Lifestyle disorders

Lifestyle disorders (LSDs) are a subset of non-communicable diseases (Chakma & Gupta, 2014)that stem from unhealthy habits such as chronic consumption of high calorie diet, physical inactivity, tobacco and alcohol abuse, chronic stress (Unwin & Alberti, 2006)and altered biological clocks (Singh, 2022). These core contributors have further roots in the frenetic lifestyle marked by a long-term ignorance on healthcare that consequently culminates in the initiation and development of LSDs (Unwin & Alberti, 2006). Since the past decade, there has been a drastic increase in these diseases. In 2005, the World Health Organization (WHO) had estimated that 61% of all deaths, i.e. 35 million and 49% of the global disease burden were attributable to LSDs (Mathers, 2020). According to this report, by 2030, the proportion of the total global deaths due to LSDs is expected to increase to 70% and the global disease burden to 56% (Mathers, 2020).

The most prevalent lifestyle disorders include obesity, diabetes, cardiovascular diseases (CVDs), chronic obstructive pulmonary disease (COPD) and even various types of cancers (Tabish, 2017)[Fig. 1]. A study in 2021 reported that the prevalence of obesity in India has risen to 40.3%, though majority of the Indian population comprises of low-income and middle-income communities (Chaudhary & Sharma, 2023). Similarly, there are an estimated 77 million people in India (above the age of 18 years) suffering from Type-2 diabetes mellitus (T2DM) and approximately 25 million individuals are prediabetics (Atre et al., 2020). These alarming statistics are further compounded by the earlier “dormant” LSDs such as Non-alcoholic fatty liver disease (NAFLD) whose prevalence has also steeply increased, with an estimated 40.8% individuals suffering from the silent disease (Riazi et al., 2022).

Despite these startling statistics, lifestyle disorders are reversible and simple modifications in an individual’s lifestyle can improve the overall health .(Tabish, 2017) However, these numbers also warrant further research into the field of these diseases, to not only understand their pathophysiology, but also to identify targets and develop effective remedies for combating LSDs.

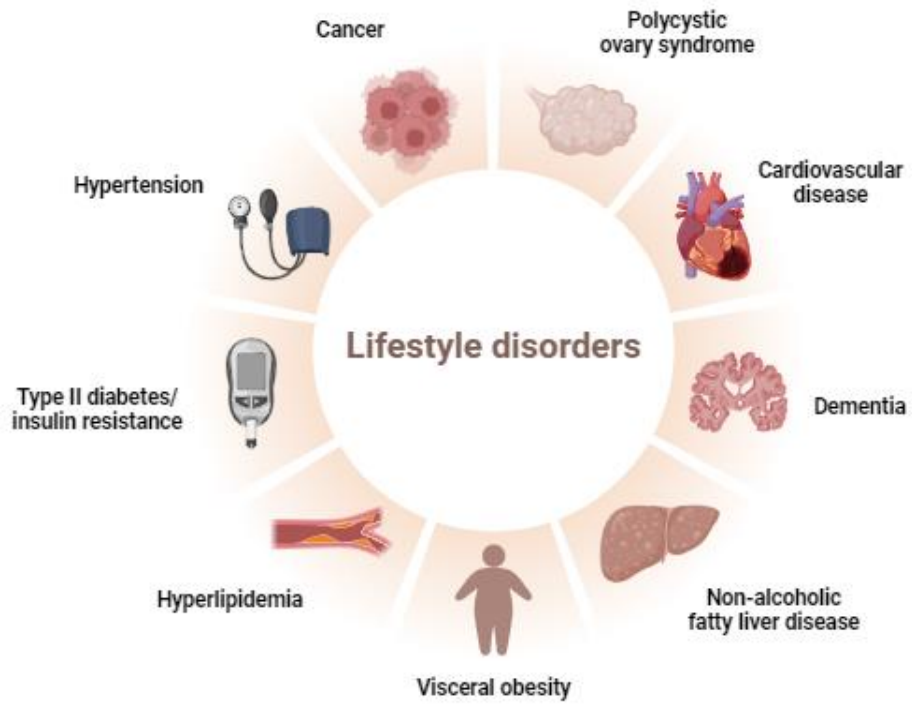


Figure 1. Several types of lifestyle disorders.

The risk factors of LSDs can be broadly classified into three categories (Sharma et al., 2014; Tabish, 2017).

(i) Modifiable behavioural risk factors:

These include excessive consumption of alcohol, poor eating habits, physical inactivity and disturbed circadian clocks. Stress due to work is also being regarded as a potent risk factor for LSDs.

(ii) Non-modifiable risk factors:

These factors cannot be controlled or modified and include age, race, gender and genetics.

(iii) Metabolic risk factors:

These comprise of causal factors such as obesity, hypertension, hyperglycemia and hyperlipidemia.

Circadian rhythms

All living organisms are exposed to 24-hours light-dark and temperature cycles that coincide with the rotation of the earth around its own axis. During evolution, most organisms, from the light-sensitive bacteria to mammals have developed an internal timekeeping system known as the "circadian clock" that allows them to anticipate the recurring daily alterations of their environment (Edery, 2000; Vitaterna et al., 2001). The word "circadian" has its origins in the Latin word "circa diem" that means "about a day" (Allada & Bass, 2021; Partch et al., 2014). Hence, even when organisms are subjected to constant conditions (i.e. constant darkness or constant temperature), they showcase cycles in behaviour and physiology of approximately 24 hours (Patke et al., 2020). In mammals, these cycles are driven by molecular oscillators in the suprachiasmatic nucleus (SCN) that is situated in the ventral hypothalamus (Takahashi, 2017). The SCN takes direct retinal innervation via the retinohypothalamic tract (RHT) that ensures its synchronization to day-night cycles. Further, the SCN projects to various brain centres, that contain local circadian clocks serving direct behavioural (such as feeding-fasting and sleep-wakefulness), autonomic as well as neuroendocrine circadian rhythms. These systemic cues coordinate tissue-specific peripheral clocks, thereby directing circadian gene expression that regulate physiological rhythms essential to wellbeing (J. S. Takahashi, 2017a). Perpetually all the physiological processes, viz. metabolism, blood pressure, mental alertness, renal function, endocrine function and even the body temperature are regulated by these biological clocks (Gachon et al., 2004).

Molecular components of the Mammalian Circadian Clock

The circadian clock circuitry comprises of a network of transcriptional-translational feedback loops which are responsible for driving rhythmic, 24h expression patterns of core clock components (Ko & Takahashi, 2006). The "core clock components" can be defined as the genes whose translated products are necessary for the generation and regulation of circadian

rhythms within all the individual cells of the organism (Borgs et al., 2009). The primary feedback loop consists of the positive elements such as members of the basic helix-loop-helix (bHLH)-PAS transcription factor family, CLOCK and BMAL1 (J. S. Takahashi, 2017a). CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (Brain and muscle Arnt-like protein) function as master clock genes and heterodimerize to initiate transcription of target genes that contain E-box cis-regulatory enhancer sequences (Buhr & Takahashi, 2013). Some of these genes include *Period* (*Per1, Per2* and *Per3)* and *Cryptochrome* (*Cry1* and *Cry2*). At a certain threshold, the PER:CRY heterodimer function in a negative feedback loop that translocate back to th

e nucleus to repress their own transcription by inhibiting the CLOCK:BMAL1 complex [Fig. 2] (Morgado et al., 2015).

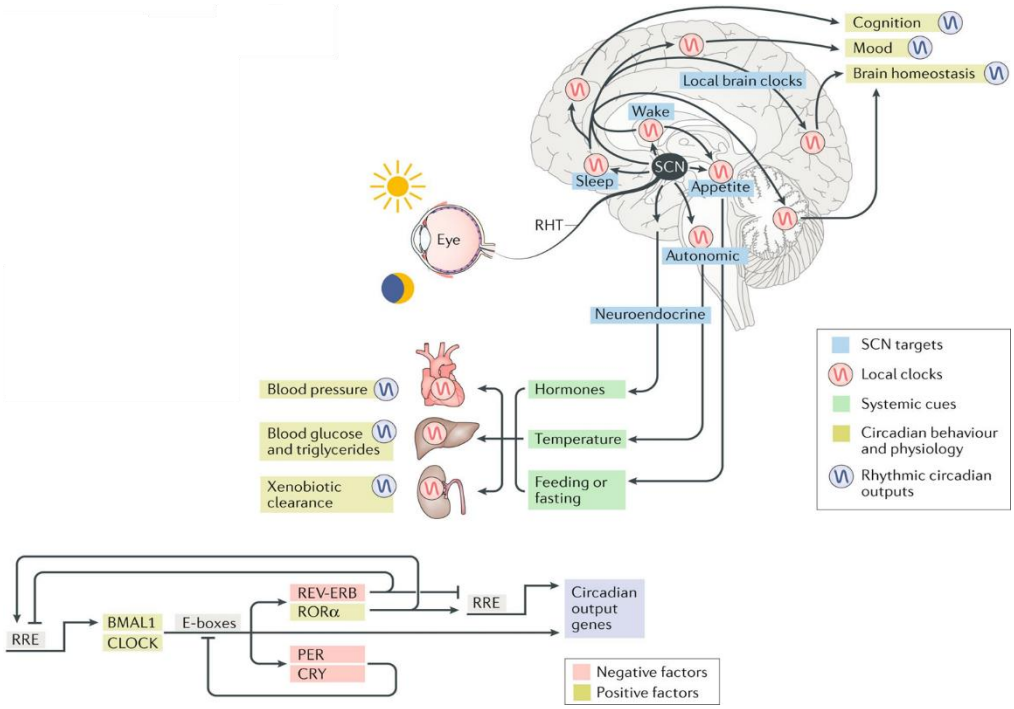


Figure 2. Schematic representation of organization of circadian clock in mammals (Morgado et al., 2015).

The CLOCK:BMAL1 heterodimer induces another regulatory loop by activating the transcription of retinoic-acid related orphan nuclear receptors, Rev-erbα and Rorα. Further, REV-ERBα and RORα compete to bind to the retinoic acid-related orphan receptor response elements (ROREs) present in the

Bmal1 promoter, thereby regulating Bmal1 expression(Ko & Takahashi, 2006). RORs activate transcription of Bmal1 whereas REV-ERBs repress Bmal1 transcription (Akashi & Takumi, 2005). These autoregulatory feedback loops take 24 h to complete a single cycle and are tightly regulated by post-translational modifications such as phosphorylation and ubiquitination (Akashi & Takumi, 2005). These processes render stability to the clock proteins as well as facilitate nuclear translocation of these proteins, thereby significantly contributing to the precision of the mammalian clock. Casein kinase 1 epsilon and Casein kinase 1 delta (*CK1ε* and *CK1δ*) play important roles in regulating the turnover of the core circadian proteins (Gallego & Virshup, 2007; Mehra et al., 2009)[Fig. 3]. Moreover, a small ubiquitin-related modifier protein modification of BMAL1 has been proposed as another level of post-translational regulation (J. Lee et al., 2008). The importance of the post-translational regulation within the molecular clock circuitry can be understood from studies that reported dramatic alterations in circadian period due to mutations in *CK1ε* and *CK1δ.* Herein, the said mutations resulted in altered kinase activities and subsequently shorter circadian periods in mammals (Mehra et al., 2009).

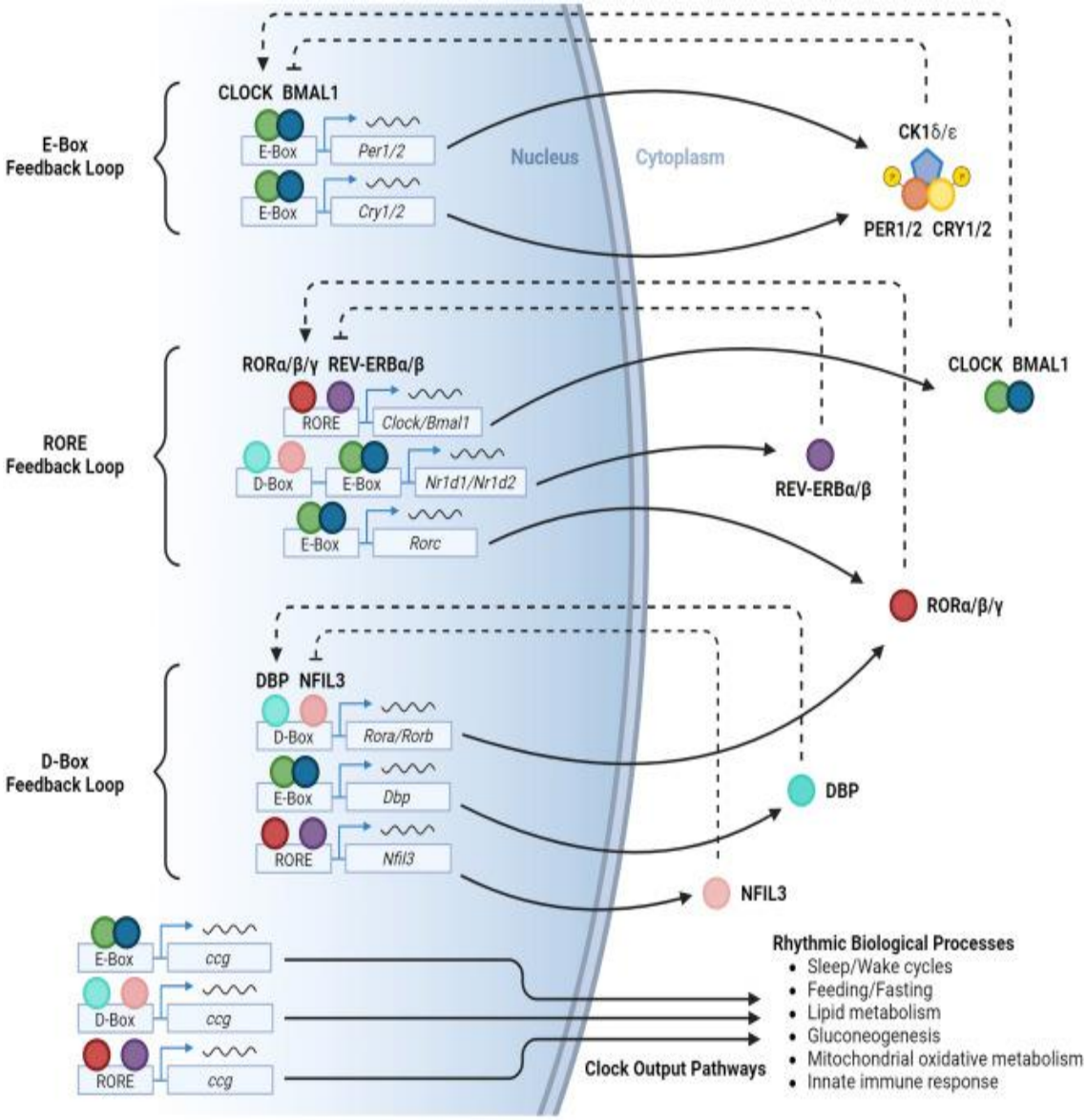


Figure 3. Components of the mammalian circadian clock (Takahashi, 2017).

Circadian rhythms in hepatic metabolism

The endogenous biological timekeeping system synchronizes a wide range of hepatic metabolic events (Reinke & Asher, 2016). These include functions such as nutrient uptake (Balakrishnan et al., 2012), processing and detoxification that align organ functions to cycle with nutrient supply and demand (Mukherji

et al., 2019). Rhythmicity of the core circadian clock is transmitted to transcriptional signals to regulate rhythmic output (Mazzoccoli et al., 2012a). To achieve this, various key metabolic enzymes are directly regulated by the core clock transcription factors (Sahar & Sassone-Corsi, 2012).

Investigations into the role role of circadian clock in liver physiology by genome-wide gene expression studies had revealed two peaks of rhythmically regulated transcripts at the end of the light and dark phases respectively in mouse liver. These peaks reflected the diverse physiological demands such as energy requirement or detoxification activity corresponding to activity or rest (Rey et al., 2011; Weger et al., 2021). Further, the rhythmicity of many transcripts corresponds with phases of the respective translated proteins along with their respective biochemical pathways (Y. H. Kim & Lazar, 2020). Notably, proteomic studies done recently have reported that while some metabolic enzymes cycle, their transcripts remain relatively constant throughout the day, suggesting the involvement of post-transcriptional mechanisms in the circadian regulation of liver functions (Lim & Allada, 2013).

Studies have shown that most of the fundamental functions of the liver are regulated rhythmically [Fig. 4]: liver regulates energy homeostasis and several key enzymes involved in metabolism are expressed in a rhythmic manner (de Assis & Oster, 2021). Expression of glucose transporters, glucagon receptors and the enzymes regulating metabolic pathways of hexose sugars have a peak expression in early evenings (Kalsbeek et al., 2014). Similarly, various enzymes of the glycerol-3-phosphate pathway (*Gpat, Agpat* and *Lipin*), that regulate glycerol and lipid metabolism as well as triglyceride (TG) accumulation, are expressed in a cyclical manner that corresponds to circadian time (Tong & Yin, 2013).

The liver is also a site of steroid hormones and cholesterol that is under circadian control (Almon et al., 2008). HMG-CoA reductase, a key protein in in cholesterol biosynthetic pathway, reaches its peak expression during the time

when cholesterol is not supplied from the diet (Davidson et al., 2004). In contrast, expression rhythms of P450 and several other cytochromes, are more evenly distributed throughout the day (Froy, 2009; T. Zhang et al., 2018). The liver is also the chief site for the thyroxine hormone production (Anastasiou et al., 2015; Ritter et al., 2020)that is regulated by rhythmic hepatic expression of deiodinase 1, that cyclically functions to deiodinize inactive T4 thyroxine to the active T3 form. Further, the peak expression of the gene for thyroid receptor α is aligned with the circadian rhythm of T3 and T4 serum levels (Bellastella et al., 2021). Moreover, various transcripts of the innate immunity proteins that are synthesized in liver have circadian rhythms (Baxter & Ray, 2020; Cox et al., 2022; Crespo et al., 2020). The liver is also a key site for the synthesis of coagulation and fibrinolytic proteins wherein; tissue factor pathway inhibitor 2 is rhythmically induced in liver (Bertolucci et al., 2005; Oishi et al., 2011). On a broader scale, circadian clock regulates the uptake and secretion of small molecules in liver via the oscillatory expression of membrane channels and transporters.(Pácha et al., 2021).

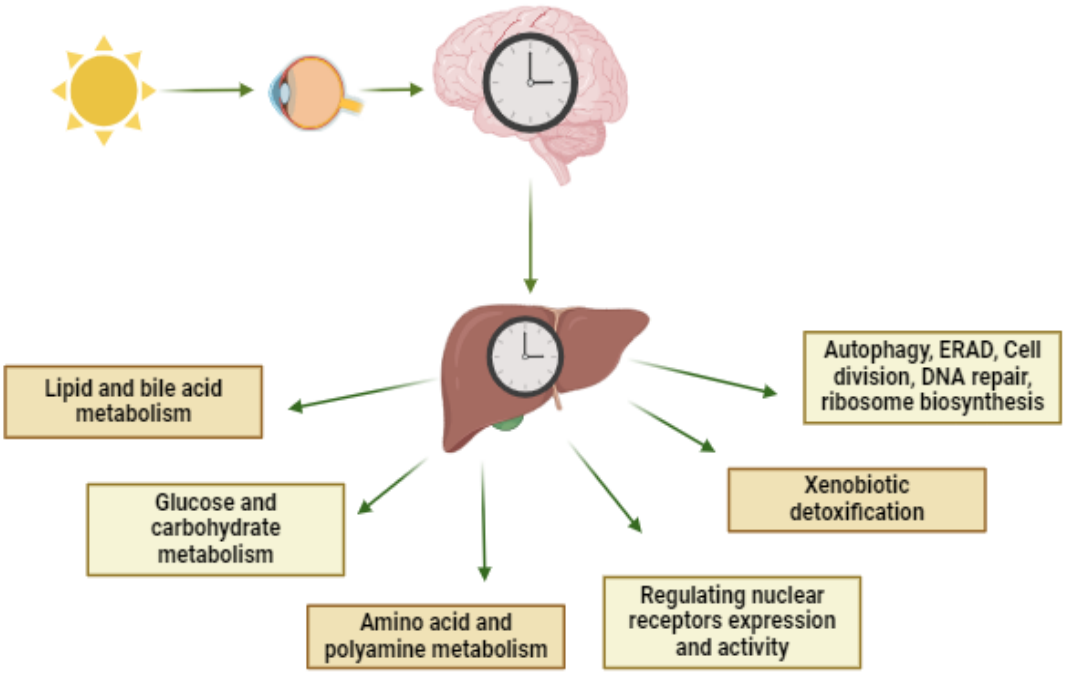


Figure 4. Regulation of hepatic processes by circadian clock.

Regulation of glucose homeostasis

Liver plays an imperative role in regulating glucose homeostasis (H.-S. Han et al., 2016)and the circadian clock appears to offer phasic baseline regulation for recurring procedures, such as the intake of food following periods of nocturnal starvation (Kalsbeek et al., 2014). Consequently, several associated processes, including insulin secretion (Sadacca et al., 2011)and glucagon (Ruiter et al., 2003; Zilstorff et al., 2024)as well as glucose production and uptake exhibit a discernible circadian component that is distinct from nutrient signalling (Peng et al., 2022). The SCN circadian clock and the liver clock employs dissimilar mechanisms thereby producing anti-phasic rhythms of glucose metabolism that combine production of almost constant blood glucose levels throughout the day (Greco & Sassone–Corsi, 2019; Y. Lee & Wisor, 2021). Furthermore, the SCN regulates rest-activity and feeding-fasting rhythms that lead to rhythmic nutrient uptake and signalling (Kalsbeek et al., 2011; Kolbe et al., 2019). Thus, the primary function of the liver clock is to mitigate the circadian fluctuations in blood glucose levels stemming from these behavioural cycles (Greco & Sassone–Corsi, 2019).

Studies had shown that disrupting hepatic *Bmal1* led to drastic fluctuations in levels of blood glucose, especially in the post-absorptive phase in mice (Ando et al., 2016). On the other hand, loss of *Clock* function globally led to minor perturbations in glucose tolerance and gluconeogenesis after insulin-induced hypoglycemia (Kennaway et al., 2007). Furthermore, several genes at the core of the circadian clock, as well as those under its control, have additional functions in glucose metabolism. Cryptochromes control hepatic gluconeogenesis through G-protein-coupled receptors (GPCRs) that block accumulation of cAMP and activation of transcription of gluconeogenic genes regulated by CREB (Surme et al., 2023). Further, overexpressing *Cry1* in liver decreased blood glucose levels and increased insulin sensitivity in diabetic mice (Tong et al., 2017). On the other hand, Cryptochromes inhibit transcription of glucocorticoid receptor and phosphoenolpyruvate carboxykinase genes that regulate gluconeogenesis (Lamia et al., 2011).

Moreover, it is known that glucocorticoids modulate glucose metabolism by inducing Per 2expression under hyperglycemic conditions and studies have shown that mice lacking an essential glucocorticoid response element in the *Per2* gene were resistant to glucose intolerance induced by a prolonged glucocorticoid treatment (So et al., 2009).

KLF10 is regulated by CLOCK:BMAL1 heterodimer and functions as mediator between the circadian clock and metabolism in a sex-specific manner. In mouse liver, KLF10 regulates glycolytic and gluconeogenic genes. (Guillaumond et al., 2010; Ruberto et al., 2021; X. Yang, Chen, et al., 2017). In male mice, KLF10 knockout accounted for post-prandial and fasting hyperglycemia while the females remained normoglycemic (Guillaumond et al., 2010).

Regulation of lipid metabolism

Liver plays a crucial role in lipid metabolism by regulating processes such as lipoprotein synthesis, lipid uptake and subsequent conversion, *de novo* synthesis as well as oxidation of fatty acids (FAs) (Nguyen et al., 2008). All the key aspects of hepatic lipid metabolism are regulated by the circadian clock, and lipid themselves have the potential to influence circadian rhythmicity (Gooley, 2016; Pan, Mota, et al., 2020). A recent lipidomic analysis comparing WT mice with CLOCK null mice provided detailed insights into the periodic accumulation of lipids in the liver and revealed that around 17% of all lipids exhibited diurnal oscillations in both WT and CLOCK-null mice. However, the composition and the circadian phase differed in CLOCK-null mice. Furthermore, when WT mice were fed exclusively during night, the circadian phase of TG accumulation shifted, thereby leading to a 50% decrement in hepatic TG levels, despite no alterations in the total calorie intake (Adamovich et al., 2014). Thus, circadian clock-dependent regulation along with feeding time governs the circadian oscillations of hepatic TGs that can persist even in the absence of a functional clock (Adamovich et al., 2014).

Not surprisingly, circadian lipid metabolism is largely governed by the clock-dependent regulation of key enzymes and transcription factors (Gooley, 2016; Pan, Mota, et al., 2020). These include biosynthetic enzymes within the glycerol-3-phosphate pathway as well as enzymes involved in FA synthesis such as ELOVL3, ELOVL6 and FAS that exhibit rhythmic expression patterns (Reinke & Asher, 2019) . Similarly, regulatory factors for lipid metabolism, such as PPARs (L. Chen & Yang, 2014a; X. Wang et al., 2013a), PGC1 (L. G. F. Rodrigues et al., 2021), SREBP1c (Gilardi et al., 2014), NR1D2 (Bolshette et al., 2023) and ROR (Takeda et al., 2012; Y. Zhang et al., 2017)demonstrate rhythmic expression in liver cells. Disruptions in the *Clock* gene in mice resulted in alterations in the expression of several lipid metabolism genes and culminated in a dysregulated accumulation of intermediates and products related to hepatic lipid metabolism (P. Chen et al., 2018). Similarly, studies have shown that mice lacking the *Per2* gene exhibit dyslipidemia (Bu et al., 2023).

An additional core clock protein, NR1D1, suppresses Apoc3 expression mRNA in liver. As a result, Apoc3 levels in serum levels and very-low-density lipoprotein (VLDL) triglycerides, are elevated in mice with alterations in *Nr1d1* (C. H. Lee et al., 2023). Further, perturbations in circadian clock can also lead to hepatic steatosis, as shown in mice with disruptions in *Clock* (Pan, Queiroz, et al., 2020a) and *Nr1d1-Nr1d2* (Na et al., 2016), or upon deletion of the histone deacetylase, HDAC3 that is recruited by NR1D1 to the lipid metabolism genes (Emmett & Lazar, 2019). In contrast, studies have revealed that restricted feeding can protect mice from hepatic steatosis (Chung et al., 2016).

Indirect evidence supporting the influence of lipids on circadian clock functions primarily arises from investigations into nuclear receptors within the core oscillator mechanism (X. Yang et al., 2006). RORA (D. Liu et al., 2024; Sato et al., 2004), RORG (Lu et al., 2023)and PPAR isoforms (A, G and D) (L. Chen & Yang, 2014b; S. Wang et al., 2022)play integral roles in the core clock by regulating *Bmal1* transcription and simultaneously contributing to the

regulation of lipid metabolism. Notably, cholesterol and specific oxysterols modulate the transcription activation potential of RORA and RORG (Boukhtouche et al., 2004), while various FAs and eicosanoids bind to PPAR isoforms (Hihi et al., 2002). Additionally, SIRT6, that in involved in hepatic clock gene transcription as well as regulation of FAs and cholesterol metabolism, is activated by long-chain FAs (Feldman et al., 2013; Jiang et al., 2013). Collectively, these findings indicate a metabolic feedback loop within the core clock mechanism involving lipid metabolic intermediates.

Regulation of bile acid metabolism

Liver serves as the primary organ for conversion of cholesterol to bile acids that aid in nutrient absorption in intestine and display paracrine and endocrine functions (Chiang & Ferrell, 2018). Bile acid (BA) homeostasis is mainly regulated by a feedback loop consisting of FXR, FGF15 and SHP (Kliewer & Mangelsdorf, 2015). However, the circadian clock adds additional regulatory layers. NR1D1 promotes circadian signalling through INSIG2-SREBP and LXR pathways, thereby promoting rhythmic expression of the rate-limiting enzyme, CYP7A1, and other genes involved in cholesterol and lipid metabolism (Xing et al., 2020). Further, the PAR-domain basic leucine zipper (PPAR bZIP) protein DBP rhythmically activates the expression of CYP7A1 in the liver (Mazzoccoli et al., 2012b). The cycling transcription factor KLF15, that functions as a suppressor of the FXR-FGF15 signalling pathway, also governs BA synthesis (G. Wang et al., 2020). Interestingly, diurnal rhythms of BA synthesis have also been demonstrated in humans (Gälman et al., 2005).

Non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is being increasingly recognised as the most common chronic liver disorder with a global prevalence of 38-40% (Le et al., 2022)[Fig. 5]. NAFLD represents the hepatic manifestation of the metabolic syndrome and is characterised by excessive lipid accumulation in

the hepatocytes (Katsiki et al., 2018). Non-alcoholic steatohepatitis (NASH) is the progressive form of NAFLD that carries the risk of progressive fibrosis, cirrhosis, and end-stage liver disease (Sheka et al., 2020). NASH etiology can be characterized by excessive triglycerides (TGs) and free fatty acid (FFA) accumulation in the liver that subsequently leads to inflammation, apoptosis and ballooning degeneration (Jahn et al., 2016).

Recently, in June 2023, the American Association for the Study of Liver Diseases (AASLD) revised the existing nomenclature of NAFLD and accordingly, "Steatotic Liver Disease (SLD)" has been chosen as an umbrella term to include the various aetiologies of steatosis. Further, non-alcoholic fatty liver disease (NAFLD) shall now be referred as "metabolic dysfunction-associated steatotic liver disease (MASLD)". MASLD shall include patients who have hepatic steatotis and have atleast one of the five cardiometabolic risk factors. A new category, termed "MetALD" shall be used to describe those with MASLD who consume greater amounts of alcohol per week (140g/week and 210g/week for females and males respectively). Metabolic dysfunction-associated steatohepatitis (MASH) is the replacement term for NASH (Malhi et al., 2023)[Fig. 6]. However, for the scope of this thesis, the older terminologies, "NAFLD" and "NASH" shall be used throughout (Ramírez-Mejía & Méndez-Sánchez, 2023; Rinella & Sookoian, 2024).

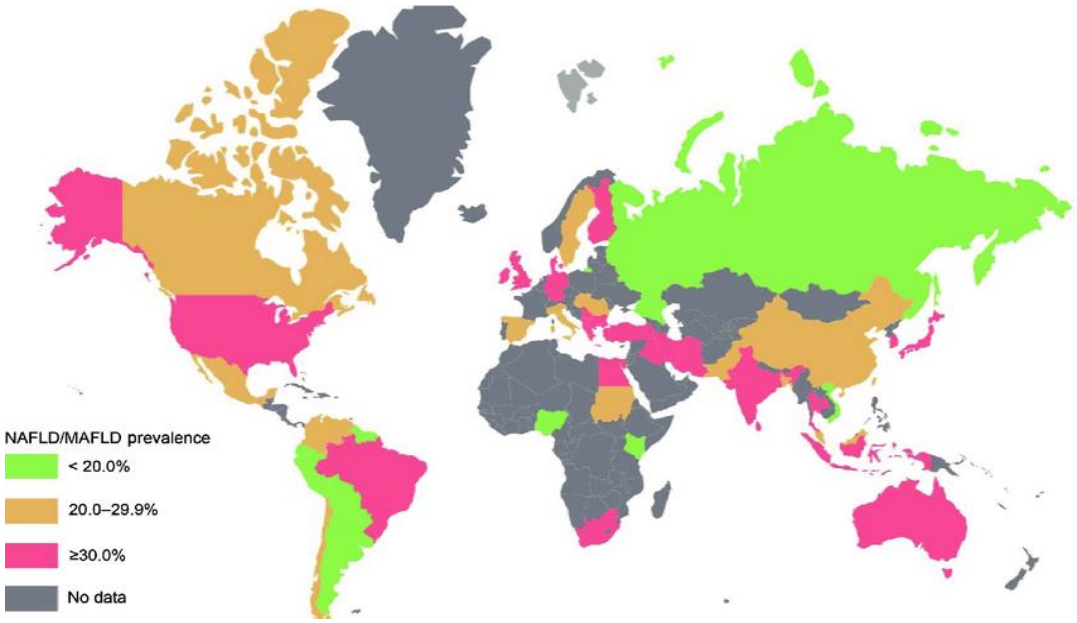


Figure 5. Global burden of Non-alcoholic fatty liver disease (NAFLD) (Le et al., 2022).

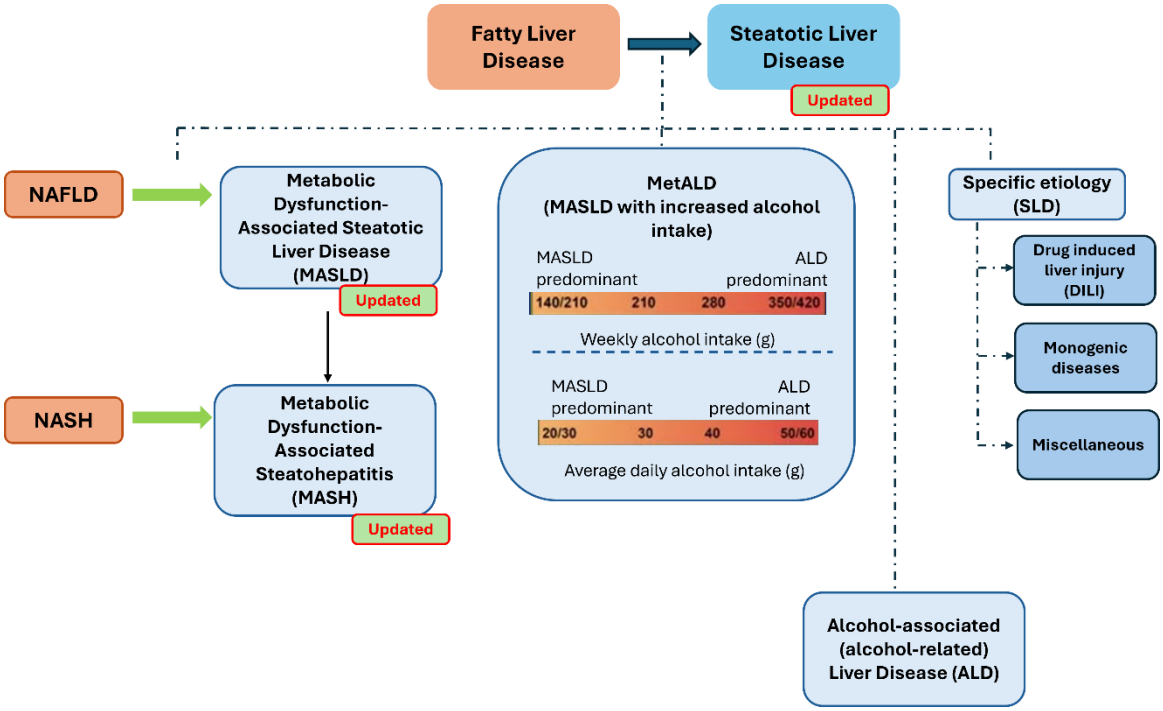


Figure 6. Flowchart representing the revised nomenclature for NAFLD and NASH (Malhi et al., 2023).

The pathophysiology of NAFLD and its progression to NASH can be explained by the "multiple parallel hits hypothesis" wherein, genetic, dietary and environmental factors lead to the development of insulin resistance (Fang et al., 2018). Insulin resistance, in turn, causes increased lipolysis and stimulates hepatic *de novo* lipogenesis (DNL) and release of adipokines such as TNF-α and IL-6 (Alam et al., 2016). As a consequence, the increased flux of hepatic FFAs induces accumulation of TGs that further causes mitochondrial dysfunction and ER stress. Intestinal permeability further participates in the activation of the hepatic inflammatory cascade. Altogether, these multiple parallel hits lead to the progression of NAFLD to NASH and subsequently to fibrosis and cirrhosis (Buzzetti et al., 2016) [Fig. 7]. The key pathophysiological events that culminate in the development of NAFLD and its subsequent progression of NASH are discussed briefly below.

Genetics

Studies have reported that offspring from parents with elevated lipid content are more susceptible to develop NAFLD and even cirrhosis (M. T. Long et al., 2019). Further, studies have shown that monozygotic twins have a higher intra-pair correlation between levels of hepatic fat and plasma ALT than dizygotic twins . Moreover, genome-wide association studies have identified various genetic factors that strongly associate with NAFLD. These include variations in genes such as transmembrane 6 superfamily member 2 (*TM6SF2*), glucokinase regulatory protein (*GCKR*) and patatin-like phospholipase domain-containing-3 (*PNPLA3*) (Kahali et al., 2015). Among these, *PNPLA3* has been classified as one of the most common genetic variations (Krawczyk et al., 2020; Romeo et al., 2008). Studies in human subjects have reported that individuals with *PNPLA3* genetic polymorphism produce a truncated form of the lipase enzyme that impedes TG breakdown and subsequently reduces hepatic TG secretion in the form of very low-density lipoproteins (VLDLs) (He et al., 2010). Further, PNPLA I248M polymorphism has been reported to be more frequent in the Hispanic population, lesser in those of European descent and the lowest in African-American populations (He et al., 2010; Martínez et

al., 2018) .However, gene polymorphisms alone are not the major contributors of NAFLD and NASH development. It is a combination of genetic predisposition and environmental factors such as obesity as well as heightened consumption of carbohydrates and sugar consumption that confers a higher risk of developing NASH (Day, 2010).

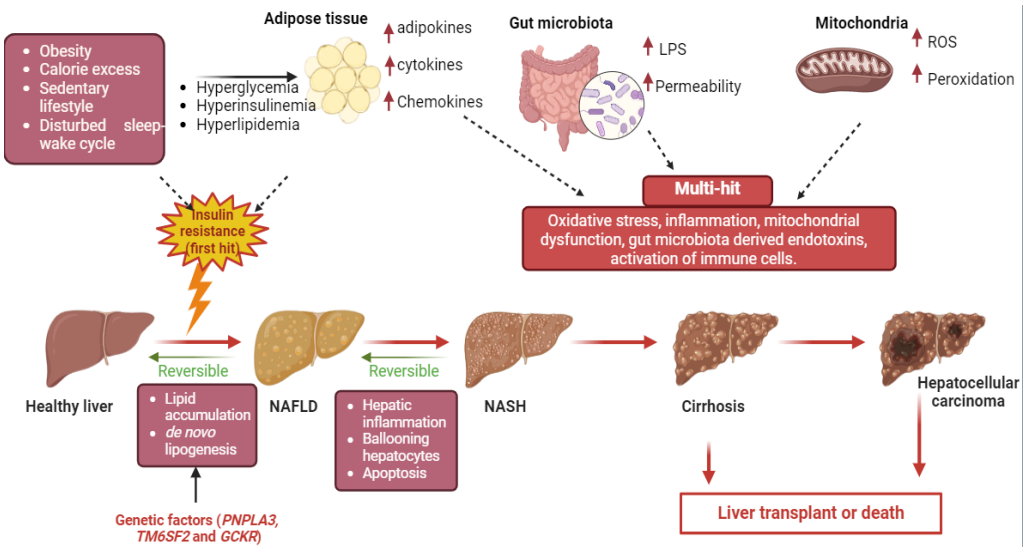


Figure 7. NAFLD/NASH pathogenesis explained by "multiple parallel hits" hypothesis.

Obesity and Systemic Insulin Resistance

Obesity and T2DM have a strong correlation with the development of NASH, thus suggesting that an insulin-resistant environment may be an important driving force for the development of NASH (Godoy-Matos et al., 2020). Excess white adipose tissue (WAT) that is known to be associated with elevated plasma and adipose tissue pro-inflammatory cytokines such as TNF-α and IL-6 have been reported in both NAFLD patients (Du Plessis et al., 2015; Mulder et al., 2016)and animal models of NAFLD (Y. Luo et al., 2016). Adipose tissue derived cytokines not only contribute to chronic low-grade systemic inflammation but also induce systemic insulin resistance by inhibiting downstream insulin signalling (Wisse, 2004). Further, studies in NAFLD patients have reported a positive correlation between insulin resistance and

increased hepatic TGs, implying that FFAs released from the adipose tissue are taken up by liver and metabolized into TGs (Fujii et al., 2020). Additionally, increased intake of diet rich in fat and carbohydrates also contribute to hepatic steatosis (Roden, 2006).

Intake of dietary fat and hepatic de novo lipogenesis

Of the total TGs found in the livers of NAFLD patients, 59% are derived from plasma FFAs while 15% and 26% are derived from dietary fat and *de novo* lipogenesis respectively (Bermúdez-Cardona & Velásquez-Rodríguez, 2016). A study reported that in human subjects on a diet rich in saturated-fats, adipose tissue TG storage and TG levels were significantly increased. (Field et al., 1985; Rosqvist et al., 2014). Further, in rodent models, chronic consumption of diets with 45-68% energy derived from fats elevated intrahepatic TGs (Peterson et al., 2013). TGs derived from *de novo lipogenesis* (DNL) were increased in subjects who were on a carbohydrate-rich diet (Parks, 2001). High fructose diet regime in rodents accounted for activation of the lipogenic transcription factor, sterol regulatory element-binding protein 1c (*Srebp1-c)* (Chyau et al., 2020; Wada et al., 2013). Thus, development of NAFLD can be attributed to hepatic FFA accumulation. However, the subsequent lipotoxicity appears to be one of the key driving factors for hepatic injury and inflammation that is distinctive of NASH (J. Zhang et al., 2014).

Oxidative Stress in Liver

Hepatocyte damage and apoptosis due to oxidative stress is one of the major drivers of hepatic tissue injury in NASH patients (Stiuso et al., 2014; Sumida et al., 2013; Tariq et al., 2014). Moreover, mitochondrial dysfunction from reactive oxygen species (ROS) has been reported in later-stage NASH patients(Begriche et al., 2006). Further, activation of NADPH oxidase 2 (NOX2) in liver-infiltrating macrophages contributed to oxidative-stress-induced hepatic damage in NAFLD (Del Ben et al., 2014). Studies have also reported a reduced activity of antioxidative enzymes such as coenzyme Q10 and superoxide dismutase in NAFLD/NASH patients(Ardekani et al., 2023;

Świderska et al., 2019). Further, in animals subjected to diet-induced NASH, reduced glutathione: oxidized glutathione (GSH: GSSG) has been reported that is suggestive of an imbalance between ROS and antioxidants (L. Li et al., 2016; Świderska et al., 2019). Reduced antioxidant activity is known to enable the generation of reactive oxygen/nitrogen species such as hydroxyl radical, superoxide anion and peroxynitrite to accumulate and react with the intracellular biomolecules such as FFAs and DNA (Mehta et al., 2002). Thus, the by-products of ROS-induced damage such as 4-hydroxynonal and 3-nitrotyrosine have been shown to be significantly increased in the plasma and liver of NAFLD and NASH patients (Saxena et al., 2019).

Hepatic inflammation

One of the key features that distinguishes NASH from NAFLD is the onset and persistence of hepatic inflammation in NASH and there are various mechanisms that play a role in triggering inflammatory response in NASH patients (Brunt et al., 2009). To begin with, adipose-tissue derived cytokines, such as TNF-α contribute to hepatic inflammation (Frances et al., 2013). Further, gut dysbiosis due to chronic high fat diet (HFD) consumption results in a "leaky gut" that enables endotoxins, such as lipopolysaccharide (LPS) to travel to the liver, thereby triggering/enhancing hepatic inflammation in NASH (Cheng et al., 2018; Kang et al., 2023). Additionally, metabolism associated molecular patterns (MAMPs), that include FFAs and cholesterol, are known to initiate inflammasome-induced inflammatory cell death in hepatocytes (X. Wang et al., 2020). The consequent danger-associated molecular patterns (DAMPs) due to inflammatory cell death further stimulate the activation of liver resident macrophages (Kupffer cells). These activated Kupffer cells secrete TNF-α that exacerbates insulin resistance and initiates NF-κB activation (G.-X. Xu et al., 2023). In addition to NF-κB activation, TNF-α is also known to induce the expression of monocyte chemoattractant protein-1 (MCP-1) which has been reported to be elevated in NASH patients (Mounika et al., 2024; Papadopoulos et al., 2022). Furthermore, neutrophil-secreted myeloperoxidase exacerbates hepatic inflammation by generating oxidative

stress (Cho et al., 2023). In agreement with these findings, studies have reported that during the early stages of NASH, neutrophils are the key contributors to hepatic inflammation (Cho et al., 2023). Mice with $LyG6^{+}$-neutrophil depletion in the early stages of NASH showed significantly lower levels of serum ALT and a reduced pro-inflammatory gene expression compared to NASH mice (Ou et al., 2017). Studies have further reported that NASH patients display a higher level of natural killer T-cells and $CD8^{+}$- T-cells. Moreover, a probable role of T-helper cells in mediating NASH progression and initiation of fibrogenesis has also been proposed (Bhattacharjee et al., 2017; Syn et al., 2010).

Interventions for managing NASH:

Lifestyle modifications remain the mainstay of NASH management, and these include regular exercise and consuming a hypocaloric diet. A reduction of ≥5-10% of body weight is necessary to achieve attenuation of NASH (Hallsworth & Adams, 2019). However, at the later stages of disease progression, lifestyle modifications alone are not sufficient to stop disease progression. Along with lifestyle changes, The only FDA-approved treatment for NASH is obeticholic acid (OCA) (Hindson, 2020). OCA is a farnesoid X receptor (FXR) agonist that regulates the expression of transcription factors that decrease synthesis of bile acids and hepatic steatosis (K. Wang et al., 2024).

Vitamin E is an anti-oxidant that reduces ROS and inflammation-induced hepatic damage. Results from placebo-controlled trial had revealed improvements of liver histology as well as inflammation, ballooning and steatosis in 43% of the non-diabetic NAFLD subjects treated with 800IU vitamin E daily as compared to 19% individuals treated with placebo. However, long-term vitamin E may be associated with hemorrhagic stroke (Ji et al., 2014; Larion & Khurana, 2018).

Pioglitazone mainly targets PPARγ receptor that improves insulin resistance (S. M. Lee et al., 2021). In a clinical trial, 30 mg of pioglitazone improved the liver histology (34%) of non-diabetic NASH patients. Further, another 18

months study showed that pioglitazone reported that pioglitazone treatment in combination with a hypocaloric diet (500kcal/day deficit) improved liver histology in 51% of NASH patients with diabetes. However, its efficacy still needs to be evaluated in larger patient cohorts (Promrat et al., 2004).

Pharmacological interventions under clinical trial

Most of the phase IIb and phase III clinical trials of NASH primarily focus on two endpoints: resolution of NASH without worsening of liver fibrosis and/or improving liver fibrosis without worsening of NASH. Herein, resolution of NASH refers to a decrease of NAS, while improvement of liver fibrosis refers to reduction in fibrosis scores by liver histology. Various pharmacological treatments are under clinical trials (Konerman et al., 2018).

1. Glucagon-like Peptide 1 receptor agonists:

Glucagon-like Peptide 1 (GLP-1) is a hormone secreted by the small intestine after a meal and functions in restoring insulin sensitivity and attenuating hyperglycemia in humans (Cani et al., 2006). Synthetic long-acting glucagon-like peptide 1 (GLP-1) receptor agonists such as liraglutide (Kalogirou et al., 2021)and semaglutide (Newsome et al., 2021)were initially approved for treating T2DM but recently, has gained adequate attention for their efficacy in attenuating insulin resistance, hyperglycemia and hepatic lipotoxicity in NASH patients.

2. De novo lipogenesis (DNL) enzyme inhibitors:

Aramchol is a synthetic molecule designed by conjugating bile acid and arachidonic acid (Gilat et al., 2001). Aramchol functions by inhibiting a key rate limiting enzyme, SCD-1 (responsible for converting FAs into TGs) (Allen et al., 2018). Further, studies have reported potent antioxidative and anti-fibrotic activity of Aramchol along with a reduction of hepatic steatosis in animal models (Golan-Gerstl et al., 2017; Ratziu et al., 2024). Moreover, a phase II clinical trial revealed that NASH patients treated with 300mg of

Arachol daily had hepatic fat reduced by 12.6-22.1% as compared to the placebo group (Ratziu et al., 2021).

3. Anti-inflammatory and anti-apoptotic drugs:

Hepatic inflammation is one of the key features of NASH pathophysiology and is one of the popular targets of pipeline drugs (Kurikawa et al., 2013). Till date, only one drug, Cenicriviroc, a CCR2/CCR5 dual-inhibitor, has been cleared in clinical trials (Tacke, 2018). Further, 20% of the patients receiving 150 mg of cenicriviroc daily reported a reduction in fibrosis as compared to only 10% of the subjects receiving placebo in its phase II clinical trial (Anstee et al., 2020; Friedman et al., 2016).

4. PPAR Agonists:

Activation of Peroxisome proliferator-activated receptor α (PPAR α) is known to improve NASH by increasing FFA β-oxidation (Sambasiva Rao & Reddy, 2004a), while PPARδ is known to improve hepatic and systemic insulin sensitivity (Nagasawa et al., 2006). Elafibranor is a PPAR α/δ dual agonist that displayed efficacy in improving NASH histology in its phase II trial with 274 patients (Ratziu et al., 2016).

In order to further bridge the gap between existing knowledge about NASH and identifying novel drug targets, more research into the pathophysiology of NASH becomes pertinent that shall pave the way for identification of novel drug targets for NASH.

Circadian desynchrony affects liver functions

The biological timekeeping system helps the body anticipate environmental cues to optimize energy utilization for the diurnal cycles of rest-activity and

feeding-fasting (Buijs et al., 2003). The central circadian pacemaker in the SCN is entrained primarily by the light-dark (LD) cycle and it promotes wakefulness during the light cycle and facilitates sleep during the dark cycle (Honma, 2020). Disruptions in these oscillations has been recognized to affect metabolic homeostasis (Barclay et al., 2012).

Cross-sectional studies have suggested an association between sleep deprivation and NAFLD. A study on approximately 8000 male government employees in Japan reported a higher prevalence of NAFLD in individuals with a short sleep duration (less than 5 h) whereas a sleep duration of greater than 7 h was associated with a decreased risk of NAFLD (Imaizumi et al., 2015). In another study of 45,000 Korean adults, short sleep duration (less than 5 h) was found to be associated with an increased risk of NAFLD in adults . However, when adjusting for BMI, this association was significant only in females (J.-H. Kim et al., 2019). Similar study on 2000 Japanese adults reported that a higher risk of NAFLD was associated with a short sleep duration of less than 6 h among females when adjusting for age, snacking, regular exercise and BMI but no such association could be established among males (A. Takahashi et al., 2020).

Research has also explored NAFLD and its connection to other disturbances in sleep patterns. Using the NHANES database, a study reviewed approximately 8000 participants and observed a higher prevalence of NAFLD among shift workers (L. Wang et al., 2024). In a middle-aged to elderly Chinese population, prevalence of NAFLD was higher among daytime nappers, with a dose-dependent association with the duration of napping (C. Hu et al., 2020). Another study reported that an increased prevalence of NAFLD was associated with a greater frequency of snacking over 24 h (Yari et al., 2020). In Basel NAFLD cohort, it was found that patients with NAFLD ate more frequent nocturnal meals when compared with healthy controls (Bernsmeier et al., 2015).

Several animal studies have linked circadian clock genes to the pathogenesis of NAFLD and its subsequent progression to NASH. It is well established that WT mice, when subjected to HFD regime, become obese, insulin resistant and develop hepatic steatosis (F. Long et al., 2023). Interestingly, mice lacking *Clock* gene globally display comparable metabolic traits and hepatic steatosis when fed a regular diet. Moreover, they demonstrate an exacerbated phenotype when fed a HFD (Meyer-Kovac et al., 2017). Thus, these findings throw light on the key role of *Clock* gene in regulating metabolic homeostasis and appears to play a protective role against NAFLD development. Male WT mice subjected to HFD for 11 months reported significant changes in the mRNA levels of the clock genes, *Bmal1, Per1, Per2, Per3, Cry1* and *Cry2*, thus implying that chronic consumption of a high-fat diet leads to aberrations in the circadian clock gene expression that can subsequently promote the development of the metabolic syndrome (Roy et al., 2022)[Table 1].

PPARα is one of the target genes of the CLOCK:BMAL1 heterodimer and is known to upregulate the expression of *Bmal1* in the peripheral tissues (Inoue et al., 2005). In *PPARα* knockout mice models, *Bmal1* mRNA expression was maintained in the SCN but was diminished in the liver when compared with WT mice (X. Wang et al., 2013b). PPARα regulates various genes involved in lipid metabolism and FA beta-oxidation and *PPARα* knockout mice displayed decreased levels of HMGCR, SREBP, and FAS (Dihingia et al., 2018). Further, prolonged fasting in *PPARα* knockout mice models, either global knockout ($PPAR\alpha^{-/-}$) (Pawlak et al., 2015)or liver-specific ($PPAR\alpha^{hep-/-}$), led to hepatic steatosis (Y. Wang et al., 2020). Both the models, $PPAR\alpha^{-/-}$ and $PPAR\alpha^{hep-/-}$, developed hepatic steatosis as they aged (> 1 year) even when fed a standard diet (Sambasiva Rao & Reddy, 2004b). Subjecting *PPARα* knockout mice to a chronic HFD regime for 6 months led to development of steatohepatitis in comparison to WT mice (Montagner et al., 2016). Methionine and choline deficient (MCD) diet is known to induce steatohepatitis along with fibrotic manifestations in WT mice that is comparable to NASH in humans (X. Li et al., 2020). However, this effect was more pronounced in *PPARα* knockout mice

models. Conversely, administering *PPARα* agonist prevented steatohepatitis and fibrosis in WT rodent models and ameliorated existing steatohepatitis (Z. Luo et al., 2014). Thus, these findings indicate that the circadian rhythm-regulated PPARα significantly contributes to the development of NAFLD and its progression of NASH.

In addition to studies in knockout mice models, the impact of circadian disruption has been explored in mice models through the induction of chronic jet lag . This was achieved by advancing the light phase early in the week, followed by a delay in the light phase mid-week. Under chronic jetlag conditions, WT mice developed insulin resistance, obesity, high body fat composition, hepatomegaly with increased hepatic TGs and FFAs, chronic hepatic inflammation and fibrosis (Zheng et al., 2023). Previous studies in our lab reported that male C57BL/6J male mice subjected to a high fat high fructose (HFHF) diet alone or in combination with photoperiodic shifts induced chronodisruption (CD) regime developed hepatic fatty manifestations and were marked by significantly altered hepatic circadian clock gene expression (Joshi et al., 2021).

Table 1. Metabolic phenotypes in clock genes mutant animal models

Animal model	Metabolic Phenotype	References
CLOCK		
Clock$^{-/-}$ mice	Decreased TG accumulation in liver with HFD.	(Pérez-Mendoza et al., 2018)
Clock Δ19/ Δ19 double mutant mice	Hepatic steatosis, obesity, hypertriglyceridemia. Aberrant expression of metabolic genes such as FABP1, HMGCR, LDLr, ACS4.	(Sookoian et al., 2007)
Clock mutant mice	Significantly higher hepatic TG with HFD.	(Pan, Queiroz, et al., 2020b)
BMAL1		
Bmal$^{-/-}$ mice	Hepatic steatosis on a regular chow diet. Decreased fat storage, increased circulating FAs and ectopic fat accumulation in liver and muscle.	(McDearmon et al., 2006)
Liver-specific Bmal1$^{-/-}$ mice	Elevated circulating FFAs and TGs, dyslipidemia.	(Gréchez-Cassiau et al., 2008)
Heart-specific Bmal1$^{-/-}$	Systemic insulin resistance, decreased insulin-induced phosphorylation of AKT in liver.	(Nakahata et al., 2018)
REV-ERBs		
Liver-specific HDAC3$^{-/-}$ mice	Exacerbated hepatic steatosis, increased *de novo* lipogenesis.	(Dizaji, 2018)
Rev-erbα$^{-/-}$ /HDAC$^{-/-}$ mice	Hepatic steatosis	(Dizaji, 2018)
Rev-erbα$^{-/-}$ mice	Increased VLDL and hepatic Apoc-III expression	(Dizaji, 2018)

Liver Rev-erbα$^{-/-}$ and Rev-erbβ deficient mice.	Hepatic steatosis	(Z. Yang et al., 2016)
NPAS2$^{-/-}$ deficiency in SHP$^{-/-}$ mice	Lipoprotein metabolism derangement, hepatic steatosis	(Schleicher et al., 2015)
Myeloid-specific RORα$^{-/-}$ mice	Increased susceptibility of liver to HFD-induced hepatic steatosis.	(Y.-H. Han et al., 2014)
Cry1/Cry2 double knockout mice	Abnormal serum and hepatic TG levels	(Chaudhari et al., 2017)

MicroRNAs

MicroRNAs (miRNAs) were first discovered in 1993 by research groups of Ambros and Ruvkun in *C. elegans* (Ambros, 2008; Pasquinelli & Ruvkun, 2002). miRNAs are a class of small non-coding RNA molecules, with an average length of 22 nucleotides (C. Lee et al., 2006). DNA sequences are transcribed into primary miRNA (pri-miRNA) transcripts that are subsequently processed to precursor miRNAs (pre-miRNAs) and thereby mature miRNAs (Ha & Kim, 2014). miRNAs interact with the 3'UTR of their target mRNAs, thereby suppressing their expression (Vishnoi & Rani, 2017). However, miRNA interactions with other regions, such as the 5'UTR, coding sequence and gene promoters have also been reported (Treiber et al., 2012). Further, certain miRNAs can also activate gene expression under specific conditions (Huang, 2017). Studies have shown that miRNAs are shuttled between subcellular compartments to regulate the rate of translation as well as transcription (Pitchiaya et al., 2017). miRNAs play critical roles in regulating gene expression and are involved in all the biological processes (Carthew, 2006). An altered miRNA expression profile is associated with the onset and progression of various diseases (Macvanin et al., 2023). Furthermore, many miRNAs are secreted into extracellular fluids and these extracellular miRNAs

have been variedly reported as potential biomarkers for several diseases where they also serve as signalling molecules to facilitate cell-cell communications (Karolina et al., 2012).

Biogenesis of miRNAs

Biogenesis of miRNAs begins with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally (Y. Lee et al., 2004). Nearly half of all the known miRNAs are intragenic (processed from introns) whereas the remaining are intergenic, transcribed independently of a host gene and regulated by their own promoters (García-López et al., 2013). At times, miRNAs can also be transcribed as a single long transcript, called "cluster" that may comprise of similar seed regions and are called "family". miRNA biogenesis pathways can be classified into canonical and non-canonical pathways (Marco et al., 2013).

Canonical pathway of miRNA biogenesis

Canonical biogenesis pathway is the predominant pathway by which miRNAs are synthesized (Chong et al., 2010). Herein, genes are transcribed into pri-miRNAs that are subsequently converted into pre-miRNAs by microprocessor complex that comprises of an RNA binding protein, Di George Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha. DGCR8 recognizes an N6-methyladenylated GGAC and other motifs within the pri-miRNA transcript whereas, Drosha cleaves the pri-miRNA duplex at the base of the characteristic hairpin structure of pri-miRNA, thereby resulting in the formation of 2 nt 3' overhang on pre-miRNA. Subsequently, pre-miRNAs are exported to cytoplasm with the help of exportin 5(XPO5)/RanGTP complex and then processed by RNase III endonuclease, Dicer, that removes the terminal loop, resulting in a mature miRNA duplex. The direction of the miRNA strand determines the name of the mature miRNA form: 5p strand arises from the 5' end of the pre-miRNA hairpin while the 3p strand originates from the 3' end. Further, both of these strands can be loaded into the Argonaute (AGO) family of proteins in an ATP-dependent manner. Usually, the strand

with lesser stability at the 5' end or 5'uracil is loaded into AGO and is called the "guide" strand. The unloaded strand (passenger) strand, is unwound from the guide strand based on the degree of complementarity. The passenger strands of miRNA with no mismatches are cleaved by AGO2 and degraded by the cellular machinery. However, miRNA duplexes with central mismatches or non-AGO2 loaded miRNAs are passively unwound and degraded (Chong et al., 2010)[Fig. 8].

Non-canonical pathway of miRNA biogenesis

Various non-canonical pathways of miRNA biogenesis have been studied and these pathways employ differing combinations of the proteins involved in the canonical pathway, i.e. Dicer, Drosha, Exportin 5 and AGO2. These non-cannonical pathways can be further categorized into Drosha/DGCR8-independent (Havens et al., 2012)and Dicer-independent pathways (Cheloufi et al., 2010). Pre-miRNAs that are synthesized by Drosha/DGCR8-independent pathway resemble Dicer substrates (J.-S. Yang & Lai, 2011)while Dicer-independent miRNAs are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts . Since these pre-miRNA transcripts are of insufficient length to be Dicer substrates, they require AGO2 to mature within the cytoplasm. This in turn facilitates loading of the entire pre-miRNA into AGO2 and AGO2- dependent slicing of the 3p strand and the 3'-5' trimming of the 5p strand completes their maturation (J.-S. Yang et al., 2012)[Fig. 8].

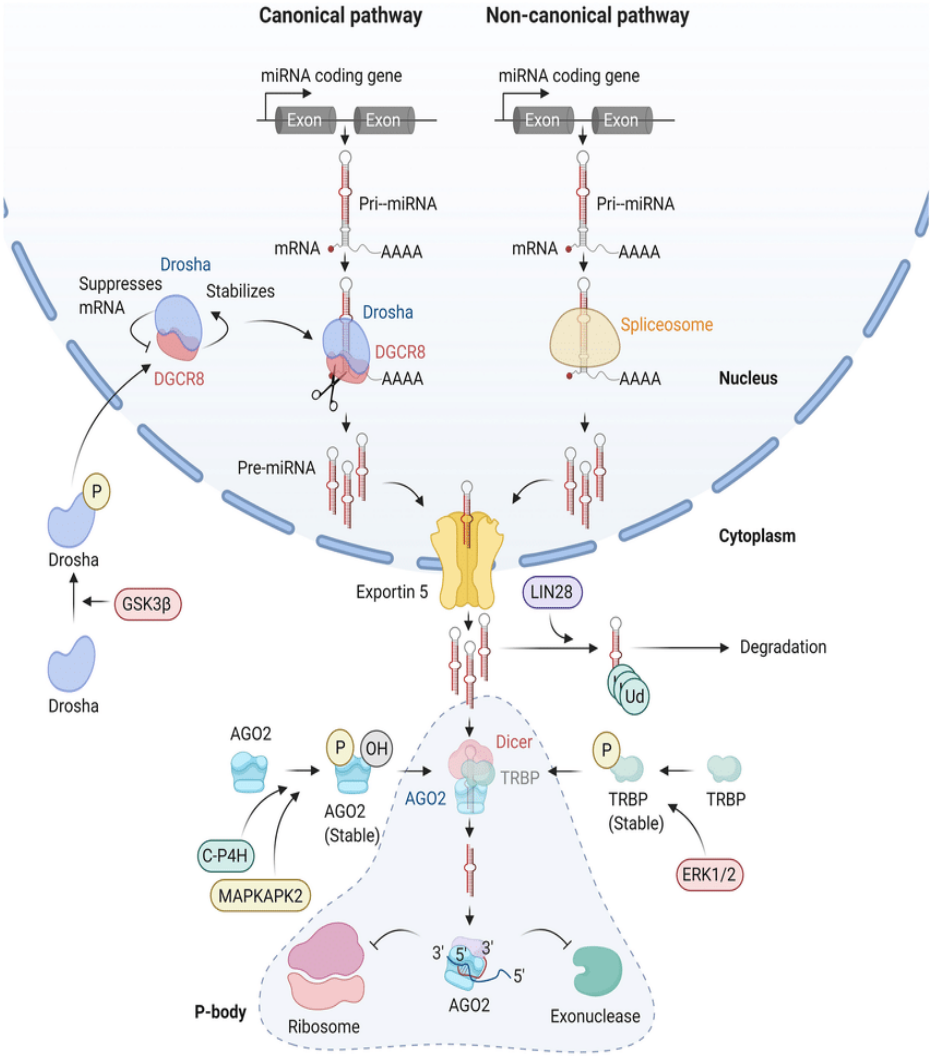


Figure 8. Cannonical and non-cannonical pathways of miRNA biogenesis.

MicroRNAs in NASH

The last few decades have witnessed a rising interest in understanding the role of small non-coding RNA molecules such as microRNAs (miRNAs) in the pathophysiology of various diseases (Price et al., 2014). A vast network of miRNAs is known to maintain hepatic homeostasis and studies have established that an altered miRNA profile is a hallmark of liver disorders such as NAFLD and NASH (X.-M. Chen, 2009). Since miRNAs tightly regulate cell survival, proliferation (Baffy, 2015) as well as the complex process of inflammation(Roy et al., 2015), understanding miRNA circuitry and their

aberrations in NASH shall promote better understanding of the disease pathology.

A study had reported that in the livers of NASH patients, 23 miRNAs were found to be under- or over-expressed as compared to healthy livers. Predicted targets of these miRNAs were genes involved in cell proliferation, apoptosis, inflammation, oxidative stress as well as metabolism. Such differential miRNA expression patterns were reported not just in the liver, but also in the visceral adipose tissue (VAT) of NAFLD patients (Cheung et al., 2008). Corroborating with the patient studies, another study had reported a differential hepatic miRNA expression in ob/ob mice wherein; 8 miRNAs (miR-34a, miR-103, miR-107, miR-194, miR-335-5p, miR-221 and miR-200a) were significantly upregulated and 3 miRNAs (miR-29c, miR-451 and miR-21) were downregulated (S. Li et al., 2009). Further, 44 upregulated and 12 downregulated miRNAs were identified in the liver tissues of rats subjected to high fat diet (HFD). Among these miRNAs, 6 miRNAs (miR-200a, miR-200b, miR-200c, miR-146a, miR-146b and miR-152) were reportedly upregulated *in vitro* in hepatocytes subjected to FFA treatment for 24 hours [Fig. 9] (Nie et al., 2018). Thus, the composition of the HFD, including its cholesterol content, influences the miRNA profile.

The methionine-choline-deficient (MCD) diet-induced NASH accounts for early hepatic inflammation, steatohepatitis and fibrosis but lacks the typical obesity and peripheral insulin resistance seen in other models (Deng et al., 2020). Studies have reported a distinct miRNA profile in the livers of MCD-diet fed mice wherein; 3% of miRNAs were upregulated and 1% were downregulated. Interestingly, five of these miRNAs (miR-182, miR-183, miR-199a-3p, miR-705 and miR-1224) were altered in both alcoholic and nonalcoholic steatohepatitis (Szabo & Csak, 2016). Another study had identified 71 miRNAs that were upregulated and 60 miRNAs that were downregulated in the MCD mouse model (Katsura et al., 2015). The choline-deficient amino acid-defined (CDAA) model that induces steatohepatitis and hepatocellular carcinoma (HCC), is also known to alter hepatic miRNA

expression. 30 miRNAs were reported to be differentially expressed at various stages of NASH and NASH-related HCC development (B. Wang et al., 2009).

In liver, miR-122 is abundantly expressed in hepatocytes, accounting for ~70% of total miRNAs and plays a key role in regulating lipid metabolism, cell cycle and HCV replication (J. Hu et al., 2012). MiR-122 regulates a plethora of genes involved in fatty acid biosynthesis (Wen & Friedman, 2012)and studies have shown that administering a miR-122 antagonist in mice led to reduced plasma cholesterol levels, decreased synthesis of hepatic fatty acids and cholesterol whereas, an increment in the hepatic fatty acid oxidation was observed(J. Hu et al., 2012).

Hepatic lipid accumulation and inflammation usually occur together and miR-155 is a key regulator of inflammation (Bala et al., 2016). Mice lacking miR-155 exhibited reduced steatosis but recorded no improvements in liver damage, following MCD diet-induced steatohepatitis . Further, decrement in liver TGs and steatosis in miR-155-deficient mice was attributed to the decreased expression of lipogenic genes such as *Adrp, Dgat2, Cpt1α, Fabp2, Ldtr, Hmgcr* and *Pparα* (X. Chen et al., 2024). Further, miR-21 has been widely studied in NASH (Benhamouche-Trouillet & Postic, 2016; Loyer et al., 2016; P. M. Rodrigues et al., 2018)and studies have reported have miR-21 deficiency reduces the expression lipogenic genes and genes involved in cell cycle progression via the p53 pathway (Wu et al., 2016). Additionally, miR-21 can restore PPARα expression in NASH, resulting in improved liver function (P. M. Rodrigues et al., 2018).

MiR-34a has been investigated as a key player in NASH pathology (Y. Xu et al., 2021). One of its primary targets I sirtuin 1 (SIRT1), a NAD-dependent deacetylase that regulates hepatic steatosis and apoptosis (Castro et al., 2013; L. Wang et al., 2020). In NASH patients, elevated levels of miR-34a were recorded in both liver tissue and serum and these levels were associated with the severity of the disease (X.-L. Liu et al., 2016).

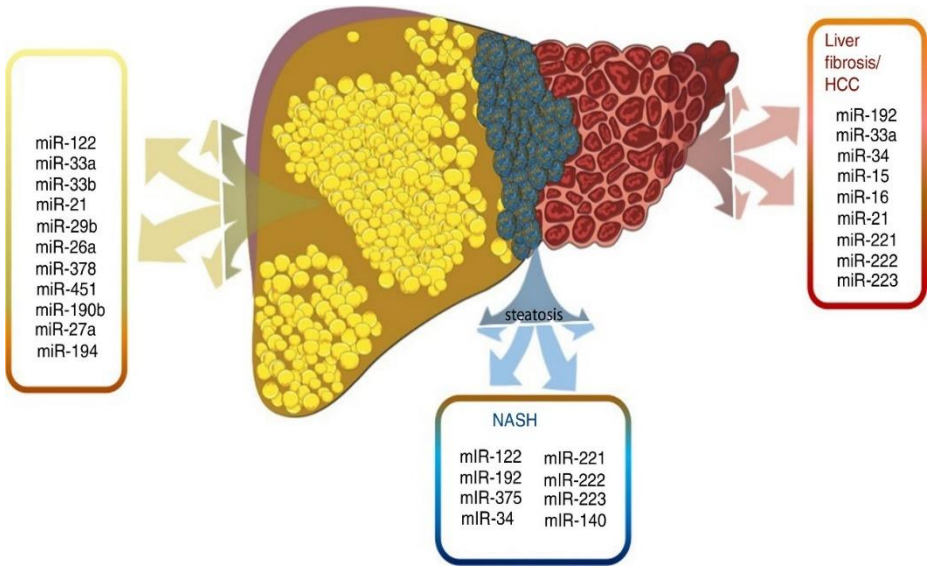


Figure 9. miRNAs implicated in NAFLD/NASH pathophysiology (López-Sánchez et al., 2021)

Nocturnin

Biological timekeeping systems regulate cellular processes via their output genes (Trott & Menet, 2018). One of the less-studied genes is Nocturnin (Noct), also known as Ccrn4l (Kulshrestha & Devkar, 2023), that belongs to the Exonuclease, Endonuclease and Phosphatase (EEP) protein family (Laothamatas et al., 2020). Noct was first discovered in *Xenopus* retinal cells wherein its rhythmic mRNA expression was identified using a differential display method (Green & Besharse, 1996). Over time, research has shown that Noct is well-conserved in yeast, plants and mammals (Hughes et al., 2018a). NOCT contains a conserved catalytic domain that shares sequential similarity with CCR4 family proteins, however, its N-terminus has only a partial structure, making NOCT functionally distinct from CCR4 family members (Abshire et al., 2018a). Due to its high sensitivity to alterations in circadian rhythms and external stimuli such as serum, mitogens, or phorbol ester 12-O-

tetracanoylphorbol-13-acetate (TPA), Noct has been classified as an Immediate Early Gene (IEG) (Garbarino-Pico et al., 2007).

Additionally, studies have highlighted a crucial role of NOCT in embryonic development in both murine models (Nishikawa et al., 2013)and *Xenopus* (Curran et al., 2008) . Silencing *Noct* mRNA resulted in a transient slowdown in early embryonic stages whereas its overexpression led to developmental arrest (Hughes et al., 2018). In the later stages, it was shown that *Noct* transcription was vital for maintaining stem cell pluripotency by inhibiting differentiation into mesoderm and endoderm lineages (Kulshrestha & Devkar, 2023). In mice, Noct is expressed in various brain regions such as the pineal gland, arcuate nucleus, hippocampus and piriform cortex (Y. Wang et al., 2001) and rhythmic patterns of *Noct* mRNA have been observed in organisms such as sea sponges (Müller et al., 2012), zebrafish (X. Yang, Fu, et al., 2017), mice (Barbot et al., 2002; Dupressoir et al., 1999; Menet et al., 2012)and humans (Perrin et al., 2018) . Research has also revealed that Noct is ubiquitously expressed in tissues such as liver, adipose tissue, pancreas, ovaries, thymus, kidneys and muscles (Y. Wang et al., 2001).

Intracellular localization of Nocturnin

Early investigations implied towards a cytoplasmic localization of NOCT in *Xenopus* photoreceptor cells (Baggs & Green, 2006). Endogenous NOCT was found to be localized in both the cytoplasmic and nuclear fractions in mouse embryos (Baggs & Green, 2003a). However, nuclear localization of NOCT orthologs was reported in *Drosophila*, *X. laevis* and *D. rerio* (Hughes et al., 2018c). Further, computational studies predicted a mitochondrial localization of NOCT in *X. tropicalis, R. norvagicus, H. sapiens, M. musculus,* and *D. rerio* (Hughes et al., 2018). Recent studies in mice had suggested a circadian regulation of the intracellular localization (cytoplasmic and mitochondrial) of Noct wherein; expression of NOCT in mitochondria was robustly rhythmic with significantly high amplitude that peaked at ZT12 (Laothamatas et al.,

2020). However, NOCT was constitutively expressed in the cytoplasm throughout the 24 h period (Laothamatas et al., 2020). Despite these findings, factors regulating circadian rhythmicity of cytosolic or mitochondrial NOCT lack clarity and warrant further investigation. Furthermore, studies had reported that cytoplasmic form of NOCT associates externally with membranes of other organelles. On the other hand, mitochondrial import of NOCT occurred due to the presence of mitochondrial target sequence (MTS) (Laothamatas et al., 2020). These findings corroborated with previous studies on murine models and *Drosophila melanogaster* (Estrella et al., 2019a) that reported NOCT localization in mitochondria and the same was attributed to the presence of MTS in NOCT. However, a later study reported that NOCT expression varies depending on the cell and tissue type and revealed that the unprocessed NOCT protein (55kDa) was exclusively found in the cytoplasm of human brain, kidney and liver tissues. In contrast, muscle tissue contained both cytoplasmic and mitochondrial isoforms of NOCT. These observations suggest the presence of an unknown mechanism that may inhibit the transport of NOCT from the cytoplasm into the mitochondria (Abshire et al., 2020).

Enzyme activity of Nocturnin

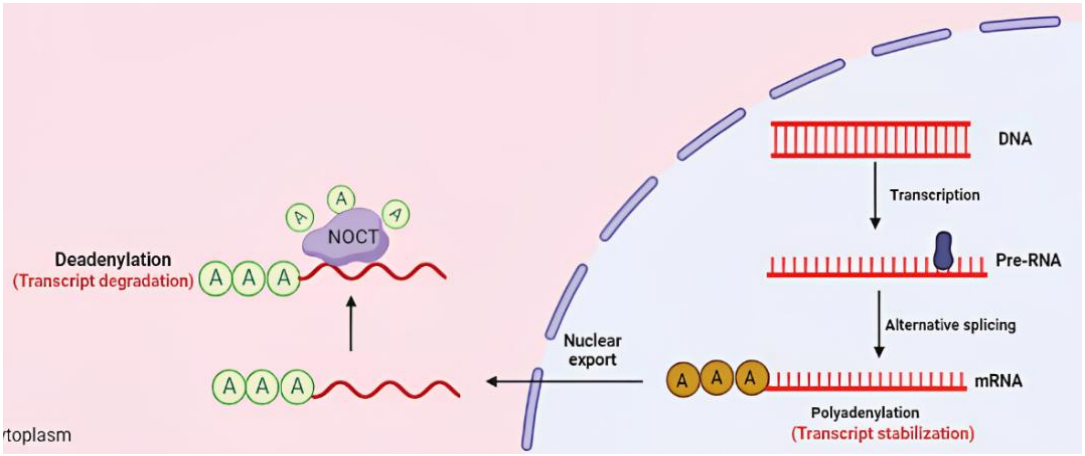
Deadenylases are magnesium-dependent enzymes that play a key role in regulating various processes essential for maintaining cellular homeostasis (Morita et al., 2019). To date, 11 deadenylases have been identified in mammals, classified into two superfamilies: (i) the DEDD (Asp-Glu-Asp-Asp) superfamily, which includes the POP2, CAF1Z, PARN, and PAN2 families, and (ii) the EEP superfamily, that comprises of CCR4, NOCT, Angel, and 2PDE families (Goldstrohm & Wickens, 2008). Deadenylase activity of NOCT has been confirmed through various experiments, demonstrating its involvement in potentially regulating insulin sensitivity (Green et al., 2007a), nitric oxide signalling (Niu et al., 2011), and lipid metabolism (Stubblefield et al., 2018). Unlike other deadenylases, NOCT exhibits a rhythmic oscillatory

pattern controlled by the circadian clock, which results in tissue-level oscillations that regulate hepatic anabolic processes (Green et al., 2007b; Kojima et al., 2010).

A "Poly(A)denylome" analysis of wild-type (WT) and *Noct* knockout (KO) mice livers revealed that in KO livers, 213 transcripts had extended poly(A) tails, suggesting that these transcripts could be probable direct targets of the enzymatic activity of NOCT. Further, these transcripts were enriched in ribosome and oxidative phosphorylation pathways (Kojima et al., 2015). Another study with Noct KO revealed that hepatic metabolic genes such as *Acly, Fdps,* and *Gpam* may be the direct targets of the deadenylase activity of NOCT since distributions of their poly(A) tail lengths shifted to longer tails in *Noct* KO livers (Stubblefield et al., 2018). Although there is literature available, the direct target genes of NOCT largely remain unknown, presenting a significant scientific gap that requires attention [Fig. 10 (a)].

Reports had shown that crude preparations of NOCT deadenylated mRNA (Baggs & Green, 2003b), whereas its purified form did not demonstrate comparable outcomes (Abshire et al., 2018b; Estrella et al., 2018). In *Drosophila*, *curled* (*Cu*, a homolog of NOCT) demonstrated phosphatase activity that accounted for NOCT-mediated conversion of NADP+ into NAD+ and NADPH to NADH (Estrella et al., 2019b). This study suggested that NOCT identified distinct ribose-phosphate backbone of NOCT-NADPH complex, thus providing evidence of NADP(H) regulation by NOCT. Furthermore, it has been reported that NOCT utilizes NADPH as a substrate and subsequently generates NADH in mice, thus, regulating oxidative stress response through its NADPH phosphatase activity (Laothamatas et al., 2020)[Fig. 10 (b)].

(a)



(b)

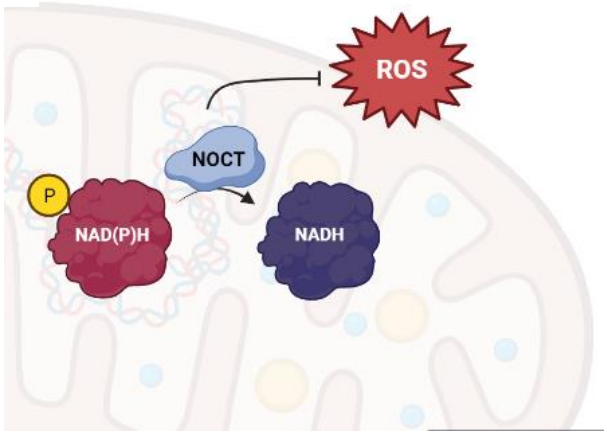


Figure 10. Enzymatic activity of Nocturnin: (a) Nocturnin as a deadenylase and (b) Nocturnin as a NADPH phosphatase (Kulshrestha & Devkar, 2023).

Nocturnin, a circadian regulator of hepatic lipid metabolism

Hepatic *Noct* expression in healthy mice peaks between ZT12-ZT18 and this peak corresponds with decreased expression of metabolic genes involved in acetyl CoA, cholesterol and TG synthesis pathways. HFD feeding to mice for 3 weeks accounted for increased expression and amplitude of hepatic *Noct* mRNA, peaking at ZT12 as compared to control mice. These results suggest that chronic HFD consumption can disrupt Noct rhythmicity in the liver, highlighting the significance of NOCT in energy homeostasis under nutritional challenges. Additionally, during fasting, *Noct* expression decreases, paralleling reduced expression of metabolic genes (*Acly, Fdps* and *Gpam*), while re-feeding leads to increased *Noct* expression alongside upregulation of metabolic genes (Stubblefield et al., 2018).

Noct KO mice demonstrate increased expression of metabolic genes (*Acly, Acss2, Hmgcs1, Pmvk, Mvd, Sqle, Lss, Dhcr7, Acaca, Fasn, Pnpla3, Fdps* and *Gpam*) at ZT18 compared to WT mice, underscoring the regulatory role of NOCT in hepatic metabolism. Furthermore, Noct KO mice fed with HFD do not exhibit anabolic changes such as body weight gain and respiratory exchange ratio observed in WT mice, attributed to the observed upregulation of genes governing hepatic metabolism in the absence of NOCT. These findings provide mechanistic insights into a previous study indicating that Noct KO mice are resistant to high-fat diet-induced hepatic steatosis (Green et al., 2007b).

Role of Nocturnin in health and disease

Early evidence suggests a potential role of Noct in the pathophysiology of cancer in humans. Computational studies indicated that the expression of several deadenylases was altered in squamous cell lung carcinoma (SCC) wherein; a differential expression of 4 deadenylases (PARN, CNOT6, CNOT7 and NOCT) was reported. Elevated expressions of PARN and NOCT in lung cancer cells accounted for deregulated genes involved in cell adhesion, cell

junctions, muscle contraction and metabolism. These findings underscore the importance of these enzymes in cancer-related phenotypes and their role in regulating gene expression in SCC (Couto et al., 2014). Additionally, another study linked the intronic *Noct* SNP rs3805213 with non-small cell lung cancer. These preliminary findings highlight the potential role of Noct in cancer biology (Maragozidis et al., 2015).

Late-onset Alzheimer's disease (LOAD) is characterized by a gradual decline in episodic memory, often accompanied by impairment in other cognitive domains. Although LOAD has a strong genetic component, only 22 genes or loci have been identified that can help assess the risk of developing the disease. Genome-wide association studies have identified four new loci related to Alzheimer's disease: *PAX3, CCRN4L, PIGQ* and *ADAM19* (X. Wang et al., 2014).

Shift-workers are at an increased risk of developing metabolic disorders such as diabetes, NAFLD and obesity (M. Sun et al., 2018). A study in shift workers reported diurnal variation in *NOCT* expression with a peak at 8:00 AM. *NOCT* expression in peripheral blood lymphocytes was higher in shift workers compared to daytime workers. Multivariate analysis confirmed that shift work independently influenced *NOCT* expression, that was associated with alterations in body mass index. Additionally, *NOCT* expression was inversely correlated with total energy expenditure in shift workers (Bracci et al., 2019).

Cisplatin-induced drug-induced liver injury (DILI) is one of the major causes of acute liver damage and can be life-threatening (Hwang et al., 2020). Since Noct plays a crucial role in liver function, alterations in its expression may have serious implications in DILI. It was reported that individual variations in hepatic *Noct* expression could be a novel factor influencing susceptibility to cisplatin-induced hepatotoxicity, potentially by regulating mitochondrial antioxidant functions in rats. Noct knockdown in WB-F344 (rat liver progenitor) cells exacerbated cisplatin-induced mitochondrial dysfunction,

with alterations in *Catalase* and apoptosis-related *Bax* expression. These initial findings implied towards a potential role for Noct in DILI (Hwang et al., 2020).

Other studies have taken a closer look at how Noct plays a role in a disease pathophysiology. Elevated levels of *Noct*, *Cyp2d26* and sphingolipid metabolism gene (*Esyt1*) were recorded in mice subjected to dextran sodium sulfate (DSS)-induced ulcerative colitis (UC), thus highlighting the involvement of circadian clock-relates genes, particularly *Noct*, in UC. However, the underlying mechanisms require further investigation (Thomas et al., 2019).

Melatonin, a circadian neurohormone

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine, mainly produced and secreted by the pineal gland during the dark phase of the day (Malpaux et al., 2001). Circadian rhythm of melatonin is very robust, and melatonin rhythm is not only a reflection of the day-night cycle but is also internal to the organism resulting from cyclical signals, possibly from the SCN (Cipolla-Neto & Amaral, 2018). Melatonin exerts receptor-mediated (Witt-Enderby et al., 2003)and non-receptor-mediated actions, with the target sites of melatonin being both central and peripheral (R. K. Xu et al., 2000). Further, melatonin is a lipophilic hormone that functions via high-affinity G-protein-coupled membrane receptors (MT_1 and MT_2) expressed in different regions of the brain such as the SCN, cerebellum, cortex, hippocampus and hypothalamus (Lacoste et al., 2015), as well as other peripheral tissues such as retina, adipose tissue and kidneys (Brydon et al., 2001). The high fat solubility of melatonin allows it to freely cross the cell and nuclear membranes (Molska et al., 2020). Furthermore, melatonin also functions as a ligand for the retinoid-related orphan receptor (ROR) (H. Ma et al., 2021)and retinoid Z receptor (RZR) family of orphan nuclear receptors.

Melatonin binds to cytosolic calmodulin thereby directly modulating calcium signalling and protecting macromolecules and other cellular structures from oxidative damage (Benítez-King et al., 2024).

In pineal gland, melatonin synthesis and release are triggered during darkness and inhibited by light. In darkness, the SCN sends electrical signals to the pineal gland through a complex autonomic neural pathway, subsequently leading to the release of norepinephrine from post-ganglionic neurons onto pinealocytes and activating melatonin production via adrenergic receptors (Zhao et al., 2019). Apart from the pineal gland, melatonin is also produced in other tissues such as retina (Tosini & Menaker, 1998), thymus (Naranjo et al., 2007), bone marrow (Conti et al., 2000), respiratory epithelium (Habtemariam et al., 2017), skin (Slominski et al., 2008), lens (Alkozi et al., 2017), intestine (Huether, 1994), reproductive organs (Reiter et al., 2014)and lymphocytes (Carrillo-Vico et al., 2004)where it may regulate various physiological functions through paracrine signalling. The primary pathway for melatonin synthesis involves hydroxylation, decarboxylation, N-acetylation, and O-methylation of tryptophan. In pineal gland, arylalkylamine N-acetyltransferase (aaNAT) is regulated by the SCN. The SCN initiates and sustains melatonin production by promoting the nocturnal release of norepinephrine onto pinealocytes (Tan et al., 2016)[Fig. 11].

The primary physiological function of melatonin is to impart information concerned with the daily cycle of light and darkness to various tissues of the body (Barrenetxe et al., 2004). Further, the circadian organization of physiological functions such as immune responses (Szczepanik, 2007), antioxidant defence (Reiter et al., 2016), glucose regulation (Garaulet et al., 2020)and energy homeostasis (Owino et al., 2019)also depend on melatonin signals. Melatonin likely enhances the overall rhythmic organization via its chronobiotic properties (Pevet et al., 2002). It is possible that melatonin can regulate the circadian time of the SCN (Pevet & Challet, 2011). Further, the

pancreatic β cells and metabolically most relevant tissues such as the liver, muscle and adipose tissue are the targets of melatonin (Farid et al., 2022).

As a potent chronobiotic, melatonin impacts the circadian timing of metabolic processes, aligning them with the activity-feeding/rest-fasting cycle (Wajid et al., 2020).

Circulating melatonin is absorbed by hepatocytes, where it is metabolized by P450 mono-oxygenase enzymes (CYP1A2, CYP1A1 and CYP1B1). It is subsequently conjugated to form 6-sulphatoxymelatonin (primary urinary metabolite), whose levels closely reflect the concentration of melatonin in the bloodstream. Melatonin is also released into the cerebrospinal fluid (CSF) where it is metabolized into N-acetyl-N-formyl-5-methoxykynuramine (AFMK) by neurons and glial cells. AFMK is further converted into N-acetyl-5-methoxykynuramine (AMK). Both AFMK and AMK are powerful free radical scavengers, with AMK being particularly effective, and these metabolites form additional compounds through interactions with reactive oxygen and nitrogen species. The free radical scavenging activity of melatonin and its metabolites is consistent across all cells in all organisms (X. Ma et al., 2005; Semak et al., 2008).

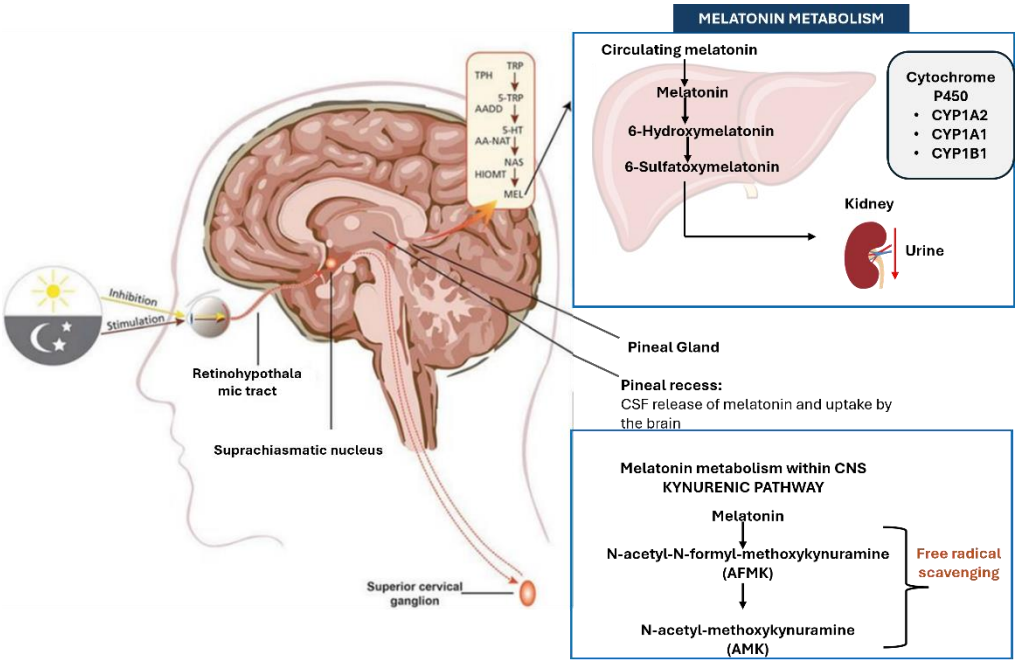


Figure 11. Synthesis and metabolism of melatonin.

Melatonin holds significance not only as a chronobiotic but also for its role as an antioxidant and several direct and indirect antioxidant actions of melatonin have been reported. Melatonin functions as an antioxidant by directly scavenging free radicals, stimulating antioxidant enzymes, increasing the efficacy of mitochondrial oxidative phosphorylation, reducing electron leakage and increasing the efficacy of other antioxidants (Zarezadeh et al., 2022). Rats subjected to subcutaneous carbon tetrachloride for 6 weeks reported hepatic fibrotic changes, increases hydroxyproline and malondialdehyde (MDA) levels and decreased glutathione peroxidase and superoxide dismutase levels whereas melatonin reversed these results. Further, melatonin inhibited the expression of NF-κB in liver tissue and decreased the production of proinflammatory cytokines such as TNF-α and IL-1β in Kupffer cells of fibrotic rats (Ohta et al., 2000, 2004; H. Wang et al., 2005). Additional physiological effects of melatonin pertain to its participation in sexual maturation and reproductive

processes (Reiter, 1998), as well as in aging mechanism, potentially exerting an oncostatic effect and bolstering immune response (Poeggeler, 2005).

Therapeutic potential of melatonin in NAFLD and NASH

Several studies support the hepatoprotective effects of melatonin in NAFLD and NASH [Fig. 12]. A study had reported that administering 5mg/kg/day melatonin in NAFLD rats had hypoglycemic and hypolipidemic effects, with a decrease in liver enzymes and MDA as well as an increase in reduced glutathione that can be attributed to the antioxidant properties of melatonin (Miguel et al., 2022). In mice subjected to HFD-induced NASH, melatonin treatment (20mg/kg body weight) for 2 weeks led to reductions in steatosis, inflammation and ballooning hepatocytes when compared with NASH group. Further, melatonin treatment attributed to a decreased lipoperoxidation (MDA), increased activity of superoxide dismutase, glutathione peroxidase and reduced catalase activity in the liver (Martínez Soriano et al., 2020). Damage index (that serves as a biomarker for DNA damage) exhibited a significantly lower level in the NASH group receiving melatonin treatment compared to the NASH group, both in blood samples and liver tissues. Melatonin directly attenuated lipid storage in primary hepatocytes and hepatic inflammation by suppressing activation of macrophages along with a reduction in liver fibrosis in a NASH mouse model (Martínez Soriano et al., 2020). Furthermore, melatonin probably decreases inflammation via the MAPK-JNK/p38 signalling pathway (H. Sun et al., 2016). Moreover, melatonin treatment significantly lowered the mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) as compared to HFD-induced NASH mice model (H. Sun et al., 2016).

Another potential mechanism by which melatonin intervenes in the pathogenesis of NAFLD/NASH is by inhibiting the expression of nuclear orphan receptor subfamily 4 group A member 1 (NR4A1) in hepatocytes, that in turn attenuates hepatic lipogenesis and fibrosis, blocks mitochondrial

fission, restores levels of antioxidant factors, alleviates mitochondrial lipid oxidation as well as represses p53 activation. Further, melatonin protected mitochondrial respiratory function via improvements in mitophagy. Therefore, through the inhibition of the NR4A1/DNA-PKcs/p53 pathway, melatonin enhances mitochondrial function and largely mitigates the progression of HFD-induced NAFLD (Zhou et al., 2018).

In db/db mice, melatonin treatment for 8 weeks significantly lowered metabolic parameters in blood, attenuated hepatic steatosis, ballooning hepatocytes, lobular inflammation, while decreasing oxidative stress and improving mitochondrial function by upregulation in the mitochondrial membrane potential (Saha et al., 2022) . These beneficial effects of melatonin relate to the significantly reduced expression of NLRP3 inflammasome-related mRNA and proteins (*caspase-1, IL-1β and IL-18*) in the liver tissues of diabetic mice (Saha et al., 2022). These results imply towards the protective role of melatonin against diabetes-associated NAFLD by inhibiting the activation of NLRP3 inflammasome. According to another study, melatonin (20mg/kg) improved inflammation and other pathophysiological alterations in HFD-induced murine model of NASH via inactivation of NLRP3 inflammasome via the suppression of TLR4/NF-κB pathway and modulation of ATP-dependent P2X7 receptor (Ramos-Tovar & Muriel, 2023). Melatonin restored impaired autophagy in high fat diet/chronic intermittent hypoxia-induced liver injury in mice by activating SIRT1 signalling (X. Li, 2013). In obese mice, oral administration of melatonin (10mg/kg) was reported to directly decrease the hepatic miR-34a-5p expression, resulting in the upregulation of its target, SIRT1. Melatonin attenuated steatosis and lipid peroxidation in the liver, reduced endoplasmic reticulum stress and lipogenesis, while recovering autophagic flux (Stacchiotti et al., 2019).

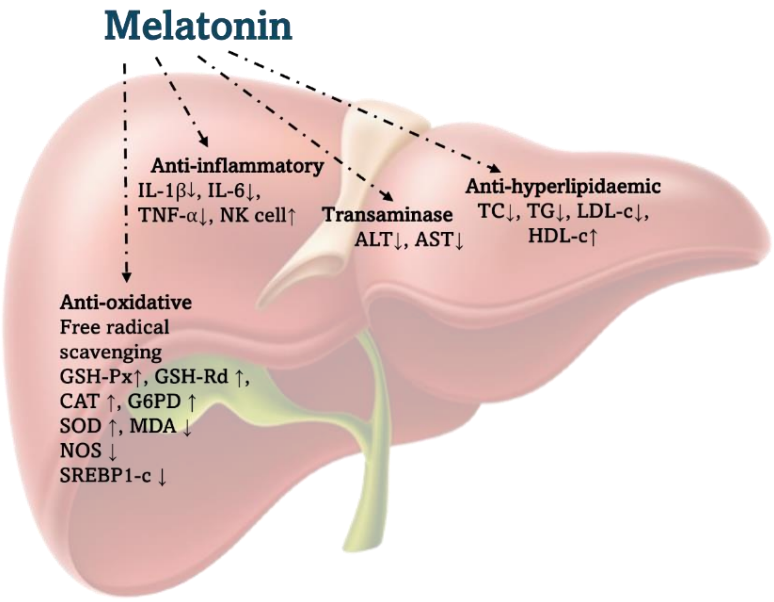


Figure 12. Hepatoprotective effects of melatonin in NAFLD/NASH (J.-J. Zhang et al., 2017).

Rationale of the study:

Alterations in biological clocks due to chronic shift work and/or consumption of high calorie diets forms the basis for the onset and progression of lifestyle disorders such as NAFLD/NASH. Nocturnin (Noct) is a circadian deadenylase whose responsiveness to the time of day and nutritional challenges highlights its potential role in maintaining energy homeostasis. Interestingly, Noct KO mice were reported to be resistant against diet-induced obesity and hepatic steatosis, further providing clues about its significance in regulating hepatic lipid metabolism. Hence, alterations in Noct expression could culminate in the development of lifestyle disorders such as NASH but studies in this regard are lacking. Therefore, it becomes pertinent to understand the role of altered Noct expression and rhythmicity in the pathophysiology of NASH. This study delves into scrutinizing hepatic Noct oscillations in photoperiodic shifts induced chronodisruption and/or high-fat-high-fructose diet-induced NASH model. This model truly mimics scenario of a lifestyle disorder and is appropriate for investigating subtle alterations in circadian oscillations of clock genes and their potential impact on hepatic Noct rhythmicity. Further, various studies, including previous studies from our laboratory, had revealed hepatoprotective effects of exogenous melatonin in experimental models of NAFLD/NASH. Interestingly, studies have shown that similar to Noct, melatonin is rhythmically robust and has a comparable phasing. Yet, there is no existing literature that has investigated melatonin-mediated regulation of hepatic Noct oscillations. miRNAs are fine tuners of gene expression, and an altered hepatic miRNA expression profile is a key feature of NASH pathology. However, the epigenetic regulation of hepatic Noct, especially under conditions of NASH pathology is elusive. This study further investigates the putative endocrine (melatonin) and epigenetic (miR-122) regulation of hepatic Noct in NASH pathology.