Review Article

Circadian rhythms and inflammatory diseases of the liver and gut☆

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A B S T R A C T

Circadian rhythms play a central role in maintaining metabolic homeostasis and orchestrating inter-organ crosstalk. Research evidence indicates that disruption to rhythms, which occurs through shift work, chronic sleep disruption, molecular clock polymorphisms, or the consumption of alcohol or high-fat diets, can influence inflammatory status and disrupt timing between the brain and periphery or between the body and the external environment. Within the liver and gut, circadian rhythms direct the timing of glucose and lipid homeostasis, bile acid and xenobiotic metabolism, and nutrient absorption, making these systems particularly susceptible to the effects of disrupted rhythms. In this review, the impacts of circadian disruption will be discussed with emphasis on inflammatory conditions affecting the liver and gut, and the potential for chronotherapy for these conditions will be explored.

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1. Introduction to circadian rhythms

Circadian rhythms refer to physiological and biological processes that occur with a repeating period of approximately 24 h. These endogenous rhythms are generated and maintained by the suprachiasmatic nucleus (SCN), a cluster of approximately 20,000 neurons within the mammalian hypothalamus. Circadian rhythms serve to temporally organize cellular function and to allow for timely adaptation to changes in the external environment. Circadian rhythms are characterized by three fundamental properties: (i) they free-run in the absence of light; (ii) they are temperature-compensated, so that the rhythm remains stable over a range of physiological temperatures; and (iii) they are entrained by external stimuli. The most powerful entraining agent is environmental light.

1.1. Mammalian circadian rhythms

In humans, the endogenous circadian period is approximately 24 h 11 min, slightly longer than the solar day. Environmental light cues received by the SCN entrain the clock to external time, thus preventing daily drift and misalignment. Intrinsically photosensitive retinal ganglion cells are specialized non-image-forming cells that transmit environmental photic information to the SCN via the retinohypothalamic tract. This photic input influences the firing rate of SCN neurons, which endogenously generate the transcriptional-translational feedback loop (TTFL) that ultimately determines molecular and cellular rhythms in the brain and periphery. The timing of photic input also influences SCN function and rhythmic behavior: light perceived early in the dark period is registered as an extension of daytime. This shifts activity of the SCN and causes downstream rhythms to occur later than expected, termed a phase delay. Conversely, light perceived later in the dark period is registered as an artificially early start of daytime, and results in a phase advance. Light perceived during the subjective day has little effect on the timing of the circadian clock in mammals, termed the "dead zone" (Fig. 1A).

The self-sustained TTFL exists in nearly all cell types and consists of a series of clock genes who products create feedback loops that regulate their own transcription (Fig. 1B). The transcription factors circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like protein 1 (Bmal1) heterodimerize and bind to E-box elements in the Period (Per1/2/3) and Cryptochrome (Cry1/2/3) promoter regions, thus driving their transcription. Per and Cry proteins then dimerize to interfere with Clock/Bmal1 activity, which ultimately suppresses *Per* and *Cry* transcription. Per and Cry proteins are also phosphorylated by casein kinase 1, which acts as a tag for ubiquitination and degradation, thus completing a 24 h cycle. The TTFL is supplemented with accessory feedback loops that serve to modulate expression of the core clock genes. The nuclear receptors Rev-erb alpha and retinoid-related orphan receptor alpha (RORα) competitively bind the ROR-response element (RORE) in the *Bmal1* promoter to suppress or induce gene transcription, respectively. This robust TFFL originates in the SCN and is propagated through neural and hormonal signals, further targeting thousands
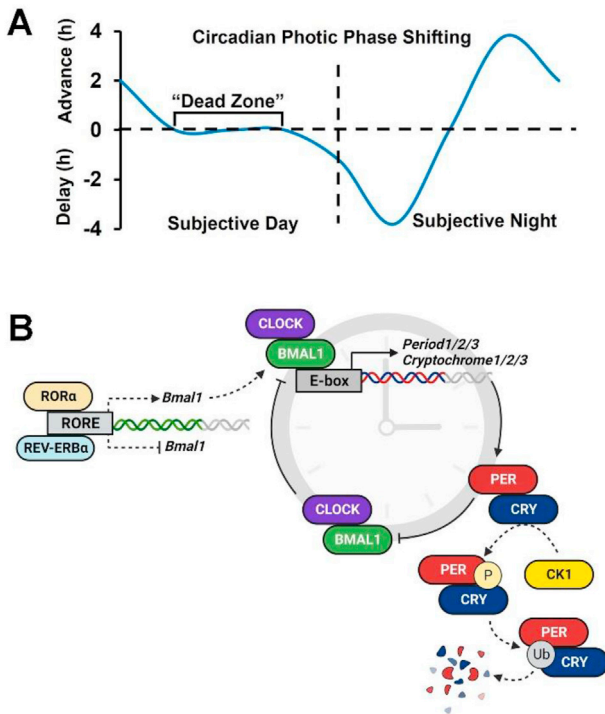
Fig. 1. The mammalian circadian clock. (A) Circadian photic phase shifting. Light perceived by the circadian clock differentially affects behavior; light perceived in the early subjective night results in a phase delay of downstream activities, while light perceived late at night results in a phase advance. Light perceived during the subjective day (termed the "dead zone") has little effect on the circadian clock or behavior. **(B)** The circadian transcriptional-translational feedback loop (TTFL). The core clock mechanism is pictured in solid lines and accessory regulation is pictured in dotted lines. The core clock proteins CLOCK and BMAL1 heterodimerize at E-box regions of the Period (*PER*) and Cryptochrome (*CRY*) gene promoters, driving their transcription. PER and CRY proteins inhibit the actions of the CLOCK-BMAL1 heterodimer, ultimately reducing their own transcription. PER and CRY are phosphorylated by casein kinase 1 (CK1), which further tags these proteins for ubiquitin-mediated degradation. The accessory proteins RORα and REV-ERBα regulate *Bmal1* transcription by competitively binding at the ROR-response element (RORE) in the *Bmal1* promoter to enhance or inhibit *Bmal1* transcription, respectively. This molecular feedback loop lasts approximately 24 h, thus completing one circadian cycle. This figure was created with BioRender.com. Abbreviations: BMAL1, brain and muscle ARNT-like protein 1; CLOCK, circadian locomotor output cycles kaput; RORα, retinoid-related orphan receptor alpha.

of clock-controlled genes to ensure synchronization of peripheral tissues with the central clock. This TTFL also exists in nearly all mammalian tissues, with conserved homologous regulation identified in bacteria and fungi, insects, and plants.[1] Overall, this synchronization ensures organisms can adapt to dynamic cellular and physiological conditions with appropriately timed responses.

1.2. Circadian disruption in the liver and gut

Energy needs oscillate over the 24 h day, and stable circadian rhythms are required to control the timing of nearly all metabolic functions, including sleep-wake cycles, body temperature and hormone fluctuations, glucose metabolism, and inflammatory responses. In the mammalian liver, the transcriptome and proteome are regulated in a rhythmic fashion due to direct circadian regulation as well as rhythmic modulation of nuclear transport and post-translational modifications.[2,3] This ensures, on a molecular and cellular level, that rhythmic influence over glucose and lipid metabolism, bile acid homeostasis, and xenobiotic processing results in favorably timed metabolic responses to nutrient availability. Disruption to circadian rhythms due to diet or alcohol, shift

work, or sleep loss can result in misalignment of the internal clock with the external environment, or a loss of central-to-peripheral temporal organization within an organism. This desynchronization contributes to the pathogenesis of cardiovascular and neurodegenerative diseases,[4,5] metabolic syndrome,[6] and many types of cancer,[7] and underlying deregulation of immune homeostasis may also be a significant contributor.[8] Cells of both the innate and adaptive immune systems exhibit rhythms in gene expression and function. Leukocyte circulation and infiltration oscillate in a circadian fashion, with the exact timing dependent on cell and tissue type.[9] Circadian mediation of the immune response may be partly mediated through clock-controlled glucocorticoid release.[10] Meanwhile, pro-inflammatory cytokines can disrupt timing of the molecular clock, and clock disruption can increase inflammation. The pro-inflammatory nuclear factor-kappa B (NF-κB) was shown to bind to the E-box region of the *Bmal1* promoter, repressing its transcription and altering period and amplitude of clock gene rhythms.[11] Conversely, deletion of *Bmal1* in macrophages increased their motility and resulted in a "preactivation" phenotype.[12] Circadian systems, sleep duration and timing, and chronotype (individual phase preference for morning *vs.* evening, i.e., morning lark *vs.* night owl) all play roles in the maintenance of homeostasis, as well as disease pathology when these systems become dysregulated (Fig. 2). Reciprocal signaling between clocks and the immune system is involved in the pathogenesis of obesity and metabolic syndrome, gut dysbiosis, and many other diseases.[13–15] Here, these interactions will be examined with emphasis on inflammatory liver and bowel diseases, as well as the therapeutic potential for time-based treatment of these disorders (chronotherapy).

2. Circadian rhythms in inflammatory liver diseases

2.1. Metabolic dysfunction-associated steatosis and steatohepatitis

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD),
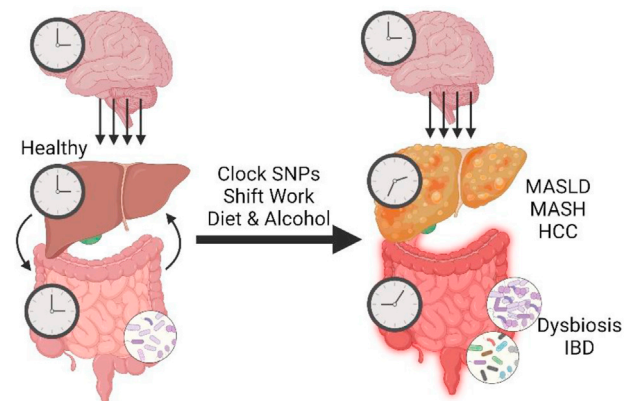

Fig. 2. Circadian disruption and pathogenesis in the liver and gut. (Left) The biological clock of the hypothalamus produces endogenous rhythms that synchronize the timing of metabolic processes in the liver and gut to the external environment and to each other. (Right) Disruptions to circadian rhythms, which can occur as clock gene single nucleotide polymorphisms (SNPs) or other genetic mutations, chronic shift work, or high-calorie diets and alcohol, desynchronize metabolic timing. This desynchronization contributes to the pathogenesis of metabolic dysfunction-associated steatotic liver disease (MASLD), metabolic dysfunction-associated steatohepatitis (MASH), and hepatocellular carcinoma (HCC) in the liver. In the gut, desynchronization alters microbe populations and can lead to dysbiosis in the form of bacterial overgrowth or disrupted microbiome composition, as well as contribute to conditions like inflammatory bowel disease (IBD). This figure was created with BioRender.com.

comprises a spectrum of disorders and is an increasing public health concern. MASLD often begins with simple steatosis (NAFL), in which more than 5% of liver weight is fat. Steatosis is estimated to occur in as much as 25% of the global population, with prevalence increasing in both women and children.[16,17] A proportion of patients with steatosis will progress to more serious forms of MASLD, including metabolic dysfunction-associated steatohepatitis (MASH), formerly known as non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, or hepatocellular carcinoma (HCC) and end-stage liver disease,[18] making MASLD one of the leading causes of chronic liver disease. MASLD is often associated with metabolic syndrome, including obesity and type 2 diabetes, though MASLD progression can occur in lean patients independent of weight.[19,20] Additionally, MASLD is associated with distinct changes in transcription factors in the liver, including sterol regulatory element-binding protein (Srebp)-mediated lipogenesis, carbohydrate-responsive element-binding protein (Chrebp)-maintained glucose homeostasis, and Nf-κb-linked inflammation.[21]

Circadian disruption, often occurring in the form of long-term shift work, can also lead to liver dysfunction and contribute to MASLD pathogenesis. Night shift work was associated with increased serum alanine transaminase (ALT) independent of MASLD status,[22] while another study demonstrated that chronic shift workers with MASLD had a 3.7-fold increased risk for elevated ALT compared to workers with non-shift schedules or those without MASLD.[23] Likewise, serum alkaline phosphatase was increased in shift workers even after adjusting for age, body mass index, and fasting blood sugar and cholesterol.[24] Another small study found that patients with MASLD exhibited poorer quality and shorter sleep coupled with shifted food intake toward nighttime, implicating a role for disrupted rhythms in MASLD.[25] Conversely, self-identified short sleep duration (<5 h) was associated with a higher risk of developing MASLD compared to long sleep duration (>7 h) in a study of more than 12,000 Japanese subjects.[26] Evening chronotype (night owl) was also associated with obesity and higher risk of MASLD in human patients.[27–29] Though can be difficult to establish a causal pathology in human patients, results from animal studies indicate that circadian desynchrony contributes to MASLD. *Clock* mutant mice have a significantly increased circadian period (approximately 27–28 h) and are both hyperphagic and obese.[30] Even on a standard diet, these mice developed steatosis with increased serum ALT by 10 months of age, which was mediated by increased hepatic lipid uptake and synthesis coupled with reduced export.[31] Feeding these mice a high-fat diet resulted in further significant increases in body weight with elevated ALT and thiobarbituric acid-reactive substances, an indicator of lipid peroxidation. The obese phenotype of *Clock* mutant mice was partially rescued with *Clock* gene delivery, which reduced body weight and food intake, and restored energy expenditure to wild type levels.[32] Mice deficient of *Bmal1* and *Apoe* were hyperlipidemic with increased very-low-density lipoprotein production and reduced biliary cholesterol efflux,[33] while adipocyte-specific *Bmal1* knockout mice were more obese than wild type controls when fed either standard or high-fat diet.[34] These mice also consumed more calories during the inactive period (daytime) compared to controls, despite the groups consuming the same number of calories overall. Analysis of human clock polymorphisms corroborates data in rodent studies. Humans with *CLOCK* gene polymorphisms are significantly more obese and are less responsive to dietary intervention compared to non-carriers.[35,36] In addition to *CLOCK*, polymorphisms in *BMAL1*, *PER2*, and *CRY2* are associated with obesity, hyperglycemia, liver triglyceride content, and other disorders in humans,[37–39] though at least one recent meta-analysis examining 13 studies with more than 17,000 subjects found that only *BMAL1* variants were associated with increased risk of metabolic

syndrome.[40] Still, these studies indicate that disruption of the circadian clock, either endogenously or exogenously, likely contributes to the pathogenesis of MASLD and related metabolic disorders.

In addition to genetic clock disruption, rodent models of shift work also provide insight into the role of the peripheral clock in mediating liver homeostasis. High-fat diet-fed mice exposed to a rotating light schedule, simulating shift work, weighed more and ate more food during the inactive period compared to mice on a standard fixed light schedule. Expression of pro-inflammatory macrophages (F4/80$^+$ CD11b$^+$ CD11c$^+$ CD206$^-$ cells) and interleukin-1beta (*Il-1β*), *Il-6*, and tumor necrosis factor-alpha (*Tnf-α*) expression in adipose tissue were also significantly induced.[41] Similarly, 8 weeks of light schedule rotation in mice caused weight gain and hepatic steatosis even on a standard chow diet, which was further exacerbated with increased ALT after feeding high-fat diet.[42] Exposure of mice to six months of rotating light schedules caused adipocyte hypertrophy and an adipose transcriptome characterized by increased inflammation, angiogenesis, and fibrosis.[43] Another study found that either constant light or a rotating light schedule induced serum levels of Tnf-α, IL-6, IL-1β, as well as serum lipids, aspartate transaminase (AST) and ALT in mice.[44]

Circadian disruption can also result from social jet lag or the twice-yearly clock changes observed with Daylight Saving Time (DST) in the United States. Social jet lag, a form of chronic circadian disruption determined by calculating the difference in sleep timing on work days *vs.* free days, may contribute to metabolic syndrome by increasing inflammatory states. One study demonstrated that social jet lag was significantly associated with metabolic obesity and increased inflammation in humans, even after accounting for sleep duration.[45] Several other studies have demonstrated similar associations between social jet lag and obesity in both adults and children,[46–48] and these associations remain after adjustment for confounders like age, sex, and sleep quality. However, further longitudinal research is required, as chronotype alone may also be a significant contributing factor.[47]

DST is an example of circadian disruption that causes acute physiological harm. In the United States, DST is a federally mandated period in which social time is advanced 1 h in March (colloquially referred to as "spring forward"). This artificial advancement of time results in 1 h of misalignment and an average of 19 min of sleep lost every night throughout the observance of DST.[49] The "spring forward" transition from natural standard time to DST is acutely harmful and is associated with increased mortality,[50] cardiovascular morbidity,[51,52] motor vehicle accidents,[53] and incidence of mood disorders.[54] Notably, many of these phenomena subsequently decrease after the autumnal transition to standard time ("fall back"), indicating these risks might be alleviated with the abolishment of DST. A recent study also identified significant increases in noninfective enteritis and colitis after the spring shift into DST,[55] suggesting effects on the gut and enteric immune system are likely understudied. As mentioned, light is the most powerful entraining agent of the circadian clock. DST artificially delays sunrise and sunset by 1 h, and late sunsets are associated with obesity, type 2 diabetes, and cardiovascular disease.[49,56] Additionally, data indicates human rhythms do not adjust to altered timing during DST, as cortisol rhythms advance by approximately 2 min rather than 60 min.[57] Together, these studies demonstrate both physiological susceptibility to insults from circadian disruption and an inability to adjust to chronic disruption; therefore, every effort should be made to mitigate the health risks associated with disruption of circadian rhythms.

The progression of MASLD to MASH was once attributed to a "two-hit hypothesis" in which steatosis represents the first

metabolic insult and increased inflammation the second. A more accurately detailed model might be described as a "multi-hit hypothesis", in which multiple risk factors including poor diet, obesity, type 2 diabetes or insulin resistance, oxidative stress, lipotoxicity, and microbe-derived endotoxin release and subsequent permeabilization of the gut all interact to contribute to disease progression. Circadian disruption is likely an additional hit that influences both lipid metabolism and inflammatory processes in the liver. Constant light exposure, a model for circadian disruption by light pollution, worsened insulin resistance and increased inflammation induced by a high fructose diet in rats.[58] Several clock proteins have been implicated in the disease progression of MASH, while restriction of food intake, known as intermittent fasting or time-restricted eating (TRE), may provide some benefit. Mice fed a high fructose diet were obese with increased hepatic expression of Clock, Bmal1, and Per1/2, and restricting access to this diet only to the active phase, one form of TRE, reversed symptoms of MASH and restored clock gene expression.[59] Non-parenchymal cells isolated from hepatocyte-specific *Rev-erbα/β* double knockout mice fed a high-fat diet had disrupted rhythms in gene expression that were rescued by the deletion of *Srebp* signaling, as were rhythms in lipid metabolites, which may be important for the progression of MASH.[60] Conversely, treatment with the Rev-erbα/β agonist SR-9009 reduced diet-induced steatosis, fibrosis, and inflammatory activity in mice.[61] Several candidate drugs in trials for the treatment of MASH target clock-controlled outputs,[62] though it's not clear that rhythmicity or dose timing is considered in trial designs. Therefore, further emphasis should be placed on determining how manipulation of the clock affects inflammatory disease progression in MASLD and MASH at both pre-clinical and clinical research levels.

2.2. Alcohol-associated liver disease (AALD)

Alcohol and rhythms exist in a complex bi-directional relationship, and alcohol consumption influences the timing of the peripheral molecular clock. Interestingly, acute application of ethanol to SCN *in vitro* slice preparations alters glutamate-induced phase changes in SCN neuronal firing activity, though ethanol alone does not alter the central clock phase.[63] Similarly, a study in mice fed an ethanol-containing diet demonstrated that ethanol-induced phase advances in clock gene expression in the liver, while gene expression in the SCN was not affected.[64] Mice fed an ethanol-containing diet for 4 weeks displayed dampened nighttime activity, suppression of rhythmic clock gene transcription in the liver, and phase-advancement of hepatic clock gene expression.[65] Another study utilizing a similar model showed that alcohol blunted rhythms in hepatic clock gene expression and this effect was worse in *Bmal1*-liver-specific knockout mice. These mice also had increased liver and plasma triglycerides and altered rhythms in the lipogenic transcription factors *Srebp1c* and *Chrebp*.[66] Likewise, a recent systematic review found that alcohol consumption altered rhythms of serum melatonin and cortisol in humans, which was dependent on acute *vs.* chronic alcohol use.[67]

Disruption to rhythms is associated with propensity for alcohol consumption or addiction. When presented with a choice between ethanol or water, *Clock* mutant mice display a significantly increased preference for ethanol consumption,[68,69] and when fed an ethanol diet these mice displayed distinct tissue-specific alterations in gene expression.[70] Many affected hepatic and colonic genes were clock-related or clock-controlled, with alcohol having only a minor effect on inflammatory gene expression in the liver and a larger effect in the colon.[70] Likewise, selective striatal ablation of *Bmal1* or *Per2* in mice increased preference for ethanol consumption.[71] Chronotype in humans may also influence alcohol

consumption, with evening preference associated with increased alcohol consumption and alcohol use disorder (AUD),[72–74] though at least one study refutes this association.[75] Therefore, additional study into clock disruption and chronotype on the influence of alcohol consumption and use disorder is warranted.

Like MASLD, AALD is a spectrum of conditions encompassing steatosis, hepatitis, fibrosis, and HCC that can result from the consumption of alcohol. The liver is the primary site of alcohol metabolism, in which ethanol is converted to acetaldehyde and acetate via NAD$^+$-dependent pathways. Excess buildup of NADH leads to inhibition of fatty acid β-oxidation while alcohol itself increases hepatic fatty acid uptake. Alcohol also influences lipogenesis via upregulation of Srebp1c and Chrebp, ultimately resulting in steatosis.[76] Hepatic lipid metabolism is coupled with the sleep-wake cycle and oscillations in feeding and fasting, making the liver highly susceptible to alterations in metabolic homeostasis due to excess alcohol consumption. Ethanol was shown to weaken Clock-Bmal1 protein interactions *in vitro*, while liver-specific deletion of *Bmal1* in mice resulted in more severe alcohol-induced liver injury, including increased lipid accumulation and injury attributed to mitochondrial damage.[77] Also in mice, chronic-plus-binge ethanol feeding phase-shifted and suppressed mRNA expression of hepatic *Clock*, *Bmal1*, and *Per2*, while administration of melatonin, a hormone secreted by the pineal gland, rescued these rhythms. Owing to its antioxidant activities, this study also demonstrated that melatonin activated anti-ferroptosis pathways via *Bmal1* in mice, thus mitigating ethanol-induced liver damage.[78] Another study using the same ethanol-feeding model confirmed ethanol suppressed *Bmal1* in mouse liver, and further discovered that liver-specific overexpression of *Bmal1* reduced steatosis and circulating triglycerides in mice. However, the effects on the peripheral clock were not addressed in this study.[79]

2.3. Cholestatic liver diseases

Cholestatic liver disease describes a range of conditions resulting from damage to bile ducts and/or impaired bile flow into the biliary system. These disorders are typically defined as intrahepatic (involving hepatocytes or intrahepatic bile ducts) or extrahepatic (involving extrahepatic bile ducts or other blockages outside the liver). Two of the most commonly diagnosed conditions that cause cholestasis, primary biliary cirrhosis (PBC), and primary sclerosing cirrhosis (PSC), are progressive chronic disorders that can ultimately lead to serious liver disease including cirrhosis, and they may involve an autoimmune pathogenesis. Many patients with PSC may have a distinct form of inflammatory bowel disease (IBD) termed PSC-IBD, and may be at higher risk for developing colorectal cancer and death.[80] This condition is characterized by a clinical profile distinct from other forms of IBD like ulcerative colitis (UC) or Crohn's disease (CD), discussed below, with a more mild disease course and higher rates of pancolitis.[81]

PBC and PSC may also involve a circadian component, as mice lacking *Per2* had significant induction of inflammatory and fibrotic gene expression after bile duct ligation (BDL, a model of cholestasis) along with increased hepatic stellate cell activation which is indicative of a fibrotic state.[82] Many patients have fatigue or sleep disorders, including delayed sleep phase cycles. A small study of patients with PBC underwent morning bright-light therapy for 2 weeks, which improved sleep quality and timing as well as robustness of melatonin rhythms.[83] Research in animal models indicates melatonin itself may have direct role in pathogenesis of cholestasis. Knockout of the melatonin 1A receptor in mice with BDL resulted in reduction of liver fibrosis and inflammation, while knockout of the melatonin 1B receptor worsened the BDL phenotype. Additionally, the beneficial effects of melatonin administration

were limited to the 1B knockout mice, indicating that melatonin affects cholestatic responses via the 1A receptor.[84] Another study demonstrated a worsened BDL phenotype after pinealectomy in rats, which was recapitulated in human hepatic stellate cells exposed to cholangiocytes from those rats.[85] Finally, a recent clinical trial examined the efficacy of melatonin in relieving cholestatic pruritus and found melatonin improved both pruritus and sleep disturbances in patients with chronic liver disease.[86] Melatonin may represent a potential therapy for cholestatic liver diseases, though further research is required to determine the effects of long-term melatonin administration on the circadian system in the brain, liver, and elsewhere.

2.4. HCC

The global landscape of primary liver cancer is changing with the advent of successful anti-viral treatments for hepatitis B and C, coupled with the rise in alcohol- and MASH-related causes. Alcohol was estimated to contribute to approximately 20% of liver cancer-related deaths in 2019,[87] with MASH and alcohol having the highest age-standardized death rates.[88] HCC accounts for approximately 75% of primary liver cancers and is often co-morbid with other liver diseases.[89]

Circadian disruption is linked to increased risk of cancer via mechanisms involving disruption of the circadian machinery, as well as induction of oncogenes and inhibition of tumor suppressor genes. In 2007, the International Agency for Research on Cancer designated shift work involving circadian disruption as a Class 2A (probable) human carcinogen, and that designation was maintained at a review conducted in 2019.[90] Position-in-time-zone studies provide an interesting perspective on the cancer-causing effects of permanent circadian disruption. Time zones are meant to be centered on longitudinal meridians, with the sun overhead at noon and the eastern and western edges of time zones no more than 30 min misaligned from environmental time. However, manipulation of time zone boundaries in the United States resulted in western edges of time zones that are misaligned by 60 min or more. This leads to misalignment of social *vs.* environmental time and is another form of chronic circadian disruption. Living at the western edge of a time zone in the United States significantly increases risk (rate ratio ~1.1 per 5° longitude difference) for liver cancer/HCC even after adjustment for obesity and lifestyle factors,[91,92] demonstrating a need for systemic monitoring of circadian health at the public health level. In fact, people living at the western edges of time zones in the United States may serve as analogues to study the detrimental effects of DST.

Patients with HCC may have disruptions in genes involved in circadian homeostasis. The clock genes *PER1* and *RORα* were differentially expressed in HCC tumor samples compared to healthy samples, and reduced expression of these genes was associated with poorer survival. Meanwhile, methylation levels of *CRY2* and *RORα* were significantly higher in HCC samples than in healthy samples.[93] Another study associated increased expression of casein kinase delta, an accessory regulator of the molecular clock, with reduced overall survival in patients with HCC.[94] At a mechanistic level, chronic circadian disruption affects glucose, lipid, and bile acid homeostasis, xenobiotic metabolism, and urea disposal. Dysregulation of these processes likely contributes to the progression of MASLD/MASH to HCC. Chronic circadian shifting that simulated jet lag for 90 weeks induced MASLD in nearly 100% of wild type mice, and HCC in nearly 9% of mice. This was attributed to bile acid-mediated activation of the nuclear receptor constitutive androstane receptor, as chronic circadian disruption in $Car^{-/-}$ mice induced liver clock disruption but failed to initiate inflammatory and oncogenic pathways.[95] Knockdown of either *Clock* or *Bmal1*

inhibited tumor growth *in vitro*, and HCC tumor growth was inhibited in mice transplanted with Hep3B cell xenografts lacking *Clock* or *Bmal1*.[96] *Per2* mutant mice with diethylnitrosamine (DEN)-induced HCC had nearly four times the number of tumors compared to wild type controls, with increased inflammation and apoptosis that occurred prior to the onset of carcinogenesis.[97] Melatonin is secreted in response to SCN neuronal regulation, which then feeds back to the SCN and other brain regions to synchronize rhythms and promote sleep. It's also a powerful antioxidant that has wide-ranging benefits against cellular damage from ischemia, heavy metals, and cancer.[98] Melatonin treatment reduced hepatocyte proliferation and oxidative stress while inducing apoptosis in DEN-treated rats.[99] Another study demonstrated that melatonin treatment in mice ameliorated DEN-induced changes in hepatic clock genes, including restoration of *Per* and *Cry* expression that were previously associated with poor outcome. This study also showed that melatonin alone, or in conjunction with the Rev-erbα agonist SR-9009, reduced hepatic cell proliferation *in vitro* and reduced expression of cyclin D1 and c-Myc.[100] Related, single nucleotide polymorphisms (SNPs) of the human melatonin receptor 1A subtype were associated with increased risk for HCC and increased metastasis in patients with HCC.[101] Altogether, these studies provide a basis for further investigation into how disrupted rhythms contribute to hepatic disease and demonstrate how targeting rhythmic processes represents a critically under-explored aspect to treatment of disease (discussed below in chronotherapy).

3. Circadian rhythms in IBD

Like MASLD/MASH, IBD comprises a spectrum of diseases with the two most common forms being CD and UC. Studies point to increasing prevalence of IBD in both industrialized and developing nations, with an estimated 6.8 million people globally already living with some form of IBD.[102] The precise cause(s) of IBD/CD/UC are mostly unknown, though genetic predisposition and innate immune problems are thought to be main contributing factors with diet, medications, infections, and other environmental factors serving as additional non-causal triggers. Both CD and UC are characterized by chronic inflammation of the gastrointestinal (GI) tract: in CD, inflammation can occur at any point in the GI tract, though it tends to be common in the distal small intestine and proximal colon, while in UC it is most often localized to the large intestine. Both conditions involve complex intestinal barrier dysfunction resulting in bacterial infiltration and dysbiosis, or disruption to the gut microbiome that can involve changes in composition or metabolic activities of the resident bacteria. This results in clinical presentations of diarrhea, weight loss, abdominal pain, and increased circulating pro-inflammatory cytokines including TNF-α.[103]

3.1. CD and UC

The intestinal barrier is a critical component to maintaining gut homeostasis, and permeability of this barrier occurring from altered tight junctions or reduced mucin protection is associated with disease states. Circadian rhythms may play a role in the regulation of permeability, as the Rev-erbα agonist SR-9009 improved lipopolysaccharide (LPS)-induced permeability through the induction of tight junction proteins, including occluding and claudin 1.[104] Mucin production and degradation also varies rhythmically in mice, with bacterial proximity to the mucosal surface increased during the active (dark) phase, thus regulating the depth of bacterial invasion in a time-dependent manner.[105] This study also elegantly demonstrated that rhythmic bacterial adherence, rather than colony numbers alone, contributed to rhythmic gene

expression in the colon. However, total fecal bacterial load was also shown to oscillate rhythmically in C57BL/6J mice, and this rhythmicity was lost in *Bmal1* knockout mice.[106] A high-fat diet fed to mice resulted in dampened rhythmic bacterial content at the phyla level, which was partially restored by TRE,[107] indicating a complex relationship between direct and indirect circadian control of the gut microbiome that deserves further study.

Circadian rhythm disruption, including sleep loss and shift work,[108,109] are linked to IBD. One prospective cohort study linked short sleep duration (self-reported <5 h/night) and daytime napping with increased risk for CD or UC after adjustment for age, sex, alcohol consumption, physical activity, and other factors.[110] Another small study demonstrated that patients with IBD had more sleep debt and social jet lag.[111] One small study conducted in 2015 concluded that patients with CD had a similar sleep duration to healthy controls but more post-onset awakenings and worse sleep quality as measured by wrist actigraphy.[112] Limitations of many of these studies are the self-reported nature of sleep timing and quality in patients with IBD and the correlative nature of the associations (i.e., do sleep disturbances worsen IBD, do IBD symptoms worsen sleep, or is there a feed-forward cycle in which both occur?), highlighting a major need for more objective quantification of the associations between disrupted rhythms, sleep, and IBD.

3.2. Alcohol-associated dysbiosis

Alcohol consumption alters the microbiome in both rodents and humans, and alcohol-associated dysbiosis is associated with altered immunity, liver disease, and neurological disorders.[113,114] Alcohol-induced dysbiosis in mice was found due to TNF-α-dependent dysfunction of the intestinal barrier,[115] though it is likely that many other metabolic factors also contribute. *Clock*-mutant mice had lower microbial diversity compared to wild type controls, and when fed an alcohol diet these mice had further alterations in community structure and dominance by *Clostridium* and *Allobaculum*.[116] In humans, plasma short chain fatty acids, important for intestinal barrier function, displayed a diurnal rhythm which was lost with either alcohol consumption or night-shift work.[117] Night shift workers who drank alcohol consecutively for 7 days also had increased colonic permeability which was not recapitulated in day workers who drank alcohol.[118] Melatonin may also play a role, as one small study demonstrated circulating melatonin was reduced in patients with AUD which was inversely correlated with severity of intestinal permeability.[119] A recent study examined rhythmicity of the gut microbiome in patients with AUD. Overall, alcohol use reduced the relative abundance of 15 genera and 16 species, and when sampled over 24 h it was found that the microbiome of patients with AUD had more pathogenic strains with daily oscillatory patterns (including Cyanobacteria and Pseudomonadaceae) and fewer beneficial strains that oscillated (including *Prevotella*).[120] These changes in rhythmicity were also dependent on amount of alcohol consumed and duration of AUD, which could further affect stability of microbiome populations or favor growth of bacteria capable of metabolizing ethanol.[121]

4. Chronotherapy for the liver and gut

Chronotherapy refers to exploiting endogenous rhythms to enhance disease treatment. This can occur through direct targeting of the circadian machinery or via indirect manipulation of the rhythms that control the absorption, distribution, metabolism, or excretion of existing drugs. Chronotherapy is already utilized for the treatment of hypertension,[122] cancer,[123] and depression,[124] and research suggests chronotherapy could be useful as adjacent treatments for MASLD, MASH, and HCC. One simple example is the

use of simvastatin, a cholesterol-reducing medication that inhibits hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme that synthesizes cholesterol. HMG-CoA reductase exhibits a diurnal rhythm in transcription and activity in the liver, with cholesterol synthesis typically peaking in the middle of the night. Simvastatin, with a half-life of approximately 2−3 h, is routinely prescribed with a recommendation to be taken in evening, maximizing its effects when HMG-CoA reductase is highly active. Indeed, studies examining the effectiveness of simvastatin time-of-administration determined greater reductions in low-density lipoprotein cholesterol with night-administrated simvastatin compared to daytime administration.[125] Other statins with longer half-lives may be taken at any time, while controlled-release simvastatin was shown to be effective both in the morning and evening.[125]

TRE is growing in popularity as a mechanism for weight loss, as it focuses on meal timing rather than caloric intake. Animal models provide strong evidence of the benefits of TRE. Mice fed a high-fat diet *ad libitum* gained more weight than mice eating an isocaloric diet restricted only to the active period. These TRE mice also had reduced hepatic triglycerides and steatosis score, and circadian expression of sirtuin 1, a major metabolic regulator, was restored to that of chow-fed mice.[126] Another study showed that after 26 weeks of *ad libitum* high-fat diet-feeding, just two weeks of isocaloric TRE decreased hepatic inflammation prior to any changes in body weight.[127] The beneficial effects of TRE may require fibroblast growth factor 21 (Fgf21), a liver-produced hormone implicated in the improvement of metabolic syndrome. TRE increased serum Fgf21 in high-fat diet-fed mice, and liver-specific *Fgf21* knockout mice were resistant to the beneficial effects of TRE.[128]

Evidence in humans is supportive of a role for TRE in improving metabolic outcomes, though most studies are lacking in sample size and generalizability. A randomized control trial demonstrated that either a 4- or 6-h daytime "eating window", in which patients could eat *ad libitum*, produced equally significant weight loss compared to control participants who ate *ad libitum* over 24 h. This was accompanied by reductions in plasma markers of oxidative stress, likely related to improved insulin resistance, while plasma markers of inflammation were unchanged.[129] Another small study demonstrated in healthy non-obese individuals that 8 h TRE increased high-density lipoprotein cholesterol and reduced C-reactive protein and total nitrate/nitrite compared to individuals eating *ad libitum* despite equal caloric intake.[130] When combined with a low-sugar diet, 8 h TRE reduced body weight, body fat, serum ALT, AST and gamma glutamyl transferase, as well as fibrosis score in patients with MASLD.[131]

The efficacy and toxicity of drugs can vary over the 24 h day, as can their metabolic actions. The metabolic functions of hepatic detoxification enzymes and transporters that process these drugs are also regulated, in part, by circadian rhythms. This presents an opportunity to pharmaceutically target drug metabolism to (i) maximize efficacy of drugs that are administered, which can lead to (ii) minimizing off-target side effects by providing the lowest effective dose at a maximally effective time (Fig. 3). This requires examination of the molecular, cellular, and tissue-level processes that contribute to xenobiotic and metabolic processing under a circadian lens.

Cell cycle proliferation and DNA repair are influenced by molecular rhythms, and time-of-treatment represents a novel approach to cancer drug development.[132] Chronomodulated administration of a combination of oxaliplatin, 5-fluoroacil, and folinic acid for colorectal cancer increased response rate by 76% and reduced toxic events compared to constant-rate infusion of the same drugs.[133] Limited chronotherapy studies have been conducted for the treatment of lung, head and neck cancers, glioma, and
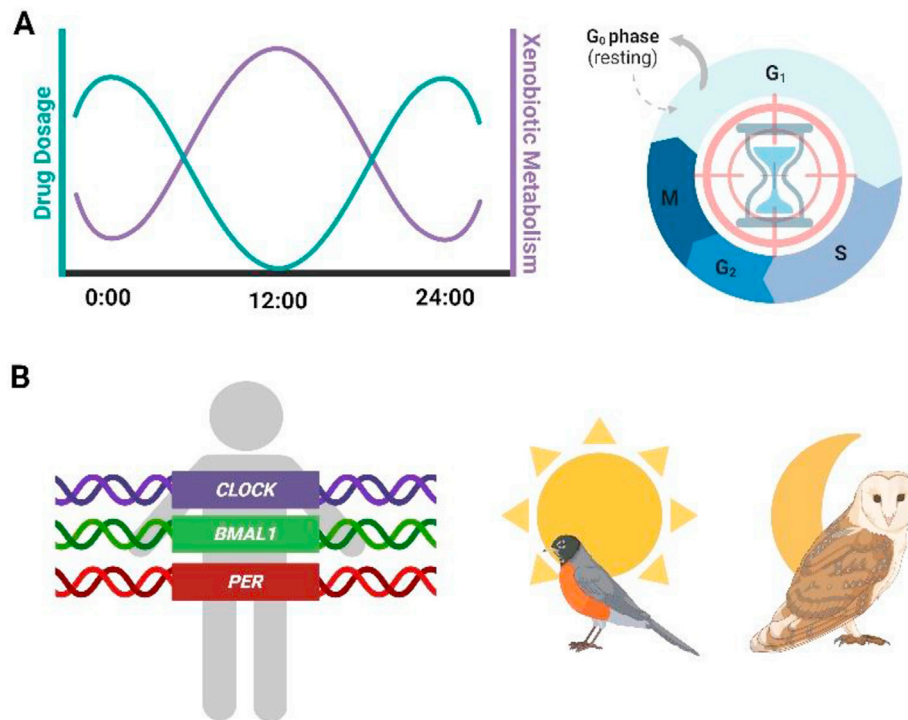
Fig. 3. Chronotherapy for drug development and disease treatment. Chronotherapy can take place in the form of generalized targeting or personalized medicine. **(A)** Administration of drugs at opportune times takes advantage of maximal efficiency of the xenobiotic enzymes that process those drugs, potentially resulting in lower effective doses required and reduction of side effects. Pre-existing drugs can be time-tested and optimized for better therapeutic results. Meanwhile, time-targeting of the cell cycle or circadian clock in HCC and other cancers can maximize cytotoxic effects of chemotherapeutic agents while minimizing damage to healthy tissue. **(B)** Progress in monitoring and understanding of patient genotype and detection of clock polymorphisms, as well as assessment of individual chronotype, will improve disease diagnosis and provide novel therapeutic targets. This figure was created with BioRender.com. Abbreviations: BMAL1, brain and muscle ARNT-like protein 1; CLOCK, circadian locomotor output cycles kaput; HCC, hepatocellular carcinoma; PER, Period.

others, though overall the concept remains poorly understudied.[134] The most common curative pathway for HCC is surgical resection followed by chemotherapy and/or radiation. Recurrence rates are high and often confounded with additional underlying conditions including alcohol consumption, viral infection or cirrhosis, coupled with the problem of chemotherapeutic agents demonstrating lower efficacy with more drug resistance in HCC than in other cancers.[135] In a c-Myc mouse model of HCC, Ki67-positive cells, a marker of hepatocyte proliferation, were highest in mid-day and mid-night.[136] Likewise, mice with DEN-induced HCC had increased cell proliferation and DNA breaks at the end of the active period, and radiation treatment applied at this time minimally affected leukocyte counts. Importantly, this timing of treatment also significantly reduced the incidence of double-strand DNA breaks in non-tumor control mice while simultaneously exerting the anticipated cellular damage in HCC mice.[137] These data provide support for further studies investigating the time-dependent efficacy of anti-mitotic cancer drugs, which could minimize damage to healthy tissues while still providing effective chemotherapy.

Melatonin has been shown to be beneficial in the treatment of HCC, though whether these effects are mediated more through the modulation of circadian rhythms or the function of melatonin as an antioxidant is not entirely clear. A recent systematic review identified studies in which melatonin, by itself or in combination with additional chemotherapeutic agents for liver cancers, inhibited proliferation and tumor growth, induced apoptosis and anti-oxidant activities, and affected the immune and circadian systems, ultimately concluding that melatonin may be beneficial for the prevention and treatment of HCC and cholangiocarcinoma.[138] In mice with DEN-induced HCC, long-term

melatonin treatment prevented HCC-induced changes in hepatic clock gene expression and induced expression of the tumor suppressor proteins p21 and p53.[100] Melatonin was also shown to enhance the cytotoxic effects of sorafenib, a first-line chemotherapeutic for HCC, by increasing apoptosis *in vitro*,[139,140] which was recapitulated in rats with DEN-induced HCC *in vivo*.[141] As mentioned, melatonin administration restored clock gene expression that was altered in HCC,[100] while several small studies in patients with MASLD demonstrated that melatonin improved liver function and steatosis scores, reduced serum AST and ALT, and reduced circulating pro-inflammatory cytokines.[142–144] Therefore, further consideration should be given to both direct and indirect modulation of the circadian clock for the treatment of chronic liver diseases.

Chronotherapy can also apply to chronic gut disorders. First line treatments for CD are typically anti-inflammatory agents, including glucocorticoids, azathioprine, and methotrexate. Time-dependent use of these agents has been demonstrated for the treatment of rheumatoid arthritis,[145,146] but evidence for the chronotherapy of IBD is lacking. A small crossover trial performed in patients with inactive IBD showed that morning administration of 6-mercaptopurine and its prodrug azathioprine resulted in significantly increased levels of therapeutic metabolites and reduced levels of toxic metabolites associated with side effects compared to administration of the same drugs and doses at other times.[147] In a mouse model of colitis, berberine was shown to inhibit *Bmal1* promoter activity through the activation of Rev-erbα. This study also demonstrated that administration of berberine near the end of the active phase of mice reduced inflammatory markers of disease compared to administration early in the active phase.[148] Animal

studies have shown beneficial effects of melatonin for the treatment of colitis in rodents, though it appears time-dependent effects were not examined. In Toll-like receptor 4 knockout mice with colitis, daily melatonin induced the production of antimicrobial peptides in colon tissue and restored the species richness and diversity indices of the microbiome.[149] Another study investigated changes in the microbiota following melatonin treatment in colitis mice and found increased abundance of the probiotic *Bifidobacterium*, while fecal microbiota transplant from melatonin-treated mice reduced Tnf-α expression and increased tight junction protein expression in the colon of colitis mice.[150] These preliminary studies form the basis for further investigation into the benefits of chronotherapy for IBD, given that circadian rhythms regulate gut motility, nutrient absorption, gut immunity, and the microbiota itself.[151]

5. Conclusions and future perspectives

Circadian rhythms serve to synchronize an organism to its external environment, allowing for anticipation of daily needs and adaptation to external stimuli. These rhythms are grounded in a conserved molecular clock that exists in nearly all tissues and cells, highlighting the importance of temporal organization at the cellular and organismal level. Disruptions to these rhythms are increasingly recognized in the progression and pathology of inflammation-related diseases, including MASLD/MASH, cholangitis, HCC, AALD, and IBD. As shift work, sleep loss, and late-night activities progressively infiltrate daily life, the physiological consequences of these disruptions will need to be addressed with research that takes circadian rhythms into account.

Questions remain unanswered with respect to implementing chronotherapy in clinical practice. Currently, clinical trials are underway for the chronotherapeutic treatment of UC (NCT05213234), IBD (NCT04304950), and AUD (NCT05684094), and several trials are examining chronotypes (NCT02895282, NCT04665336) and TRE (NCT05031429, NCT04618133) for obesity and weight loss. Still, these and other trials that incorporate rhythms into interventions and outcomes comprise only a small percentage of total active trials. The influence of circadian rhythms in healthy and diseased physiology is still understudied, and the opportunity for time-targeted treatment is even less recognized. A study in 2014 indicated that more than half of the best-selling drugs in the United States targeted a product of a circadian gene, and nearly half of those drugs had half-lives short enough to be amenable to modulation of the time of administration, i.e., chronotherapy.[152] Can currently-approved drugs have more favorable outcomes by studying time-of-administration? What influence do circadian rhythms have in disease diagnosis (*e.g.*, time of biopsy, rhythms of biomarkers)? Can modulation of metabolic rhythms curtail the projected increased incidence of obesity, type 2 diabetes, and MASLD/MASH around the world? More funding and pre-clinical research are needed to answer these fundamental questions and to make personalized and chronotherapeutic medicine a clinical reality.

Author's contributions

Jessica M. Ferrell developed the concept and wrote the manuscript. The author read and approved the final version of this manuscript.

Declaration of competing interest

The author declares that there is no conflicts of interest.

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