regulate their metabolic homeostasis [26]. The latter is achieved throughout the intracellular activity of clock genes which are ubiquitously expressed in all body cells of mammals and form a self-regulatory transcription/translation feedback loop, producing daily fluctuations of intracellular protein levels and activity [27]. The circadian fluctuation of clock genes rhythmically represses the activity of the peroxisome proliferator-activated receptors (PPARs), key transcription factors that control the expression of hundreds of genes involved in lipid biosynthesis, fatty acid metabolism, and mitochondrial biogenesis [28]. In turn, the activity and expression of clock genes is mediated by the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), a transcriptional coactivator that also participates in glucose and fatty acid metabolism (Fig. 2) [29].

2.2 Circadian Rhythmicity of Key Enzymes and Transport Proteins that Regulate Fatty Acid Metabolism

At present, only the circadian rhythm of hormone sensitive lipase (HSL) has been characterized ex vivo by using adipose cells extracted from subcutaneous adipose tissue in humans [30]. This lipolytic enzyme catalyzes the hydrolysis of diacylglycerol molecules stored in lipid droplets, and its activity gradually increases from 1200 to 2400 h (Fig. 3a). Recent studies in healthy men and women reported that MFO is positively related to the abundance of HSL in adipose tissue and skeletal muscle [31, 32]. Hence, one may assume that MFO would be higher in the early evening due to an elevated activity of HSL, which would increase the availability of free fatty acids during exercise. The circulating levels of free fatty acids, nevertheless, have not been assessed in previous studies reporting a diurnal variation in MFO. Additionally, the availability of free fatty acids is two-fold higher during the morning than in the early evening (Fig. 3b).

As we discuss in detail in the following section, the circadian rhythmicity of several hormones that regulate HSL activity does not support the notion that MFO is higher during the evening due to an elevated adipose tissue lipolysis. In fact, the concentration of endocrine hormones that activate HSL is higher during the morning than in the early evening or the afternoon (Fig. 4). Such discrepancies between HSL activity and free fatty acid availability might be explained by the fact that HSL activity was characterized from adipocytes donated by subjects with obesity whereas free fatty acid and endocrine hormone rhythmicity has been mainly characterized in lean healthy individuals. Thus, future studies assessing both HSL activity and free fatty acid availability are necessary to fully explain the molecular mechanisms that regulate MFO at different times of the day and across varying metabolic states.

The abundance of carnitine palmitoyl transferase 1 (CPT1)—the rate-limiting enzyme controlling the transport of fatty acids into the mitochondrial matrix—and long-chain fatty acid transport proteins (i.e., CD36, FATB) have also shown positive correlations with MFO in young trained and untrained men [31, 33]. Nevertheless, no previous study has investigated the circadian rhythmicity of these enzymes and transport proteins in human skeletal muscle. Only one previous study in young overweight women reported that CPT1 shows a diurnal variation in skeletal muscle, with a higher expression in the morning versus the afternoon that was associated with variations in PERIOD proteins [34]. Such findings, nonetheless, require further analyses to confirm if a higher fat oxidation during exercise might be achieved in the early morning or the evening due to a superior mobilization and oxidation of fatty acids within skeletal muscle.

2.3 Circadian Rhythmicity of Endocrine Hormones Involved in Fatty Acid Metabolism

During FATmax exercise, free fatty acids released from adipose tissue and intramuscular triglycerides provide most of the required energy in skeletal muscle [35]. As we previously discussed, the availability of free fatty acid peaks in the early morning (0800 h) and shows a gradual reduction until the late evening (2200 h) [13]. This circadian pattern is modulated by variations in several hormones that activate or inhibit HSL in adipose tissue [36]. Additionally, many of

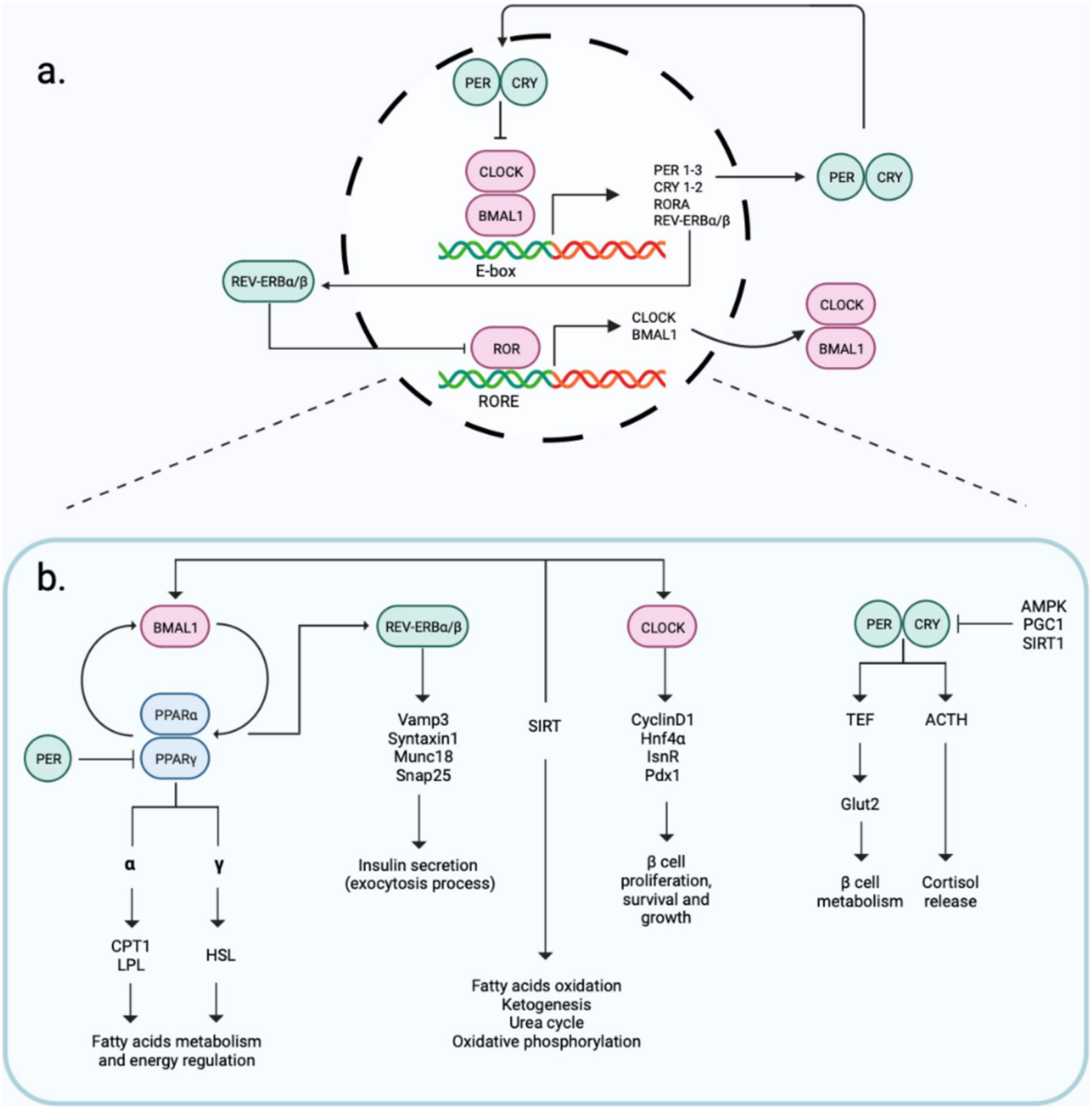


Fig. 2 Circadian molecular mechanism and signaling involved in fatty acid oxidation in mammals. *Arrows* indicate activation and *bars* indicate inactivation. **a** Circadian oscillations are generated by transcriptional–translational feedback loops, involving a core set of clock genes and proteins that regulate gene networks that oscillate with a 24-h cycle. The clock proteins include CLOCK and BMAL1 (activators), and PER1-3, CRY1-2 (repressors). CLOCK/BMAL1 begins with transcription of PER/CRY, then these proteins build up in the cytoplasm, where they form a complex that is transported to the nucleus, binding to the BMAL1 promoter to turn off their own expression, inhibiting the transcription of CLOCK/BMAL1. The second transcriptional loop involves the CLOCK/BMAL1 complex and the REV-ERBα/β nuclear receptors, which compete with the RORα/β/γ receptor for binding sites on the BMAL1 gene. REV-ERB has a negative effect of transcription while ROR has a positive effect. Although the expression of heterodimer CLOCK/BMAL is not limited to a specific time of day, it exhibits a 24-h variation. In mice, CLOCK/BMAL1 proteins are activated during the day, leading to the production of PER/CRY in the afternoon. Nevertheless, in humans the regulation cycle of these proteins is flipped [13], because humans have different natural body rhythms with most people being active during the day. The circadian clock plays an important role in energy metabolism through the interaction of genes that control this metabolism. **b** Diagram showing the molecular mechanisms that control the circadian clock and how it interacts with metabolism around fatty acid oxidation. *BMAL* basic helix-loop-helix ARNT-like 1, *CLOCK* circadian locomotor output cycles protein kaput, *Cry* cryptochrome circadian regulators, *PER* period circadian regulators, *REV-ERBα/β* Rev-erb nuclear receptors alpha/beta, *RORα/β/γ* nuclear receptor ROR-alpha/beta/gamma

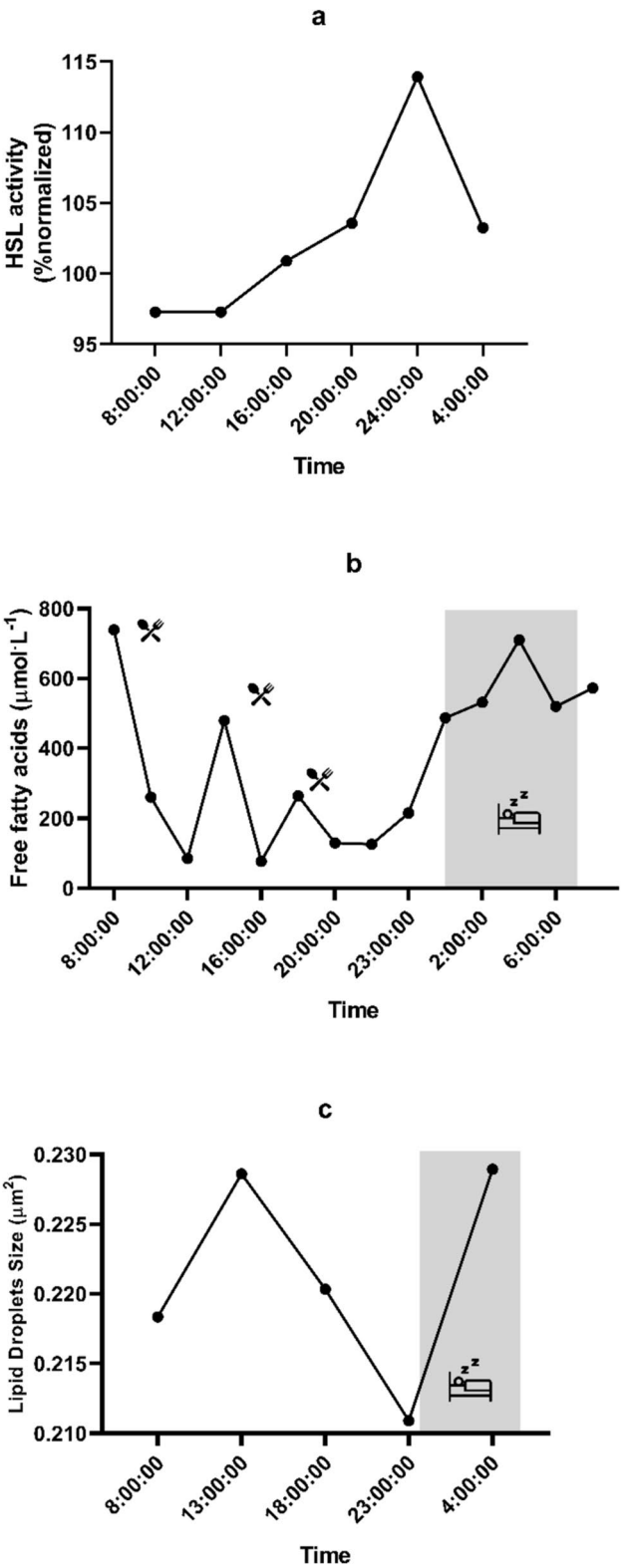


Fig. 3 Circadian rhythmicity of free fatty acids (**a**), intramuscular lipid droplets size (**b**) and hormone sensitive lipase measured ex vivo in adipose tissue (**c**). All kinetics were rebuilt in WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/) based on the data reported by Arredondo-Amador et al. [30], Held et al. [44], and Wefers et al. [13]. Sleep period is highlighted with *grey shadows* based on the protocol reported by each study. Meal timing was only available for free fatty acid availability. The figures were elaborated in GraphPad Prism v.8.1. *HSL* hormone-sensitive lipase

these hormones regulate the activity of CPT1 and fatty acid transport proteins in skeletal muscle, affecting the circadian rhythmicity of the fat oxidation rate [36].

Input signals arriving at endocrine glands via the hypothalamic–pituitary–adrenal axis (HPA) and the hypothalamic-pituitary-thyroid axis modulate hormone secretion and activity in a circadian manner [37, 38]. Conversely, these hormones provide feedback signals to the SCN, thus modulating the circadian rhythms [38].

Insulin is an anabolic hormone released by pancreatic beta cells that inhibits adipose tissue lipolysis and reduces mitochondrial β-oxidation in skeletal muscle cells through the inhibition of HSL and CPT1, respectively [18]. Under energy balance conditions, peak insulin levels are often observed during the late evening in healthy male individuals (Fig. 4a), concurring with the nadir of free fatty acid (FFA) availability and fat oxidation (~ 2200 h) (Fig. 3b) [13]. Conversely, the nadir of insulin precedes the zenith of free fatty acids and fat oxidation (~ 0400 h) [13, 14].

According to Frandsen et al. [39], augmenting insulin levels in the morning by the consumption of a high carbohydrate meal (73% of energy from carbohydrates) decreases free fatty acid availability and MFO in healthy men and women. This finding concurs with the negative correlation between insulin levels and MFO reported by Robinson et al. [15] and agrees with consistent reports of lipolysis downregulation 1–6 h after consuming a high carbohydrate meal [18]. Given that all previous studies assessing MFO diurnal variation provided high carbohydrate meals to their participants before the evening exercise trial, it does not make sense to observe a higher MFO in the late part of the day. Indeed, the analysis of insulin 24-h kinetics clearly shows that carbohydrate intake in the evening stimulates a larger insulin peak when compared with the early morning (Fig. 1a), thus resulting in a lower availability of free fatty acids (Fig. 3b) and the nadir of fat oxidation (Fig. 1b).

In contrast to the insulin circadian pattern, cortisol and epinephrine reach their zenith during the early morning after awakening (0700–1000 h) and continuously decrease until the late evening (~ 2400 h) (Fig. 4b, c) [28, 40]. These hormones stimulate adipose tissue lipolysis by promoting the activation of HSL after binding a G-coupled membrane receptor [41]. In fact, the epinephrine and cortisol peak are close to the nadir of insulin and the zenith of free fatty acid availability and fat oxidation (0700–0800 h), revealing an orchestrated synchronization of fatty acid metabolism. According to Chávez-Guevara et al. [42], no previous study has investigated the correlation of cortisol and epinephrine

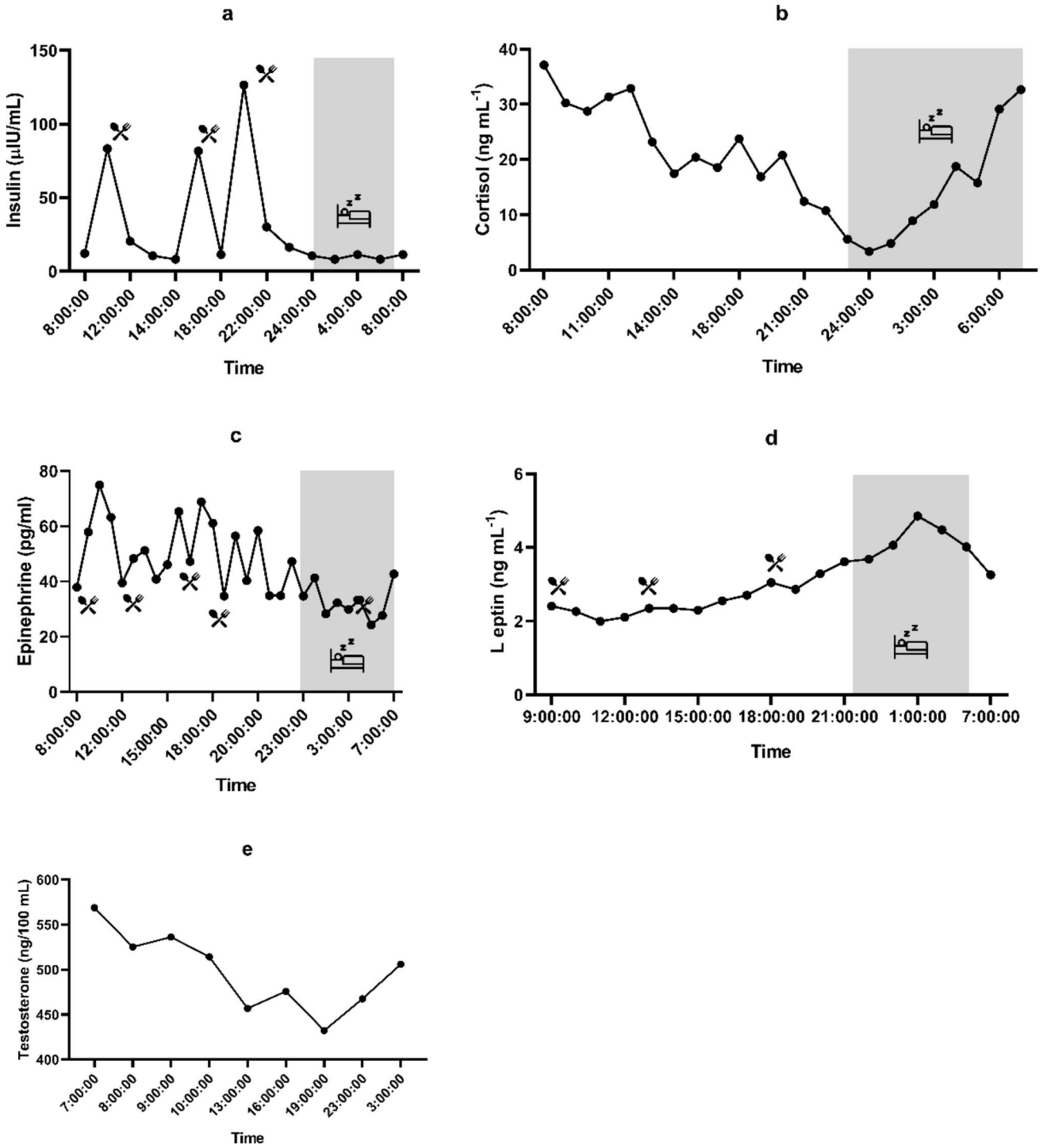


Fig. 4 Circadian rhythmicity of insulin (**a**), cortisol (**b**), epinephrine (**c**), leptin (**d**), and testosterone (**e**) recorded in young men. All kinetics were rebuilt in WebplotDigitizer (https://automeris.io/WebPlotDigitizer/) based on the data reported by Gooley [28], Linsell et al. [40], Wefers et al. [13], and van Aggel-Leijssen et al. [50]. Sleep period is highlighted with *grey shadows* based on the protocol reported by each study. Meal timing is also represented for cortisol, epinephrine, insulin, and leptin, while the molecular mechanisms associated with each hormone rhythmicity are provided in detail in supplementary file 3 (see electronic supplementary material). The figures were elaborated in GraphPad Prism v.8.1

with MFO in humans. However, the circadian pattern of these hormones suggests that free fatty acid concentration and MFO should be higher during the early morning and not the evening or the afternoon.

On the other hand, although epinephrine stimulates the partitioning of intramuscular triglycerides by upregulating the activity of HSL [43], the peak of epinephrine levels does not concur with the zenith of skeletal muscle lipid droplets size (1000 h) (Fig. 3c) [44]. Furthermore, the nadir of lipid droplets size concurs with the nadir of free fatty acid availability (2200 h), which is far removed from the zenith of fat oxidation (0800 h). Taken together, these data suggest that intramuscular triglycerides reach a low level during the late evening due to a low rate of fatty acid release from adipose tissue rather than a higher rate of fat oxidation in skeletal muscle. In this sense, it should be mentioned that the size of lipid droplets is similar between the early morning and the early evening (0800 vs 1800 h). Hence, there is no reason to believe, based on available evidence, that a higher MFO during exercise would be favored in the latter time of day (it is important to note that previous studies have mainly compared MFO in the early morning vs early afternoon). In fact, the intramuscular concentration of lipids has been unrelated to MFO in endurance trained individuals and subjects with obesity [45, 46]. Thus, lower availability of free fatty acids during the early evening and a similar utilization of intramuscular triglycerides in comparison with the morning refute the idea that exercise fat oxidation would be higher in the afternoon or the evening as reported by previous investigations.

Adipose tissue lipolysis and muscle fat oxidation are also regulated by leptin, an adipokine released from white adipose tissue in proportion to fat mass [47]. The binding of leptin to OB-R (membrane receptor) in myocytes promotes the activation of AMP-activated protein kinase, an energy sensor protein that promotes intramuscular triglyceride hydrolysis and fatty acid oxidation by activating HSL and CPT1, respectively [47]. Additionally, leptin increases adipose tissue lipolysis, diminishes fatty acid esterification to triglycerides, and attenuates de novo lipogenesis by reducing adipocyte sensitivity to insulin and insulin secretion from beta pancreatic cells [48, 49]. Under energy balance conditions, leptin levels show an amplitude of 21%, zenith at 0100 h, and nadir between 0900 and 1100 h (Fig. 4d) [49, 50]. Furthermore, the peak of leptin levels agrees with the nadir of insulin and the augmentation of fat oxidation during the sleep period, preceding the zenith of fat oxidation rate during the morning (Fig. 1b).

The rise of plasma leptin concentration seems to precede the gradual increment in free fatty acid availability observed during the sleep period before awakening (Fig. 3b). Therefore, exercising in the morning would be a more convenient time to optimize fat oxidation during exercise, if we consider the circadian behavior of leptin levels. It should be mentioned that no association between fasting leptin levels and MFO was noted in young adults with obesity in the morning [51], whereas a small but negative association was reported between MFO and plasma leptin levels in young adults [52]. Further studies are necessary to elucidate if circadian variation of leptin could impact exercise fat oxidation.

Testosterone is a steroid hormone synthesized in the gonads that enhances epinephrine-stimulated lipolysis in adipose cells and decreases leptin secretion from human adipocytes [53]. Peak testosterone levels are observed during the early morning hours (0700–0800 h) (Fig. 4e) [54], concurring with the zenith of free fatty acid availability and fat oxidation rate. Conversely, the nadir of testosterone levels has been recorded during the early evening (1900–2000 h), immediately before the exponential rise of leptin concentration (1900–2400 h). The morning concentration of testosterone levels under fasting conditions showed a modest positive correlation with MFO in young men [55], strengthening the idea that higher fat oxidation would be achieved during morning exercise.

The circadian rhythmicity of endocrine hormones that regulate fatty acid partitioning in skeletal muscle and free fatty acid mobilization from adipose tissue suggests that exercise fat oxidation would be higher during the morning and not the evening or the afternoon, rebutting the findings reported by several investigations. It should be mentioned that fatty acid metabolism is also regulated by many other endocrine hormones such as glucagon, estrogens, thyroxine, and triiodothyronine [42]. However, their circadian rhythmicity in humans remains unexplored.

2.4 Circadian Rhythmicity of Mitochondrial Dynamics and Respiration

Mitochondrion is a cellular organelle that contributes to ATP resynthesis and cellular energy homeostasis, oxidizing a plethora of energy substrates including fatty acids. This organelle is highly dynamic and undergoes morphological and functional modifications depending on nutrient availability and energy requirements [56]. In this regard, mitochondrial fragmentation index—an index of mitochondrial integrity and mitochondrial fission/fusion balance—reaches its nadir during the afternoon (1800 h) in type I and type II skeletal muscle fibers of lean healthy men (Fig. 5a), preceding the zenith of mitochondrial ADP-stimulated respiration and the exponential drop in energy expenditure (2300 h) (Figs. 5b; 1b) [13]. Based on these previous findings, Gemmink et al. [57] proposed a link between the fused mitochondrial network and mitochondrial respiration. However, the abundance of mitochondrial fusion and fission proteins display their zenith around ~ 1300 h (Fig. 5c, d) [13]. Furthermore, the abundance of oxidative phosphorylation

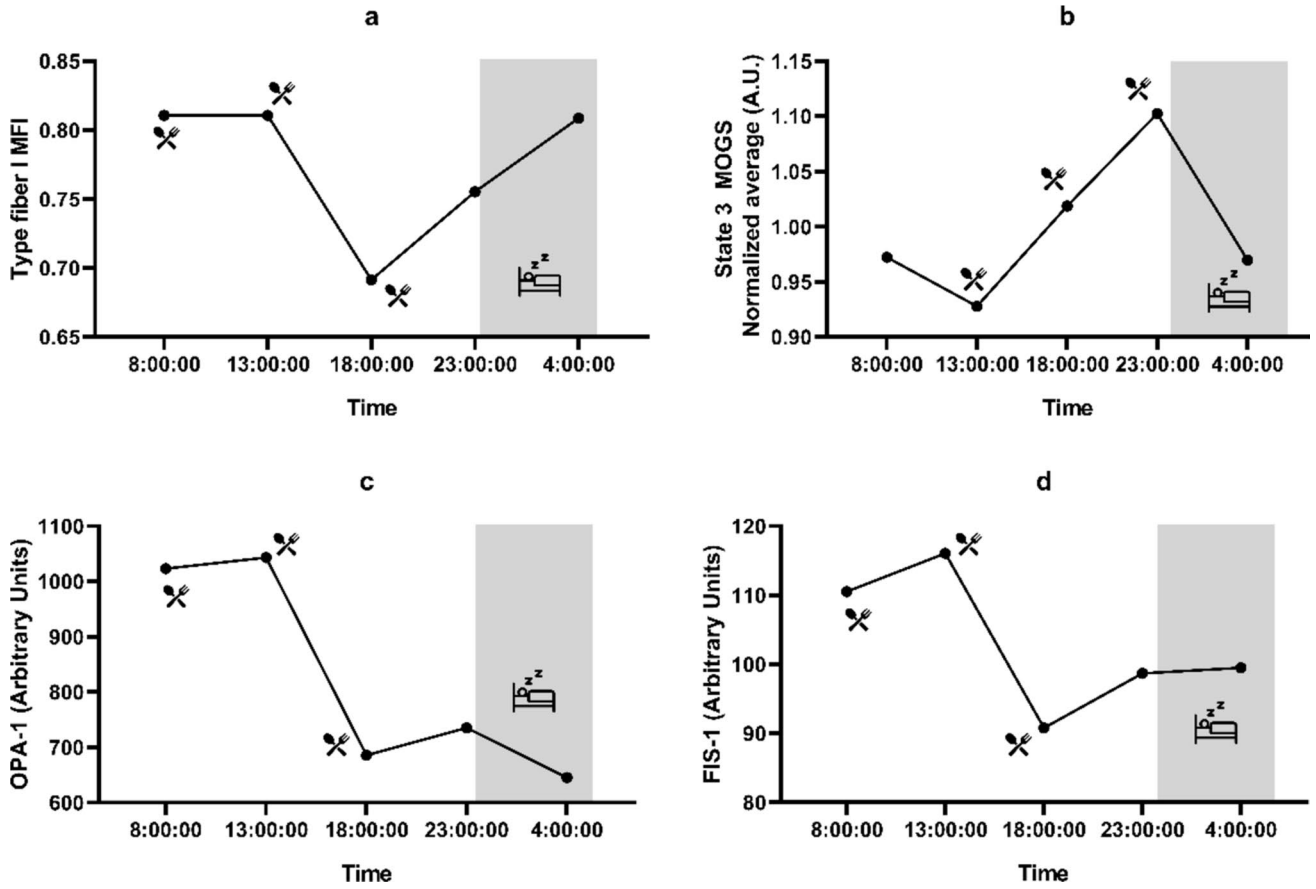


Fig. 5 Circadian rhythmicity of mitochondrial fragmentation index in type I muscle fibers (**a**), state 3 mitochondrial ADP-stimulated respiration (**b**), mitochondrial dynamin-like 120 kDa protein (**c**), and mitochondrial fission 1 protein (**d**). All kinetics were rebuilt in WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/) based on the data reported by Gemmink et al. [57], and Wefers et al. [13]. Sleep period is highlighted with *grey shadows* based on the protocol reported by each study. Meal timing is also represented for all molecular parameters. The figures were elaborated in GraphPad Prism v.8.1. *FIS-1* mitochondrial fission 1 protein, *MFI* mitochondrial fragmentation index, *MOGS* mitochondrial ADP-stimulated respiration, *OPA-1* mitochondrial dynamin-like 120 kDa protein

complexes embedded in the inner-mitochondrial membrane does not show a circadian pattern [13], limiting our comprehension of the molecular mechanisms associated with higher mitochondrial respiration during the late evening. It should be noted that enhanced enzymatic/protein activity instead of higher expression levels might be related to a superior mitochondrial fusion and oxidative phosphorylation capacity. However, this hypothesis warrants further investigation. Another plausible explanation for an elevated mitochondrial respiration during the late evening may be an enhanced mitochondrial biogenesis of skeletal muscle (i.e., higher mitochondrial volume density). Nevertheless, van Moorsel et al. [58] did not observe a circadian rhythmicity in expression levels of PGC1α, one of the major regulators of mitochondrial biogenesis.

According to Dandanell et al. [46] and Lambert et al. [59], the MFO is moderately correlated with mitochondrial volume density, oxidative phosphorylation capacity, and fat oxidation measured ex vivo in muscle biopsies donated by healthy men and subjects with type 2 diabetes. Moreover, Maunder et al. [60] reported that citrate synthase activity predicted MFO in trained men, whereas Shaw et al. [32] found a significant relationship between MFO and mitochondrial respiratory chain complexes II, III, IV, and V when combining the data from trained and untrained individuals. Comparing these findings with the aforementioned rhythmicity of mitochondrial biomarkers, we cannot argue that evening exercise would be more convenient for maximizing fat oxidation. Indeed, expression levels of oxidative phosphorylation complexes and mitochondrial biogenesis do not seem to exhibit a circadian pattern [13, 58], whereas biomarkers of mitochondrial fusion and fission peak between 0800 and 1300 h (Fig. 5c, d) [13]. Furthermore, the zenith of mitochondrial ADP-stimulated respiration seems to occur after the time window where previous studies have examined MFO (1700–2300 h) (Fig. 5b) [13].

The fact that mitochondrial respiratory capacity does not agree with the peak of resting fat oxidation or free fatty

acid availability in humans also weakens the hypothesis that mitochondrial fat oxidation would be enhanced in the evening. In fact, it seems intriguing that peak mitochondrial respiration is close to the zenith of insulin levels and the nadir of cortisol concentration, which indicates an anabolic state. Of course, the rhythmicity of mitochondrial biomarkers recently reported by Gemmink et al. [57] and Wefers et al. [13] needs to be replicated by future studies in diverse populations, particularly given that a small number of subjects were analyzed in each study ($n = 12$ per group). Furthermore, as mentioned, mitochondrial enzymatic and protein activity, rather than merely expression levels, may be the key drivers of circadian regulation of mitochondrial function. This perspective aligns with the challenges often faced in detecting changes in mitochondrial gene expression, as some mitochondrial genes are frequently used as housekeeping genes in RT-qPCR due to their consistent expression levels [61].

3 Applications to Exercise Prescription

Maximizing fat oxidation during exercise is a time-efficient therapeutic approach for improving physical fitness and cardiometabolic health in patients with obesity, type 2 diabetes, and metabolic syndrome [1–3]. For this reason, the best strategies to enhance fat oxidation during exercise have been examined over the past 20 years, resulting in specific recommendations of exercise type, intensity, and volume for healthy individuals and subjects with chronic diseases [4, 62]. Previous studies investigating which is the best time window for maximizing fat oxidation during exercise consistently reported that MFO values are higher during the afternoon or the evening (1700–2000 h) than in the early morning (0800–1100 h) (Fig. 1a) [5–7]. The analysis provided herein, however, refutes such hypothesis by demonstrating that (i) resting fat oxidation peaks in the early morning, (ii) circulating levels of endocrine hormones that stimulate adipose tissue lipolysis and intramuscular triglyceride utilization peak in the morning, (iii) the size of lipid droplets within skeletal muscle exhibit an oscillatory pattern with a nadir occurring in the late evening, and (iv) biomarkers of mitochondrial biogenesis and oxidative phosphorylation do not exhibit a circadian pattern, whereas mitochondrial ADP-stimulated respiration reaches its zenith around midnight.

Considering the evidence discussed in this review, it seems that physiological conditions in the morning would be most conducive to maximizing fat oxidation during exercise. In fact, although most of the biomarkers discussed herein have been assessed under energy balance conditions in young healthy individuals, it should be stated that peak fat oxidation and the zenith of free fatty acid availability also occur in the morning in overweight subjects and those with insulin resistance [13]. Furthermore, in this same population, the mitochondrial respiration and oxygen consumption rates do not peak in the evening [13], opposing the findings of Mohebbi et al. [6], and Methani et al. [7] who reported that MFO would be higher in the evening than in the morning in subjects with obesity and metabolic syndrome.

Consistent with these findings, Toyoka et al. [63] reported that 60 min of fasting exercise at an intensity that is close to FATmax in endurance-trained individuals (50–60% VO_{2max}) stimulated a higher increment in catecholamine levels, fat oxidation, and free fatty acid availability compared with postprandial exercise performed in the evening (4 h after a standard meal). It should be noted, however, that such differences between morning and evening exercise were reversed when morning exercise was performed in postprandial conditions. A later study by Kim et al. [64] supported these findings after observing that a single bout of moderate intensity exercise in the evening (~60% VO_{2max}; 1700–1800 h) raised epinephrine and cortisol concentration to a higher extent than morning exercise if both exercise trials are performed in a postprandial state (3 h before eating a high carbohydrate meal). Interestingly, that same study reported that free fatty acid concentration rose to a similar level in morning and evening exercise trials. Future studies investigating the circadian metabolic response to FATmax exercise are thus necessary to elucidate which time of the day (morning vs midday vs evening vs late night), and under which metabolic status (fasting vs postprandial), fat oxidation can be enhanced. To that purpose, studies must analyze the response in endocrine hormones, adipose tissue lipolysis, fat oxidation, and mitochondrial respiration during steady-state exercise at FATmax, moving beyond a single graded exercise test. A key consideration is that MFO recorded during a graded exercise test overestimates total fat utilization during 60 min of FATmax in subjects with obesity (~46%) [8], and is not sustained during 40 min of FATmax exercise in lean individuals [65].

Paying attention to post-exercise fat oxidation would be also relevant as morning exercise performed within the FATmax zone (50% VO_{2max}; 60 min) enhances 24-h fat oxidation whereas isocaloric exercise performed in the afternoon or the evening does not seem to alter 24-h fat utilization in healthy men and women [66]. According to Iwayama et al. [66], such benefits of morning exercise are attributed to a higher glycogen depletion in the fasted state, which may activate adipose tissue lipolysis and intramuscular triglyceride oxidation for maintaining energy homeostasis (catabolic effect). Hence, morning exercise performed under fasting conditions would not only enhance fat oxidation during exercise but would also have a residual effect that increases fat oxidation for the rest of the day.

In support of this hypothesis, Alizadeh et al. [67] reported that 6 weeks of treadmill running at the intensity of the ventilatory threshold in the morning (0800–1000 h) resulted in a larger body weight and body mass index reduction in inactive overweight women when compared with the same exercise protocol performed in the evening. Moreover, Willis et al. [68] reported that 10 weeks of treadmill walking/jogging in the morning (0700–1159 h) was more efficient than walking in the evening (≥ 1500 h) for reducing body weight and fat mass in physically inactive individuals who are overweight or obese. These studies applied an exercise intensity that was close to FATmax, since the ventilatory threshold is located within the FATmax zone in overweight women [69], whereas treadmill speed at FATmax ranges between 4.0 to 5.1 km/h in subjects with obesity (walking effort) [4]. Nevertheless, further studies are still necessary to confirm if morning exercise at FATmax would elicit a superior fat oxidation and body fat reduction in patients with chronic diseases. Most importantly, (i) exercise timing, but not the participants' metabolic status (fasting or postprandial), was considered in both clinical trials [67, 68], and (ii) the ~ 350-kcal reduction in energy intake reported by Alizadeh et al. [67] in the morning exercise group was not accounted for as a covariate, and these may have contributed to the observed weight loss.

Another point to highlight is that neither Alizadeh et al. [67] nor Willis et al. [68] examined if morning exercise was more effective in improving insulin sensitivity and cardiovascular function. In this regard, maximizing fat oxidation through exercise has been proposed as an efficient strategy to counteract the ectopic deposition of lipids which is related to insulin resistance and cardiovascular disorders [1–3]. Nonetheless, a recent meta-analysis by Sevilla-Lorente et al. [70] showed that exercise training in the morning was equally as effective as evening exercise for reducing fasting glucose concentration and resting blood pressure. The authors of that meta-analysis also highlighted that further clinical trials properly controlling for participants' chronotype, metabolic status, and biological sex are still needed. Hence, we are still a long way from establishing the best exercise time for counteracting cardiometabolic diseases.

4 Current Gaps and Research Agenda

Finding the best time window for maximizing fat oxidation during exercise may contribute to the treatment and prevention of chronic diseases. However, it is important to recognize that exercise-induced perturbations in energy metabolism can also impact circadian rhythms. For instance, the binding of epinephrine and norepinephrine to β-adrenergic receptors modulates the expression of PGC1α [71], a critical transcriptional coactivator that influences clock genes, enzymes, and transport proteins associated with mitochondrial function and fat oxidation. Additionally, the interaction of leptin with cell membrane receptors in the suprachiasmatic nucleus (SCN) helps synchronize energy balance with the body's activity patterns [72], while insulin may regulate CLOCK protein activity through the Akt signaling pathway, which phosphorylates CLOCK at the Ser-845 site [73].

Alterations in mitochondrial activity may also disrupt circadian rhythms, as cells with impaired mitochondrial dynamics exhibit weakened circadian oscillations [74]. Conversely, mitochondrial metabolites such as nicotinamide adenine dinucleotide (NAD+) trials [67, 68], can influence the activity of circadian clock proteins, further connecting mitochondrial function with circadian regulation [72]. Thus, exercise-induced modifications in endocrine hormones and mitochondrial dynamics could modify the optimal time window for maximizing fat oxidation.

Previous research has shown a ~ 0.6-h phase advance in circadian rhythms among young sedentary adults after five sessions of moderate-intensity exercise in the morning (30 min; 70% VO_{2max}) [75]. This observation suggests that acrophase and batiphase of fatty acid metabolism biomarkers may be altered by exercise in the morning, a hypothesis that merits further investigation. In addition, evening exercise in DB/DB mice—equivalent to morning exercise in humans—decreased the expression levels of circadian locomotor output cycles protein kaput (CLOCK) in skeletal muscle, elevating the abundance of mitochondrial dynamin-like 120 kDa protein and mitochondrial fission 1 protein [76]. Such molecular adaptations were not stimulated by morning exercise in the same animal model, indicating that exercise timing would also modify the circadian rhythmicity of mitochondrial fusion and oxidative phosphorylation capacity.

In a recent study, Harmsen et al. [77] reported that 12 weeks of high-intensity interval training performed in the afternoon (1400–1800 h) modified the diurnal variation of muscle clock gene expression in elderly males with overweight and obesity (body fat: 36.8 ± 4.9%). Nevertheless, the circadian rhythmicity of mitochondrial respiratory capacity and fat oxidation remained unchanged. Further molecular studies are needed to clarify if exercise timing alters the circadian rhythmicity of fat oxidation and its related biomarkers (e.g., fatty acid availability, mitochondrial respiration, epinephrine, PGC1α).

Additionally, the influence of age, sex, diet, and the sleep–wake cycle on the circadian regulation of fat oxidation requires further investigation, considering that these factors are related to MFO and circadian patterns (Fig. 6).

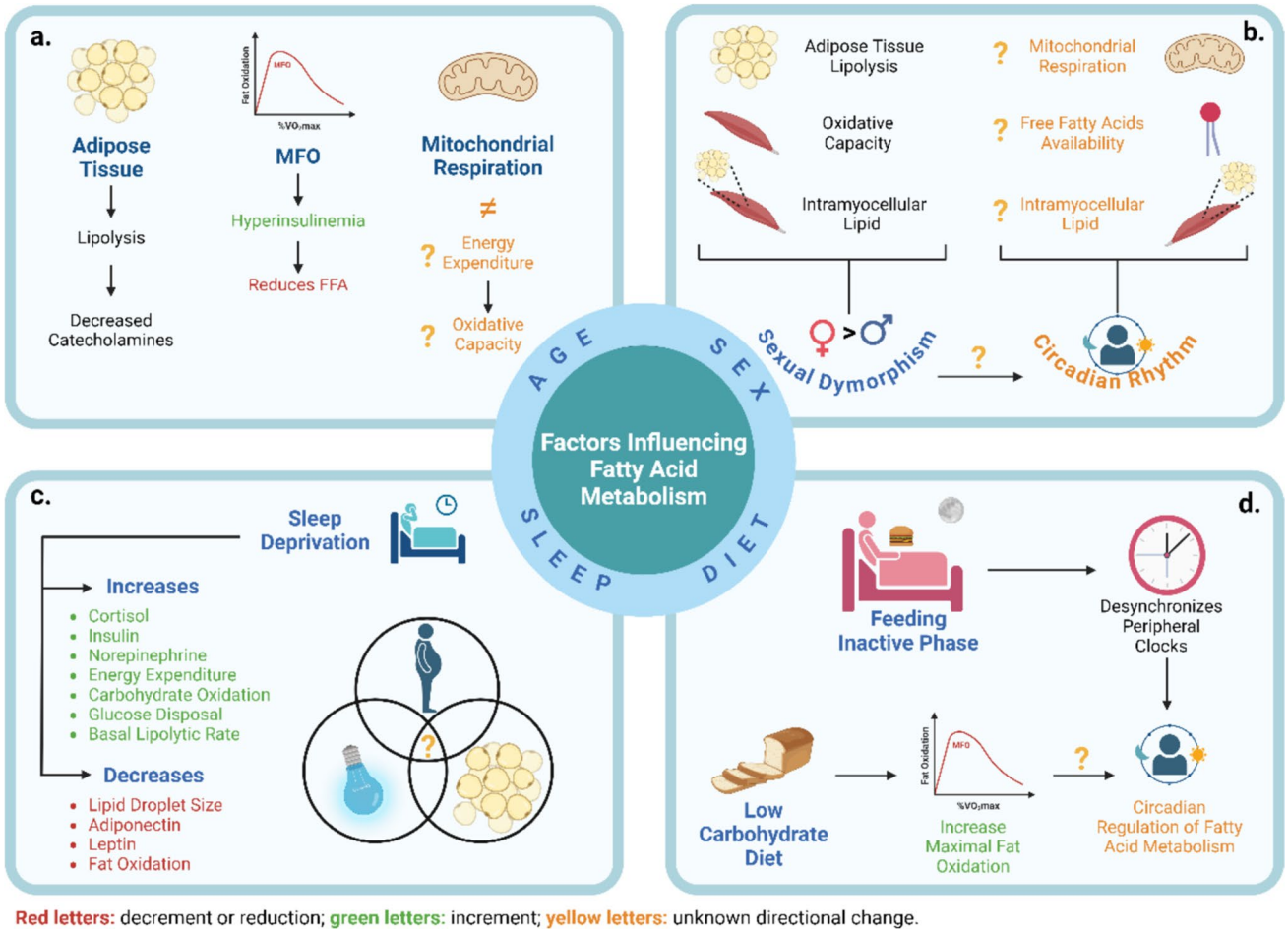


Fig. 6 Factors influencing fatty acid metabolism. **a** Age has been inversely associated with MFO whereas resting fat oxidation shows a lower amplitude and a delayed phase shift in elderly adults [4, 13, 14]. These metabolic transitions have been related to impaired adipose tissue lipolysis, resulting from elevated insulin levels and reduced catecholamine levels [78]. Mitochondrial respiration is also lower in older individuals compared with young adults and does not increase in proportion to energy expenditure [13]. **b** MFO is higher in young women than in men [8]. This has been attributed to elevated adipose tissue lipolysis, increased intramyocellular lipid concentrations, and greater skeletal muscle oxidative capacity [79]. However, the sexual dimorphism in the circadian rhythmicity of the aforementioned parameters remains unclear. **c** Circadian misalignment, sleep deprivation, and changes in environmental light exposure could alter the production and rhythmicity of endocrine hormones, modifying energy expenditure and substrate availability [80–83]. Moreover, changes in environmental light exposure alter adipose tissue lipolysis [84]. Additional research is necessary to investigate if these factors influence fat oxidation during exercise as neither sleep quality nor a single night of sleep deprivation influenced MFO in young adults [85, 86]. **d** Diet is a critical moderator of MFO, with carbohydrate intake showing an inverse association with MFO [11]. Low-carbohydrate ketogenic diets duplicate MFO after 7 days [86, 87]; however, their effects on the circadian regulation of fatty acid metabolism remain unexplored. Feeding patterns also synchronize circadian rhythms, as meal frequency, distribution, and content can disrupt or enhance the internal clock system [89]. Future studies investigating the influence of distinct dietary phenotypes on the rhythmicity of fatty acid metabolism are needed. *MFO* maximal fat oxidation rate

5 Conclusion

The analysis of 24-h resting fat oxidation rhythmicity in young healthy men reveals that fat utilization is higher during the early morning due to an enhanced fatty acid availability and concentration of lipolytic hormones. Nevertheless, this is decoupled from the peak of mitochondrial respiration and intramuscular triglyceride utilization. These findings refute the hypothesis of higher exercise fat oxidation in the evening, and strengthen the observations of larger fat mass reductions when exercise training close to FATmax is performed in the morning. Although it is tempting to propose that morning exercise would be more convenient for maximizing exercise fat oxidation and preventing cardiometabolic disorders, we are some distance from establishing time-specific recommendations for counteracting chronic diseases. Future studies must investigate the role of age, sex, nutrition, training status, and sleep quality on the physiological patterns discussed here, assessing the influence of each factor on the acute

response and long-term adaptations of FATmax exercise performed in the morning or the evening.

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Declarations

CRediT authorship contribution statement Investigation: MRV, FJAG, ARJ, IACG; conceptualization and methodology: MRV, FJAG, SAC, JAPL, IACG; resources and data curation: MRV, SAC, IACG; formal analysis: MRV, ARJ, IACG; supervision and project administration: JAPL, IACG. All the authors have read, edited and approved the final version of the manuscript and agreed with the order of presentation of the authors.

Conflict of interest The authors report there are no competing interests to declare.

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