Circadian and epigenetic control of Nocturnin in Nonalcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is a common liver disorder that represents the hepatic manifestation of the metabolic syndrome, a variably defined aggregate of disorders related to obesity, insulin resistance, hypertension and hyperlipidaemia. Epidemiological studies report the prevalence of NAFLD in 9% to 32% of the general Indian population with the global prevalence being 25%, making it a major health concern. Nonalcoholic steatohepatitis (NASH) is the progressive form of NAFLD which carries the risk of progressive fibrosis, cirrhosis, and end-stage liver disease. NASH etiology can be characterised by excessive triglycerides (TGs) and free fatty acid (FFA) accumulation in the liver which subsequently leads to inflammation, apoptosis, and ballooning degeneration.

One of the major factors contributing to the onset of NAFLD and its subsequent progression to NASH is circadian desynchrony. Studies provide a strong link between circadian rhythm disruption and the onset of a variety of human diseases. Desynchrony of circadian rhythms and the external environment, such as shift work, chronic jet lag, intentional sleep restriction, deprivation and night eating can markedly contribute to increased morbidity. Shift workers for example, exhibit a higher prevalence of obesity and associated disorders, such as NASH. A connection between the circadian rhythm and NASH has relatively recently been proposed. All the mechanisms involved in NAFLD pathogenesis and its evolution to NASH are under regulation of the molecular clockwork. These include endoplasmic reticulum (ER) stress, lipotoxicity, insulin resistance, mitochondrial dysfunction, oxidative stress, adipose tissue dysfunction, deranged control of innate immunity, and cytokine secretion as well as alterations in the gut–liver axis.

The biological timekeeping system tightly regulates physiological processes via the circadian clock gene output. One of the recently discovered and lesser explored circadian clock output genes is Nocturnin (Noct) or Ccrn4l, that belongs to the Exonuclease, Endonuclease and Phosphatase (EEP) family of proteins. Noct was first discovered in *Xenopus laevis* retinal cells wherein its rhythmic mRNA levels were reported. Over the years, studies have established

shown that Nocturnin functions as a circadian deadenylase, regulating gene expression by removing the poly(A) tails of its target mRNAs. Studies in mice had revealed a ubiquitous expression in liver, adipose tissue, pancreas, ovary, kidneys and muscles. Further, Noct was reported to be highly responsive towards any significant perturbation in circadian rhythm as evidenced in cells treated with serum, mitogens or lipopolysaccharide. Due to these credentials, Noct is also regarded as an immediate early gene (IEG). Multiple facets of Noct have been investigated till date that include adipogenesis, osteogenesis and lipid metabolism. Further, studies had reported that absence of Noct imparted resistance to hepatic steatosis and diet-induced obesity, Thus, Noct appears to be an important circadian clock associated player that could have significant implications in the initiation, progression, and pathogenesis of lifestyle disorders such as NAFLD and NASH.

Melatonin is a neurohormone secreted by the pineal gland during the dark phase of the day and functions as a key regulator of energy balance, carbohydrate and lipid metabolism. Melatonin not only regulates the alignment of the hepatic circadian cycles, but also imparts hepatoprotective effects in NAFLD and NASH that have been varied reported by several research groups. Previous studies in our laboratory had reported that exogenous melatonin improved H and/or CD induced NAFLD in mice via re-entrainment of the hepatic clock genes and corrections in the Nrf2-HO1 pathway genes. Interestingly, similar to Noct, melatonin is also robustly rhythmic and shares a similar phasing as Noct. MicroRNAs (miRNAs) are small non-coding RNA molecules that function as fine tuners of gene expression. In liver, a vast network of miRNAs function to maintain homeostasis. An altered miRNA expression profile (miRNome) is associated with NASH. However, the miRNA-mediated regulation of hepatic Noct, particularly under conditions of NASH, lacks clarity.

Despite the available literature, the direct target genes of Noct, especially in liver, remain unexplored. Further, systemic symptoms of lifestyle disorders and the extent of severity has been known to be amplified in conditions of circadian desynchrony. Additionally, though various aspects of Noct have been

investigated till date, regulators of Noct remain elusive and warrant detailed investigation. The present study was aimed at elucidating the merits and role of Noct in the pathophysiology of diet and/or chronodisruption induced NASH pathology. Further, the roles of melatonin and an epigenetic factor (miR-122) are scrutinized under the said pathology. This thesis is divided into three chapters wherein; the circadian, endocrine (melatonin) and epigenetic regulation of Noct is investigated in experimental models of NASH.

Chapter 1 was aimed at investigating the potential targets of Noct and subsequently deciphering the role of circadian desynchrony in modulating hepatic Noct oscillations in experimentally induced NASH pathology. To identify the targets of Noct, RNA Seq GEO dataset of WT and *Noct*$^{-/-}$ A549 cells was scrutinized by differential gene expression analysis (DGEA) by DESeq2. From a total of 17,428 genes, 71 genes were found to be differentially expressed. These differentially expressed genes (DEGs) were further scrutinized by gene ontology (GO) analysis wherein; physiological processes such as regulation of lipid metabolism and response to xenobiotic stimulus were significantly upregulated and processes such as angiogenesis were significantly downregulated, implying that Noct plays a crucial role in regulating metabolic processes. Protein-protein interaction analysis identified metabolic genes such as HNF4A and SCD as potential targets of Noct, further underscoring the metabolic role of Noct in liver. Further, male C57BL/6J mice were subjected to high-fat-high-fructose (H) diet alone or in combination with photoperiodic shifts induced chronodisruption (CD) regime for 16 weeks to induced NASH. H and/or CD mice were characterized by significant increment in serum liver function markers (ALT and AST) and circulating lipids and cholesterol. Additionally, histological evaluation of the liver tissues revealed significant hepatic lipid accumulation and hepatocyte ballooning. Hepatic *Noct* expression was assessed at ZT12 and our findings revealed a significant increment in *Noct* mRNA expression in H, CD and HCD groups as compared to Control. Additionally, hepatic *Noct* oscillations were assessed at 5 timepoints (ZT=0,6,12,18 and 24) wherein; a significant upregulation at ZT12 was recorded in H group.

Interestingly, CD and HCD groups recorded a shift in peak from ZT12 (in control) to ZT6. CircWave analysis revealed significant perturbations in hepatic *Noct* mRNA rhythmicity that was evident from significant increment in percentage (%) relative amplitude, shifts in peak time and cumulative *Noct* expression over 24 h.

These *in vivo* findings were put to scrutiny in HepG2 cells subjected to oleic acid (OA). The concentration of OA was standardized by MTT assay wherein; 0.5 mM concentration was chosen for all the further experiments. This concentration was in compliance with the previously published reports from our laboratory. HepG2 cells treated with 0.5 mM OA for 24 h recorded a significant upregulation in Noct mRNA and protein. Further, time-point based study revealed a significant increment in *NOCT* mRNA at 36 h. CircWave analysis revealed significant increments in relative amplitude, shifts in CoG (from 36 h in control to ~32 h) and cumulative *NOCT* expression. NOCT protein oscillations also displayed aberrant NOCT oscillations with a shift in peak (from 48 h in control to 36 h) and significant decrement in NOCT expression at 24 h, 32 h and 48 h in OA-treated cells. Additionally, a significant increment in the % relative amplitude was recorded in OA group. However, cumulative NOCT expression showed a non-significant decrement in OA group as compared to control. These findings revealed a previously unknown role of altered Noct rhythmicity in diet and/or CD induced NASH pathology.

Since CD had a profound impact on hepatic Noct oscillations, it was considered to pertinent to investigate the possible interactions of exogenous melatonin and Noct in the said pathology. **Chapter 2** was aimed at understanding the possible melatonin-Noct synergy in NASH pathology. The full-length protein structure of mouse-NOCT (mmu-NOCT) was generated using computational tools and validated by Ramachandran Plot analysis. Subsequently, molecular docking between mmu-NOCT and melatonin was performed that revealed a strong interaction between the two (high binding energy; -7kcal/mol and RMSD 0). Further, H and/or CD mice were subjected to exogenous melatonin (10mg/kg body weight) intraperitoneally from the beginning of the 9th week till the end of

16 weeks. Melatonin-treated groups (HM, CDM and HCDM) recorded a significant improvement in liver function markers (ALT and AST), lipid profile and hepatic lipid accumulation when compared with the disease control (H, CD and HCD) groups. Melatonin administration in H group (HM group) recorded a significant decrement in hepatic *Noct* mRNA. CDM group recorded a non-significant decrement whereas HCDM group recorded a significant increment that was contradictory to other melatonin-treated groups. Further, HM group recorded hepatic *Noct* mRNA expression at ZT12, however, this increment was lower than that observed in the H group. Similarly, in CD and CDM groups, a shift in the *Noct* mRNA peak from ZT12 to ZT6 was observed. Although this peak persisted in CDM group, a significant decrement in the oscillation at ZT6 was recorded. In HCDM group, *Noct* mRNA expression showed a corrective shift in peak expression from ZT6 to ZT12, aligning with Control levels. CircWave analysis revealed subtle modulations in hepatic *Noct* rhythmicity that was evident from moderate to significant decrements in % relative amplitude, shifts in CoG and cumulative *Noct* expression.

HepG2 cells subjected to a combination of OA (0.5 mM) and melatonin (100μM; deduced from MTT assay) recorded a significant decrement in intracellular lipid accumulation as compared to OA group. These findings corroborated with a significantly lower Noct (mRNA and protein) expression when compared to OA group. Further, in serum-synchronized HepG2 cells subjected to OA, melatonin treatment accounted for a notable corrective effect on *NOCT* mRNA expression over time that could be deduced from alterations in % relative amplitude, shifts in CoG and lowered cumulative *NOCT* expression, implying that melatonin has a consistent dampening effect on *NOCT* expression over time. However, melatonin treatment did not account for a profound impact on NOCT protein oscillations. Though the % relative amplitude was significantly decreased in OA +Mel. treated cells, the cumulative NOCT expression was significantly increased as compared to OA group.

In **Chapter 3,** the possible epigenetic regulation of Noct in NASH was put to scrutiny. Computational algorithmic studies identified miR-122 as a potent

epigenetic regulator of Noct whose seed sequence in Noct gene was found to be conserved across mammalian species. These findings were assessed in H and/or CD mice wherein; hepatic miR-122 and Noct recorded an inverse correlation, implying towards miR-122-mediated regulation of Noct in NASH. Further, HepG2 cells were treated with OA alone or in combination with a synthetic miR-122 inhibitor that inhibits mature miR-122 synthesis by inhibiting pre- and pri-miR-122 transcripts. The dose of the inhibitor was standardized by MTT assay and 10 μM concentration was chosen for further experiments. miR-122 inhibitor (10 μM) along with OA (0.5 mM) accounted for a significant inhibition of intracellular miR-122 in HepG2 cells. Further, a time-point based study revealed a significant increment in *NOCT* mRNA expression and disruptions in *NOCT* rhythmicity that was evident from increments in % relative amplitude, shifts in CoG (to ~40 h from 36 h in control and 32 h in OA group) and cumulative *NOCT* expression. On the other hand, NOCT protein expression did not record significant increments in OA + Inhibitor treated HepG2 cells as compared to OA group, albeit the increments were significant when compared to Control group. However, significant perturbations in NOCT protein rhythmicity were observed that could be elucidated from a significant increment in % relative amplitude as compared to Control and shifts in peak time to ~36 h (from 48 h in control and ~38 h in OA group.

Taken together, this study provides novel insights into previously unknown mechanisms operational in NASH pathology and report that: (i) Chronodisruption due to diet and/or photoperiodic shifts alters hepatic Noct oscillations; (ii) melatonin interacts with hepatic Noct and partially restores Noct oscillations and (iii) miR-122 plays a crucial role in regulating hepatic Noct rhythmicity in NASH, though miR-122 does not have a notable role in regulating hepatic Noct expression.