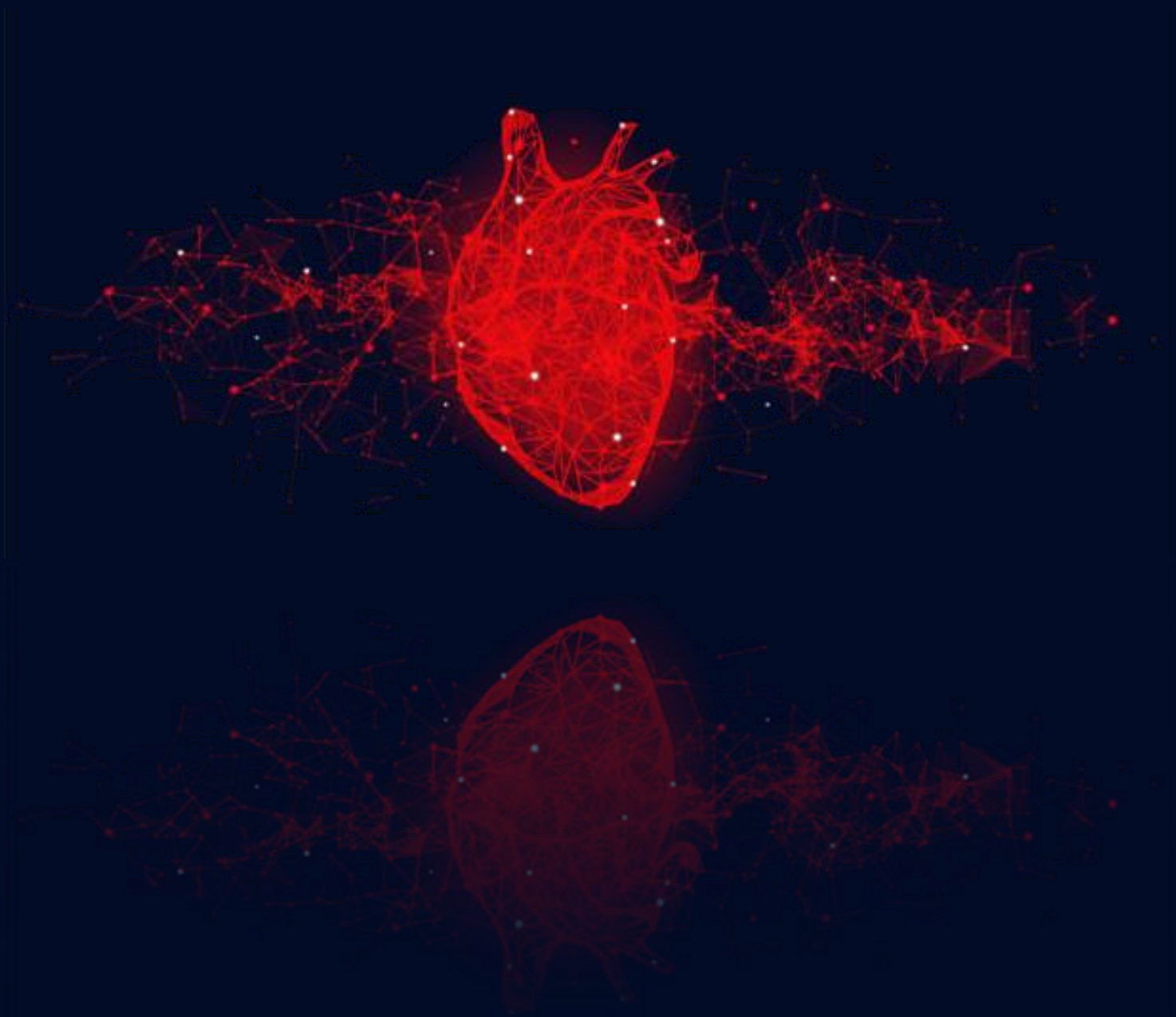
Concise Summary



Concise Summary

Modern lifestyle has subjected people to succumb to a plethora of lifestyle disorders. Disturbed photoperiodic regimen for a prolonged period of time culminates to circadian desynchrony (chronodisruption; CD) that predisposes an individual to metabolic and subsequent cardiovascular disorders (CVD). Biological system of all the organisms is entrained to environmental ques of light and dark in the span of about 24h, transpiring from earth's axial rotation. All the physiological processes taking place in the body are associated directly or indirectly to this light/dark cycles, identified as circadian rhythms. Recent times have brought about massive changes in lifestyle of the people pertaining to shift job works, transcontinental travelling, exposure to artificial light at night (via electronic devices), untimed and disproportionate food intake, consumption of empty calories, excessive caffeine, alcohol, tobacco abuse and so on and so forth. Cumulatively, these factors render an individual to orchestration of CVDs. Amongst all, atherosclerosis is one of the major CVD claiming millions of lives each year. Atherosclerosis is a multifactorial disorder and chronodisruption has been studied as one of the key causative agents since decades. However, a complete clarity on cause-consequence relation of the two lacks clarity.

Atherosclerosis is a complex pathophysiology encompassing multiple cell types at different stages of progression. Atherogenic initiation is marked by endothelial cell (EC) activation in the vascular intima, monocyte recruitment and infiltration that progresses into formation of a lesion and eventually an atheromatous plaque. Atherosclerosis encompasses a plethora of biomolecules including genes, proteins, cytokines, lipids, free radicals, microRNAs (miRNA), endogenous gasotransmitters etc. Past decade has unveiled major potential of miRNAs (20-22 nt repeats of non-

coding RNA) pertaining to operation of basic physiological processes and pathogenesis. miRNAs have been well documented for their vital role in regulation of circadian rhythms. Alterations in some of these miRNAs is shown to potentially change the period of circadian rhythms. miRNAs have also been explored for their functional role in atherosclerosis and some are suggested as potential biomarkers.

Furthermore, atherogenic pathophysiology is also closely associated to endogenous gasotransmitters pertaining to several processes like gene/protein expression, vasoconstriction, vasodilation etc. Carbon monoxide (CO) is one of the major gasotransmitters that is highly functional in regulating these processes. CO is a byproduct of heme degradation and is shown to be closely associated with smooth operation of circadian rhythms, circadian clock associated gene expression as well as with metabolic regulation and mitochondrial functions. CO can easily diffuse across the cellular and biological membrane, facilitating rapid functioning and impact.

Pertaining to the enlisted biological interactions and implications, this study is an attempt to decipher a link between CD induced atherosclerosis. Herein, we try to decipher circadian clock associated miRNA and its implications in atherogenic manifestations and progression. Moreover, an attempt is made to assess role of CO (administered via CORM-A1) in altering the miRNAs and subsequent atherogenic pathophysiology. The study identifies miR34a-5p as a possible circadian associated miRNA and CORM-A1 as a promising anti-atherogenic therapeutant against CD induced atherosclerosis.

Aim of the study

Aim of the study is to assess a mechanistic link between chronodisruption induced atherosclerosis pertaining to systemic regulation of Clock associated miRNAs.

Objectives:

1. **Deciphering Clock gene associated miRNAs**

In-silico algorithms were employed to deduce Clock associated miRNAs. These shortlisted miRNAs were further subjected to analyze their role in CVDs. miR34a-5p was construed from the data analysis and the same was validated in cellular (serum synchronized HUVEC) and chronodisruptive rodent model system (male C57BL/6J mice). Confirming on to miR34a-5p – CLOCK association, the miRNA was further looked upon for it atherogenic target genes that are studied in detail in further chapters.

2. **Elucidating mechanism of miR34a-5p expression in experimental atherogenic models and its modulation via CORM-A1.**

miR34a-5p expression was assessed in experimental atherogenic cellular (HUVEC & MDMs) and rodent models (male Sprague Dawley rats). The elevated expression was further investigated for mechanistic details in the pathological milieu. Furthermore, CORM-A1 meditated alterations in miR34a-5p expression were also assessed that formed the bases for detailed investigation of miR34a-5p functions in atherogenic systems.

3. **To assess implication of miR34a-5p – KLF4 axis pertaining to inflammation in atherogenic milieu.**

In-silico target prediction software showed presence of Watson-Crick base pair complementary seed sequence for miR34a-5p, in 3'UTR of KLF4 gene. The same was validated in cellular system (HUVEC & MDMs). Further an attempt to assess role of miR34a-5p in KLF4 – NF-κB mediated inflammatory response in atherogenic conditions was studied in cell specific manner. CORM-A1 mediated downregulation of miR34a-5p and overall impact on atherogenicity was also studied herein.

4. **To assess implication of miR34a-5p – SIRT1 axis pertaining to mitochondrial biogenesis and function in atherogenic milieu.**

Improving mitochondrial health is a current hotspot as therapeutic target in atherosclerosis. Mitochondrial damage dictates cellular health in a potent manner. miR34a-5p is well documented to regulate SIRT1 expression by inhibiting its 3'UTR activity. Herein, the same was validated in our model systems. Further this section studies CORM-A1 mediated improvement in mitochondrial function and biogenesis in miR34a-5p dependent and independent manner.

The study initiates with identification of Clock gene associated miRNAs using computational algorithms for miRNA – mRNA interactions, miRDB V 6.0 and TargetScan V 7.0, covered in **Chapter 1**. These interactions deploy under defined physiological conditions including miRNA interaction to the 3'UTR of mRNA exhibiting Watson & Crick base pair complementary seed sequence that is further followed with conditions like miRNA/mRNA concentrations, free energy of binding (-ΔG), cellular compartmentalization/location, cell type and competitive inhibitions etc. Clock complementary miRNAs identified for human and mice were assessed for common overlapping miRNAs (86 miRNAs were identified). These preferential miRNAs were further subjected to FuncMir test with primary objective of their underlying cardiovascular implications. Selected miR34a-5p was assessed further with KEGG database for its gene targets and consequential pathological implications. The results displayed its role in over hundreds of pathogenic conditions including fatty liver, CVD, cancers, Parkinson's disease, Alzheimer's disease, vasculitis, obesity, heart failure, atherosclerosis, diabetes mellitus and rheumatoid arthritis etc. Detailed scrutiny with PubMed published literature unveiled functional role of about 39.9% of miR34a-5p target genes in CVD making it a potential candidate linking chronodisruption to onset and progression of CVD. Thereby, we decided to primarily validate the chronobiological role of miR34a-5p in invitro and in-vivo model systems followed by investigating in atherogenic implications in forthcoming chapters.

In-silico based chronobiological implications of miR34a-5p were further scrutinized and validated in biological system in **chapter 2**, wherein thoracic aortae of male C57BL/6J mice and serum synchronized HUVEC cells (endothelial cells) were used as experimental model systems. Primarily, potential of miR34a-5p to inhibit 3'UTR functioning of CLOCK gene was validated in biological system by Luciferase reporter

gene assay performed in HUVECs. The assay confirmed miR34a-5p mediated impending of CLOCK gene and protein expression, qualifying itself as a chronodisruptive miRNA.

Further, physiological circadian association of miR34a-5p was assessed in the thoracic aorta and HUVEC cells at five different time points ZT 0, 6, 12, 18 & 24 along with expression of its target genes, CLOCK and SIRT1. This study is the first to report the cyclic expression of miR34a-5p in synchronized HUVEC cells. We report a reciprocal oscillatory relationship between *CLOCK* / *SIRT1* and miR34a-5p. The peak timings of *CLOCK* / *SIRT1* corresponded to the ebb of miR34a-5p and vis-à-vis. Contrary to this observation, a similar oscillatory pattern of miR34a-5p was not seen in thoracic aorta. Dampened oscillations of miR34a-5p recorded in thoracic aorta is possibly attributable to it complex tissue architecture comprising of diverse cell types in which, the tunica intima contributes to a small subset of the total mass and fail to put out the sole cellular settings.

Further expression/role of miR34a-5p was assessed in chronodisruptive C57BL/6J mice developed by subjecting it to constant a phase advance/phase delay photoperiodic manipulation for 18 weeks. Assessment of chronodisruption was done by evaluating circadian clock gene transcripts and protein content in the thoracic aorta of C57BL/6J mice. Levels of miR34a-5p were found to be elevated in CD mice as compared to that of the control. In order to elucidate the mechanism of elevated miR34a-5p levels we evaluated the methylation pattern in its promoter region, at about 300 bp upstream of the transcription start site. Wherein, alteration in form of hypomethylation in the thoracic aorta of CD mice was identified, explaining the higher transcription rate and thus the elevated levels of miR34a-5p in aortae of CD mice.

CD predisposes an individual to pro-atherogenic manifestation that has been known since decades. SIRT1 is a 'chrono-metabolic' gene that not only regulates smooth operation of circadian rhythms, but also is vital in operation of several metabolic processes. miR34a-5p has been reported to inhibit SIRT1 expression by inhibiting its 3'UTR function. The plausible implications of pro-atherogenic manifestation were assessed in thoracic aortae of mice, wherein the aortae of CD mice exhibited fibrillar derangement, elastin fragmentation, intimal-media thickening, elevated collagen/elastin ratio and elevated expression of adhesion molecules as compared to that of the control aortae. This data was coupled with proatherogenic changes observed in serum of CD mice that were calculated and represented as Atherogenic Index of Plasma (AIP) and Cardiac Risk Ratio (CRR). Overall, this data confirms the CD induces expression of miR34a-5p, its direct interaction with clock gene impending circadian rhythms coupled with inhibited SIRT1 expression that predisposes CD mice to pro-atherogenic manifestations.

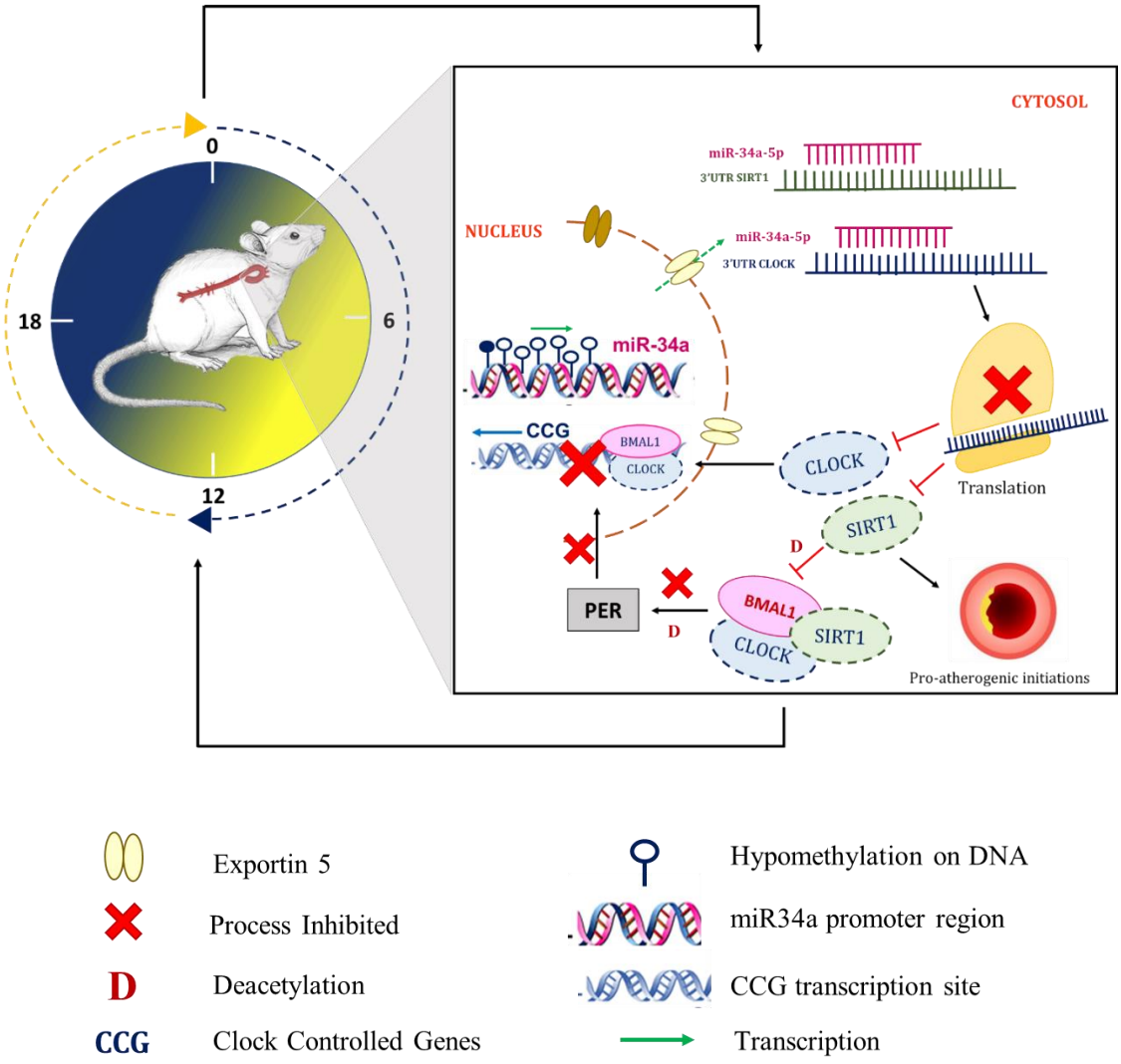


Fig S1: Photoperiodic manipulation induced chronodisruption in male C57BL/6J mice showed elevated miR34a-5p titers in the thoracic aortae pertaining to hypomethylation in its promoter region. Elevated miR34a-5p inactivated 3'UTR function of CLOCK gene, impending its expression, facilitating chronodisruption. miR34a-5p also inhibited SIRT1 expression aiding to failure in operation of physiological chrono-metabolic functions, promoting pro-atherogenic manifestations.

Atherogenic implications of miR34a-5p are talked in detail in **chapter 3**. In human, miR34a-5p is documented to be elevated in atheromatous plaque as well as in circulation. However, the mechanism/reason for this phenomenon is yet unexplored. CO has been studied for its varied functions, on one end it encompasses regulation of circadian functioning and on other is reported to regulate vasomotor tone. A study conducted in patients with CVD showed elevated titers of CO as compared to the control patients. In our system, an indirect approximation of CO quantity was made using titers of bilirubin (produced in equal proportions as CO in the process of heme degradation) that were elevated in ath diet fed SD rats. Hypothesizing elevated CO as a biological coping mechanism, we decided to test impact of CO in the regulation of higher miR34a-5p expression in pro atherogenic condition.

To address the question, an in-vivo atherogenic model was developed in male SD rats. The same was validated by serum lipid profile, en face assay, arterial stiffening, H&E staining, intimal-media thickening assessment, elastin fragmentation, fibrillar derangement, collagen to elastin ratios, gene, and protein expression studies etc. wherein the group of rats dosed with CORM-A1 (2mg/Kg BW; *i.p.*) exhibited anti-atherogenic changes. In-vitro atherogenic models were developed using ox-LDL (80 µg/mL) in HUVEC and THP1 derived macrophages, MDMs (using PMA, 100 nM), with an aim of studying detailed events of pro-atherogenic manifestation.

miR34a-5p levels were elevated in atherogenic conditions, as anticipated. However, the same were depleted on CORM-A1 treatment. Transcription factors of this intergenic miRNA, *P53* and *NF-κB* were assessed that were found to be elevated in atherogenic conditions and lowered on CORM-A1 treatment. Further transcription inhibitors of miR34a-5p, *Zeb1, Snai1* and *Stat3* were also assessed. Interestingly, these genes were

differentially expressed in cell specific manner. However, CORM-A1 treatment had elevated expression of *Zeb1, Snai1* and *Stat3* in all the three disease models.

Aberrant DNA methylation is a physiological response to stout external stimuli. Altered circadian rhythms have been shown to manifest DNA methylation in biological systems (as witnessed in chapter 2) and manipulate gene expression. Thus, herein we aimed to assess the plausible alterations in DNA methylation patterns in the promoter region of miR34a-5p about 300bp upstream of the transcription start site. The atherogenic cells had shown elevating unmethylation in the promoter region and complementing lowered methylation implying to facilitated increase in rate of miRNA transcription. Cells co-treated with CORM-A1 showed opposing results to that of the disease conditions. The methylation pattern was significantly reverted, comparable to the control.

Further we attempted to understand if CO physically associated with any of the transcription factor to impend the miR34a-5p transcription. The data obtained for molecular docking and relative free binding energies from MMGBSA analysis showed CO binding relatively strongly to P53 as compared to NF-κB. It would be rather interesting to further assess if CO docking were inside any of the identified DNA binding domain of these proteins. Overall, this data indicates CORM-A1 mediated lowering of miR34a-5p expression by altering its transcription factors and reverting the hypo-methylation in its promoter region.

Inflammation and mitochondrial dysfunction are two preliminary events happening at time of atherogenic manifestations. Insilico data has shown miR34a-5p potentially binding to 3'UTR of KLF4 and SIRT1, impending their expression. **Chapter 4** is divided into **chapter 4a** and **chapter 4b** wherein a detailed mechanism of miR34a-5p

– KLF4 axis regulating inflammatory events and mi34a-5p – SIRT1 axis regulating mitochondrial biogenesis and function has been studied in detail, respectively.

Chapter 4a studied miR34a-5p pertaining to KLF4 mediated inflammatory changes in cell specific manner. KLF4 is a stress responsive gene that is lowered in atherogenic conditions promoting detrimental effects. KLF4 is vital transcriptional factor and inhibitor that is studied to be observing a tight feed-back loop with P53 in physiological conditions. *KLF4* is also an anti-inflammatory gene that inhibits NF-κB expression to lower inflammatory response in atherogenic conditions. Contemplating these facts, we looked up plausible interaction of miR34a-5p with 3'UTR of *KLF4*. The complementary seed sequence indicated miR34a5-p mediated plausible inhibition of 3'UTR function of *KLF4* that was further assessed in HUVEC and MDMs. Cells transfected with miR34a-5p antagomir when dosed with ox-LDL did not show decrement in *KLF4* expression comparable to that of ox-LDL treated non-transfected cells, implying towards miR34a-5p mediated down regulation of *KLF4* expression. The same was further observed in CORM-A1 co-treated cells, harbouring lowered miR34a-5p.

Elevated miR34a-5p expression by *NF-κB* and consequently lowered *KLF4* expression in HUVECs lead to endothelial cell activation and expression of adhesion molecule (*ICAM1* & *VCAM1*) that promoted monocyte adhesion. Similar observation was made in the IHC stained (ICAM1 & $CD68^{+}$) thoracic aortae of SD rats. Inflammatory response pertaining to gene pool expression is cell type specific and critical in pathological conditions. Monocytes adhered to endothelial cells are internalized to luminal and sub-luminal region of vasculature, wherein lipid accumulation and cocktail of cell secreted cytokines endorse cells to differentiate into macrophages and polarise into either M1 or M2 type. Dipped KLF4 expression in atherogenic conditions elevated

NF-κB and subsequent pro-inflammatory changes that promoted M1 macrophage polarization. CORM-A1 co-treated cells harbouring lowered miR34a-5p, exhibited lowered M1 polarization.

Chapter 4b focuses on miR34a-5p – SIRT1 axis mediated mitochondrial health in atherogenic milieu. Herein, we show that CORM-A1 regulates miR34a-5p – SIRT-1 axis that facilitates a cellular redox shift and improvement in atherogenic conditions. CORM-A1 treatment results in recovery of 3'UTR activity of SIRT-1 that facilitates mitochondrial biogenesis as evidenced by MitoTraker Red and mtDNA copy number. oxLDL accumulation incurs ROS production, mitochondrial hyperpolarization and disrupted MMP that was effectively restored on CORM-A1 treatment, as evidenced by DCFDA and JC-1 staining in HUVECs and MDMs. Cells harbouring damaged mitochondria are exposed to the risk of mitochondrial homed HSP60 leaking out into the cytosol. Excessive HSP60 leak into the cytosol is proatherogenic trait that promotes endothelial dysfunction and cell death. IHC staining showed CORM-A1 mediated lowering of HSP60 in luminal and sub-luminal region of atherogenic aortae promoting anti-atherogenic manifestations. Mitochondrial redox status, regulated by *TrxR2* and *SOD2*, dampened in atherogenic conditions is also shown to restore on CORM-A1 treatment. miR34a-5p binds to 3'UTR of *TrxR2* and halts the protein expression, depressing mitochondrial status. Herein, ox-LDL/ ath diet group with elevated miR34a-5p showed lowered expression of *TrxR2* and *SOD2* that were restored on CORM-A1 treatment. Increased mitochondrial number and health imply towards efficient mitochondrial function that was accessed by ATP and seahorse assay. Higher ox-LDL accumulation in atherogenic cells, promote mitochondrial hyperpolarization and OXPHOS dysfunction leading to elevated cellular ROS. Ox-LDL treatment augmented depression in BRC & MRC coupled with lowered ATP production in

atherogenic HUVECs and MDMs. CO is reported to orchestrate mitochondrial uncoupling via elevated proton leak, shifting cellular energy production from glycolysis back to OXPHOS. CORM-A1 treatment mitigated BRC and MRC in atherogenic HUVEC and MDMs further culminating in elevated ATP production. Mechanistic counteractive impact of CORM-A1 was further assessed in IB cells wherein MDMs showed maximal CORM-A1 operation via miR34a-5p. HUVECs exhibit corrective variance in IB and non-IB cells, implying towards a plausible mechanism wherein CORM-A1 mitigates via an alternative pathway along with miR34a-5p. Further, the data shows that CORM-A1 boosts overall cellular respiration in HUVECs whereas, a shift is observed in cellular energetics dependency from non-mitochondrial to mitochondrial respiration in case of MDMs. However, it is evident from the data that CORM-A1 mitigates mitochondrial respiration majorly via miR34a-5p in atherogenic HUVEC and MDMs.

Taken together, this compile of investigation reports circadian rhythm mediated miRNA alterations in atherosclerotic system. Herein we first-time report (i) circadian expression of miR34a-5p in endothelial cells. (ii) CD mediated elevation in miR34a-5p levels in thoracic aortae of mice, pertaining to epigenetic modifications in its promoter region. (iii) Elevated miR34a-5p inhibits *CLOCK* gene expression by inactivating its 3'UTR activity. These results qualify miR34a-5p as chronodisruptive miRNA. Further detailed investigation in atherogenic rat model, HUVEC and MDMs showed expression regulation of miR34a-5p in atherogenic milieu by altered transcription factors and inhibitors, coupled with epigenetic modifications. Also, CORM-A1 mediated lowering in miRNA and anti-atherogenic manifestations opens new therapeutic avenues for atherosclerosis. Further the study matures into finding miR34a-5p mediated depletion of *KLF4* and *SIRT1* affecting inflammatory changes and mitochondrial biogenesis and

function in atherogenic milieu, respectively. The coupled effect of both the pathways plays a vital role in progression of atherogenic pathology. Cumulatively suggesting miR34a-5p as a possible biomarker and therapeutic target against chronodisruption induced atherogenic manifestation.

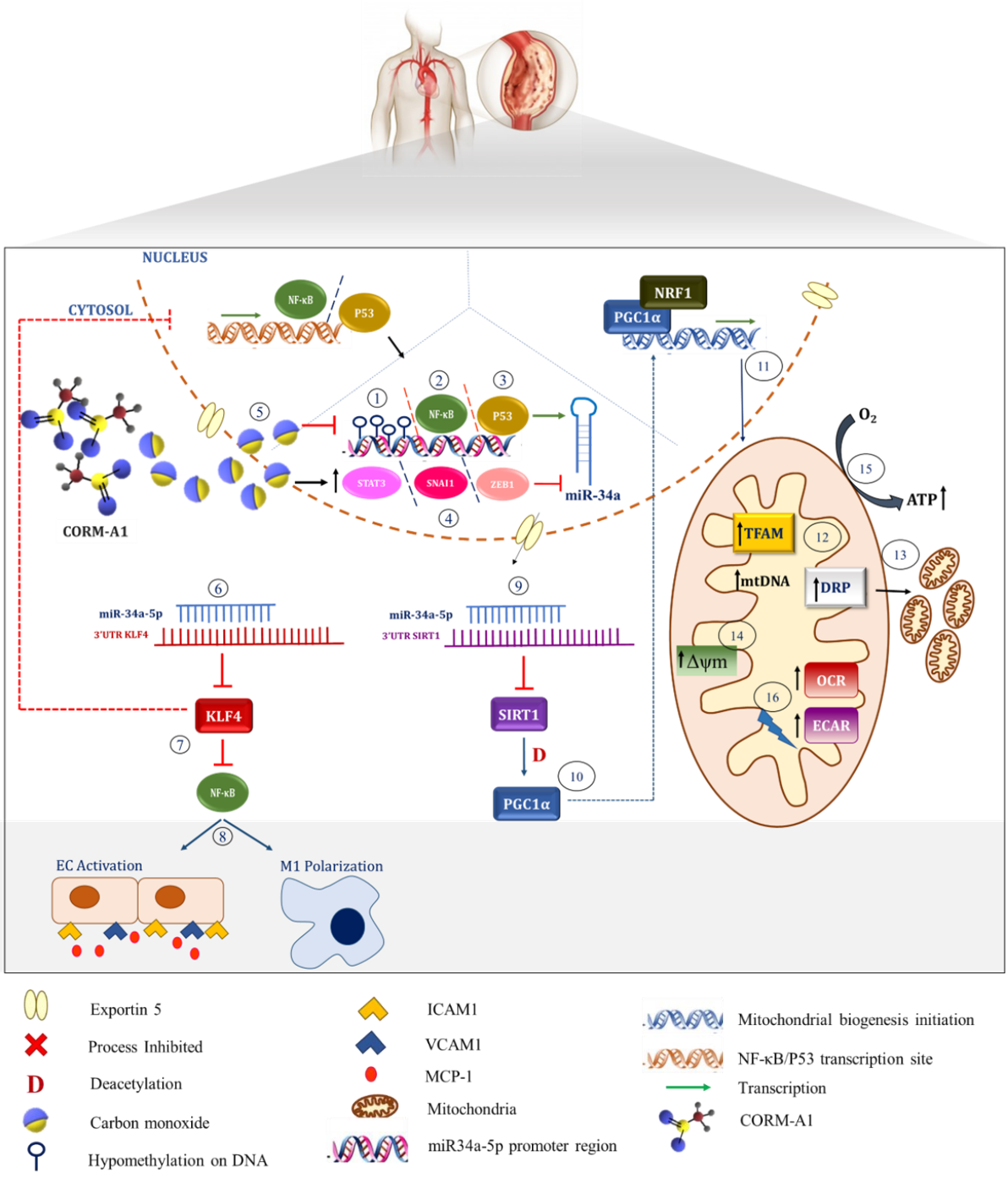


Fig S2: Atherogenic milieu exhibits elevated miR34a-5p pertaining to hypomethylation in the promoter region and alteration in its transcriptional factors. CORM-A1 mediated modifications lowered miR34a-5p expression and orchestrated anti-atherogenic effect. Study showcases 2 main, miR34a-5p – KLF4 and miR34a-5p – SIRT1 axis regulation. All the steps numbered in the image have been described in detail below.

Number Key:

1. Hypomethylation in the promoter region of miR34a-5p in atherogenic condition.
2. NF-κB mediated transcription of miR34a-5p.
3. P53 mediated transcription of miR34a-5p.
4. Transcription inhibition of miR34a-5p by ZEB1, SNAI1, STAT3 respectively.
5. CORM-A1 mediated inhibition of miR34a-5p transcription by lowering NF-κB & P53 and elevating ZEB1, SNAI1 & STAT3.
6. miR34a-5p mediated inhibition of KLF4 expression.
7. KLF4 mediated inhibition of NF-κB & P53 in normal physiological conditions with lowered miR34a-5p.
8. NF-κB mediated Endothelial cell activation (expression of adhesion molecules & MCP1 production) and M1 macrophage polarization.
9. miR34a-5p mediated inhibition of SIRT1 expression.
10. PGC1α deacetylation by SIRT1 and migration to nucleus.
11. Mitochondrial Biogenesis by PGC1α and NRF1.
12. Elevated TFAM and mtDNA levels in cells harboring CORM-A1 mediated lowered miR34a-5p.
13. DRP1 induced mitochondrial fission processes in cells harboring CORM-A1 mediated lowered miR34a-5p.
14. Improved Mitochondrial Membrane Potential in cells harboring CORM-A1 mediated lowered miR34a-5p.
15. Enhanced ATP Production in cells harboring CORM-A1 mediated lowered miR34a-5p.
16. Improved mitochondrial function assessed as OCR and ECAR in cells harboring CORM-A1 mediated lowered miR34a-5p.