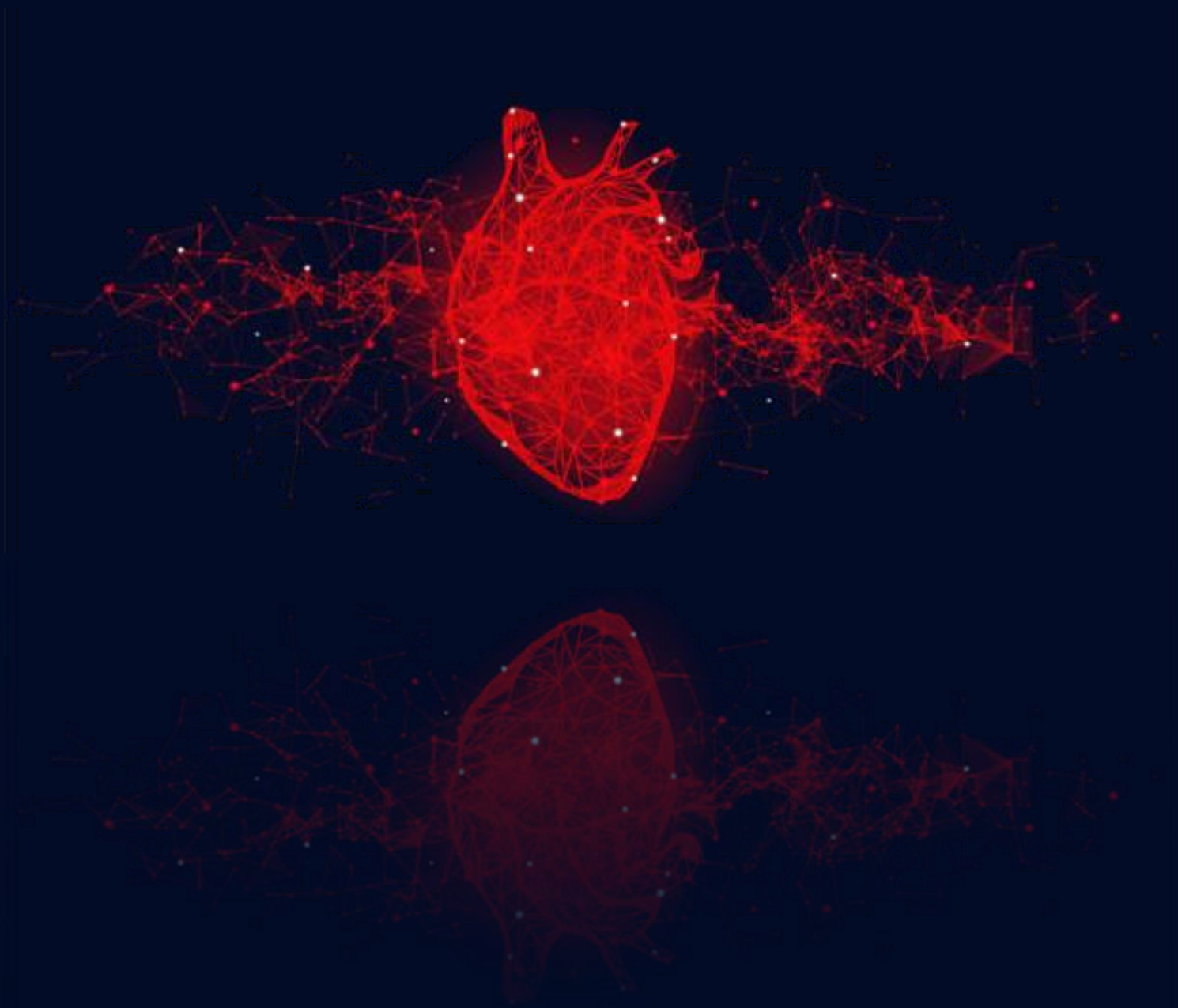
Chapter 4a

Inhibition of miR34a-5p ameliorates KLF4 mediated inflammatory progression in atherogenic conditions



Introduction

Marking of initiation of atherogenic etiology yet has varied perspectives. However, a profound understating pertaining to the role of inflammation in initiation and progression of the disease have indisputably been reported. Inflammatory milieu in vasculature facilitates endothelial cell activation, innate immunity operation, monocyte recruitment, ROS generation and lesion progression (Szmitko *et al.*, 2003). Cellular inflammatory response is a cumulative action of several genes, wherein Kruppel-like factor 4 (KLF4) potentially functions as a 'molecular switch' and is also proposed as a vital therapeutic target by several research groups. KLF4 is an important transcription factor for cell growth, differentiation, proliferation, and has been shown to substantially affect the regulation of cardiovascular disease (Yang *et al.*, 2021). High-shear stress at the endothelial surface induces the transcription factors Kruppel-like factor 2 (KLF2) and KLF4, which promote a quiescent EC phenotype characterized by a low turnover rate, tight intercellular junctions with low permeability, reduced inflammatory activation and antithrombotic properties (Van Agtmaal *et al.*, 2012). Disturbed flow at arterial bifurcations constantly damages the endothelium by activating the endoplasmic reticulum stress response, suppresses EC-specific transcriptional programmes by downregulating KLF2/4, and increases the activity of the proinflammatory transcription factor nuclear factor-κB (NF-κB) (Jha and Das, 2017). KLF4 – NF-kB circuit regulates vital physiological processes like EC activation by triggering expression of adhesion molecules (ICAM-1, VCAM-1) and M1- M2 macrophage polarization in atherogenic pathology (Liao *et al.*, 2011).

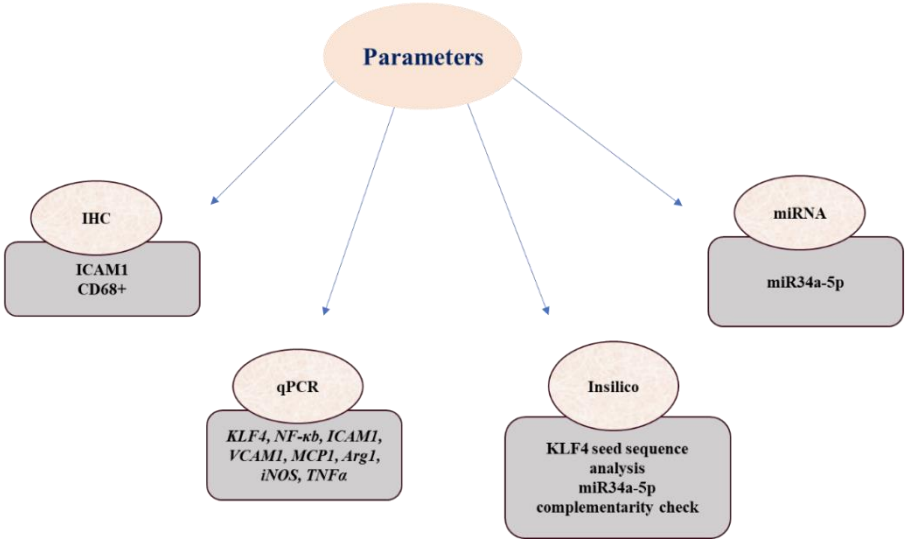
KLF 4 has also been studies for its expressional regulation by several miRNAs in various pathological systems. miR103 mediated repression of KLF4 expression has

been documented to promote maladaptation of endothelial cells and atherosclerosis (Hartmann *et al.*, 2016). miR1 mediated repression of KLF4 regulates smooth muscle cell differentiation (Xie *et al.*, 2011). miR375/KLF4 pathway functionally regulates macrophage polarization in atherogenic pathology (Chen *et al.*, 2019b).

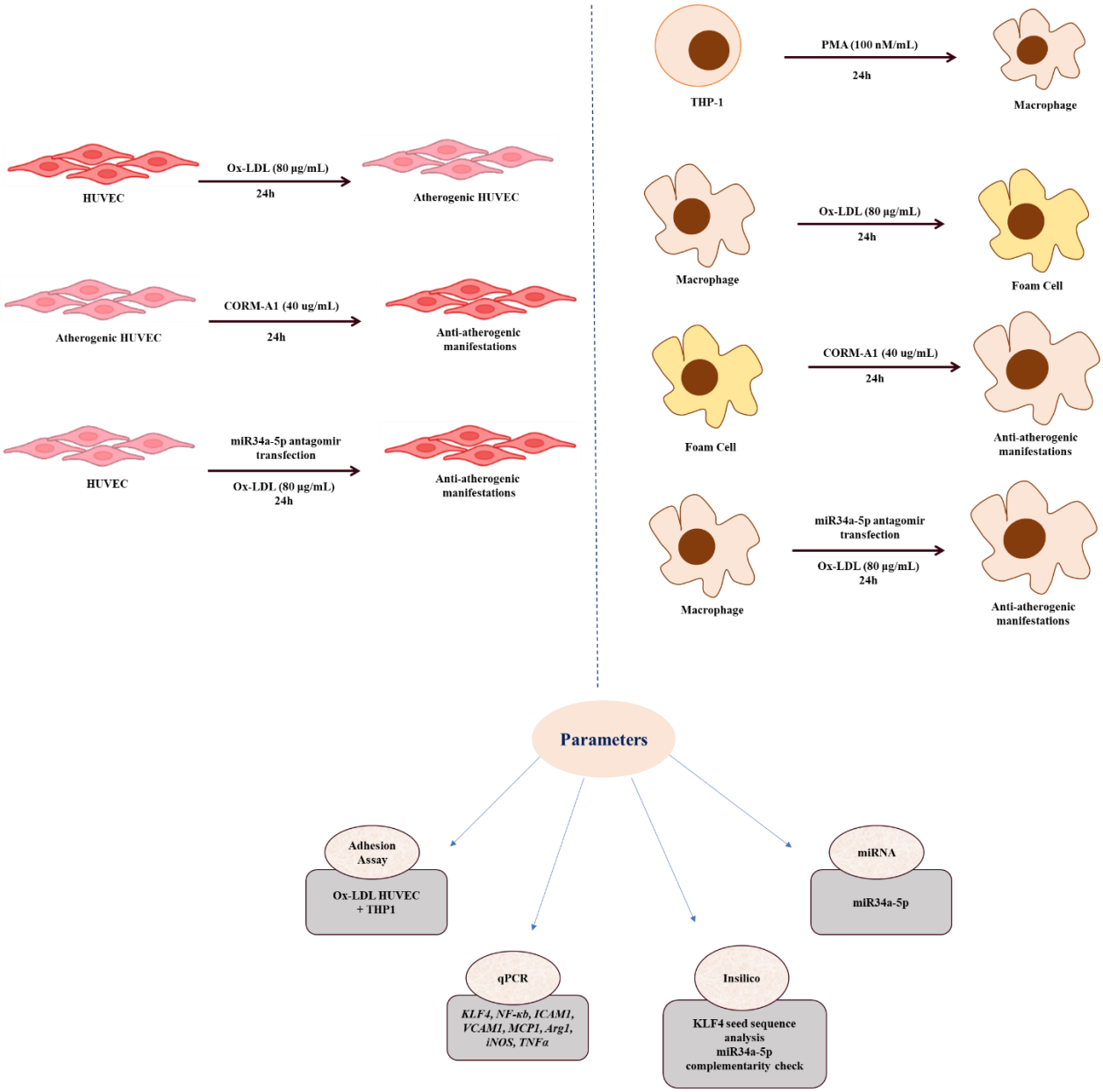
P53 and NF-κB the transcription factors of miR34a-5p are also reported to have transcriptional regulation on expression of KLF4. P53 co-exist in a tight feedback loop with KLF4 (Stöger *et al.*, 2012) as well as miR34a-5p. This chapter makes an attempt to assess miR34a-5p mediated plausible regulation of KLF4 expression in atherosclerosis. It further digs into looking the KLF4 mediated transcriptional inhibition of NF-κB and its role in endothelial cells and macrophage polarization using HUVEC and MDMs, respectively. CORM-A1 mediated inhibition of miR34a-5p expression and consequential changes in KLF4 – NF-κB axis have also been studied in this compile of work.

Methodology

In-vivo Experimentations



In-vitro Experimentations



Results

Depleted KLF4 titers are restored on CORM-A1 treatment in atherogenic conditions

KLF4 is a vital transcriptional factor, reported to be depleted in atherogenic conditions. Herein, we assessed the thoracic aortae of ath diet fed SD rats that exhibited similar significant depletion in KLF4 titers as compared to that of the control. However, the aortae of CORM-A1 treated animals exhibited significant restoration in levels of KLF4, implying to anti-atherogenic manifestations in the system. Further, in-vitro experimentation with ox-LDL treated HUVEC and MDMs also displayed similar depletion of KLF4 as compared to that of the control. Cells co-treated with CORM-A1 exhibited inverse effect, the KLF4 titers were significantly restored and were comparable to that of the control (Fig 4a.1).

miR34a-5p regulates KLF4 expression in atherogenic HUVEC and MDMs

Cells treated with CORM-A1 harboring lowered miR34a-5p titers showed restoration in KLF4 expression. In-silico analysis of KLF4 transcript revealed a complementary seed sequence indicative of miR34a-5p mediated loss of 3'UTR function and consequent *KLF4* expression (Table 4a.1; Fig 4a.2a). To validate the same, HUVECs and MDMs were transfected with miR34a-5p antagomir (IB HUVEC or IB MDMs). IB cells treated with ox-LDL showed higher *KLF4* titers as compared to the ones observed in ox-LDL treated untransfected cells. ox-LDL treatment to HUVEC showed lowering in *KLF4* as compared to that of the control cells, whereas MDMs exhibited elevated titers when compared to control. *KLF4* expression in CORM-A1 treated cells was comparable to that of the IB HUVEC, implying towards CORM-A1 mediated

lowering of miR34a-5p as a causation for *KLF4* restoration. Whereas CORM-A1 treated MDMs exhibited *KLF4* titers comparable to the control MDMs (Fig 4a.2b).

Lowered *KLF4* correlates with higher *NF-κB* titers and cell adhesion molecules in atherogenic HUVEC.

KLF4 regulates expression of multiple genes and physiological process including NF-κB mediated inflammatory pathways. Atherogenic aortae and cells harbouring lowered KLF4 promotes NF-κB expression (Fig 4a.3). Herein, elevated *NF-κB* expression in endothelial cells leads to endothelial cell activation as witnessed by expression of elevated adhesion molecule (*ICAM1 & VCAM1*). Adhesion molecules in activated endothelial layers recruits circulating monocytes that are internalized forming atherogenic lesions. Herein, we assessed the same with immunohistochemical analysis of ICAM1 and $CD68^{+}$ that were observed to be significantly high in ath diet group and the same was reverted in aorta of CORM-A1 treated rats (Fig 4a.4a-c). Further qPCR analysis for the gene expression studies of *Icam-1* and *Vcam-1* normalized with 18s rRNA also exhibited similar decrement in CORM-A1 treated aortae (Fig 4a.4d).

Elevated NF-κB expression culminates in higher transcription of Monocyte chemoattractant protein (MCP1) that is functional in monocyte recruitment to the location. And these monocytes adhere to adhesion molecule and are internalized manifesting plaque development. Herein, we found ox-LDL treated HUVEC exhibiting elevated titers of *MCP1* gene that facilitates monocyte recruitment to the cells. Along with expression of *ICAM1* and *VCAM1* that were lowered in CORM-A1 cotreated cells (Fig 4a.6).

To further assess the monocyte recruitment activity, we performed adhesion assay. HUVECs, at about 70% confluency, were treated with ox-LDL (80 µg/mL) for 24h. THP-1 cells were stained with 1µg/mL Hoechst 33342 in an incomplete medium for 15 min in dark and were laid over atherogenic HUVEC and incubated for 30 min. ox-LDL treated HUVECs showed higher number of adhered monocytes as compared to that of the control and CORM-A1 co-treated cells on microscopic evaluation. The cells were then counted and quantified revealing about 20% adhesion in control, >80% in ox-LDL treated cells and about 60% in the CORM-A1 co-treated cells (Fig 4a.5). The data herein cumulatively indicates CORM-A1 mediated lowering of endothelial cell activation and monocyte recruitment orchestrating its antiatherogenic effect.

Cells harboring lowered KLF4, promote M1 polarization in atherogenic MDMs

Macrophages have vital role in atherogenic pathophysiology and functions as both pro- and anti-atherogenic factors. Macrophages differentiate to M1 or M2 type based on the external stimuli. M1 macrophages are pro-inflammatory in nature and promotes plaque development (Stöger *et al.*, 2012). Herein Thp-1 derived MDMs used for experimentations were in M_0 state. Ox-LDL treated MDMs exhibited elevated NF-κB, TNFα and iNOS along with lowered Arg1 indicating towards M1 macrophage polarization. CORM-A1 co-treated cells showed lowered levels of NF-κB, TNFα and iNOS and elevated Arg1 impending M1 macrophage polarization and thus having anti-atherogenic impact (Fig 4a.7).

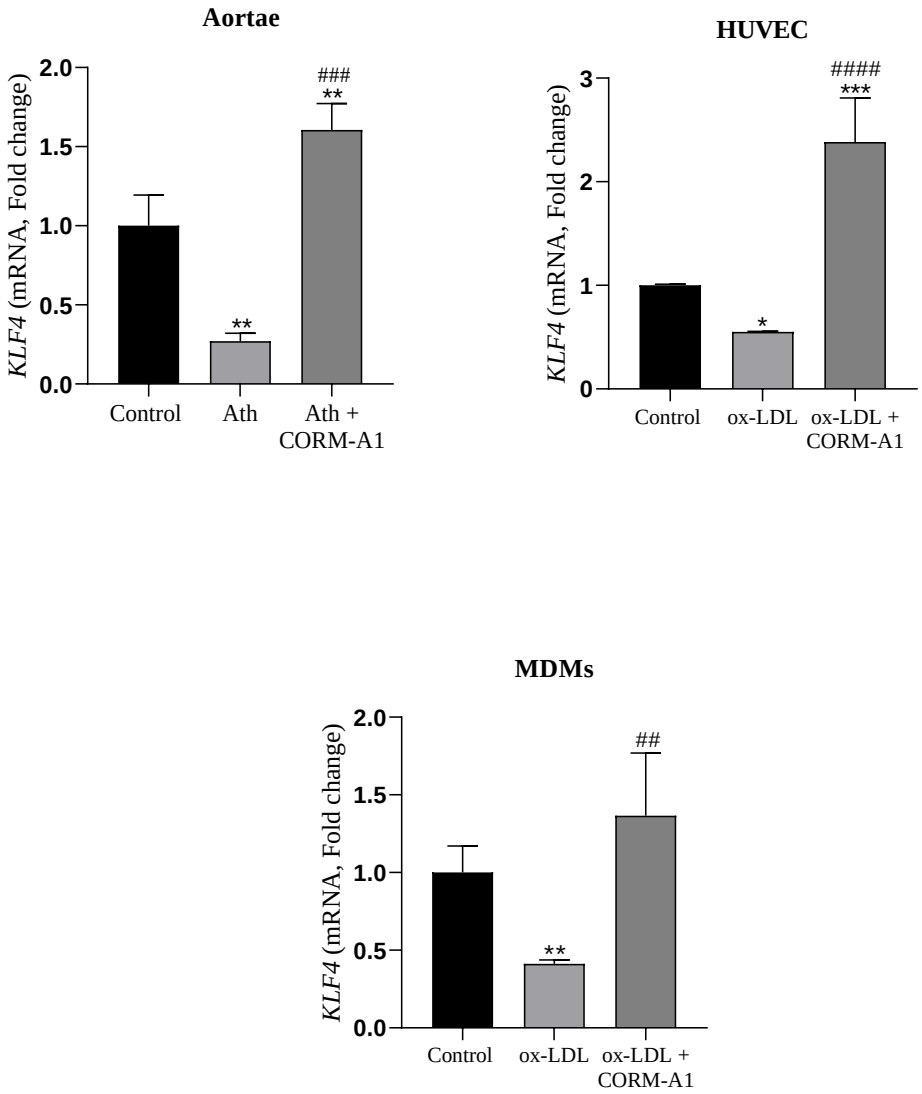
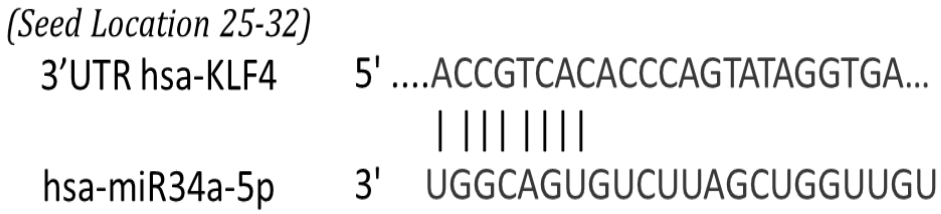


Fig. 4a.1: mRNA quantification of *KLF4* gene in thoracic aortae of SD rats (n=4), HUVEC and MDMs (n=3). Results are expressed as mean ± S.E.M. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 or ****p < 0.0001 is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}$p < 0.0001 when compared to atherogenic group.

Sr. No.	Species	Gene	miRNA	Dock Score	Seed location	Length of 3'UTR region
1.	*Homo sapiens*	KLF4	hsa-miR 34a-5p	86	25	924 nt
2.	*Mus musculus*	KLF4	mmu-miR 34a-5p	83	25	1001 nt
3.	*Rattus norvegicus*	KLF4	rno-miR 34a-5p	77	25	868 nt

Table 4a.1: Information of miR34a-5p – KLF4 docking were studied using computational algorithms in human, mice, and rats. The dock score based on miRNA mRNA interactions, seed locations in the 3'UTR and the length of 3'UTR has been documented in the above table.

(a)



(b)

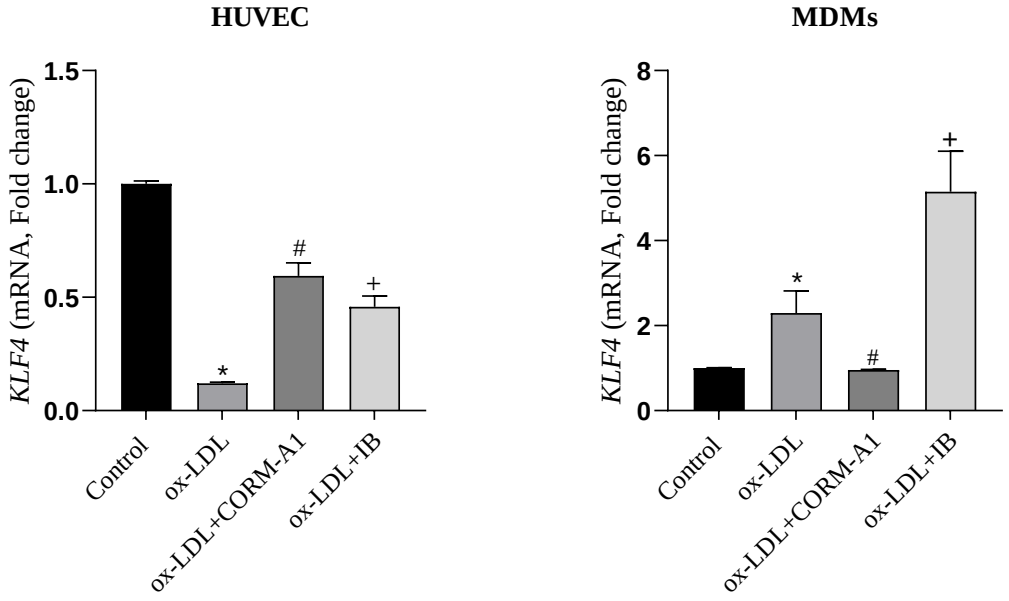


Fig. 4a.2: KLF4 interaction with miR34a-5p (a) Schematic representation of miR34a-5p complementary seed sequences in 3'UTR of KLF4 gene. (b) mRNA quantification of *KLF4* and comparison between atherogenic, CORM-A1 co-treated and atherogenic IB (miR34a-5p antagomir transfected) cells. Results are expressed as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$ is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}p < 0.0001$ when compared to atherogenic group.

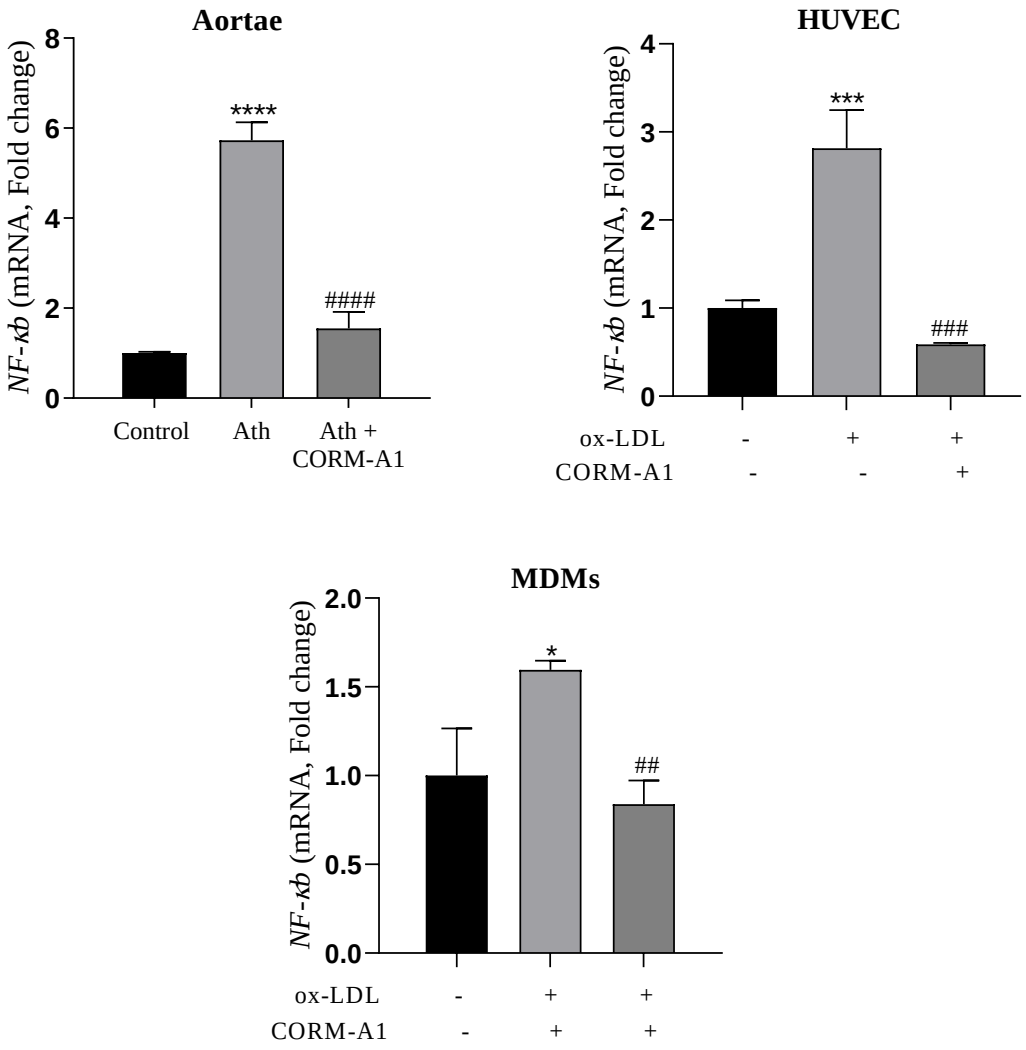


Fig. 4a.3: Transcriptomal quantification of *NF-κB* gene in thoracic aortae of SD rats (n=4), HUVEC and MDMs (n=3). Results are expressed as mean ± S.E.M. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 or ****p < 0.0001 is when compared to control group and $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 or $^{\#\#\#\#}$p < 0.0001 when compared to atherogenic group.

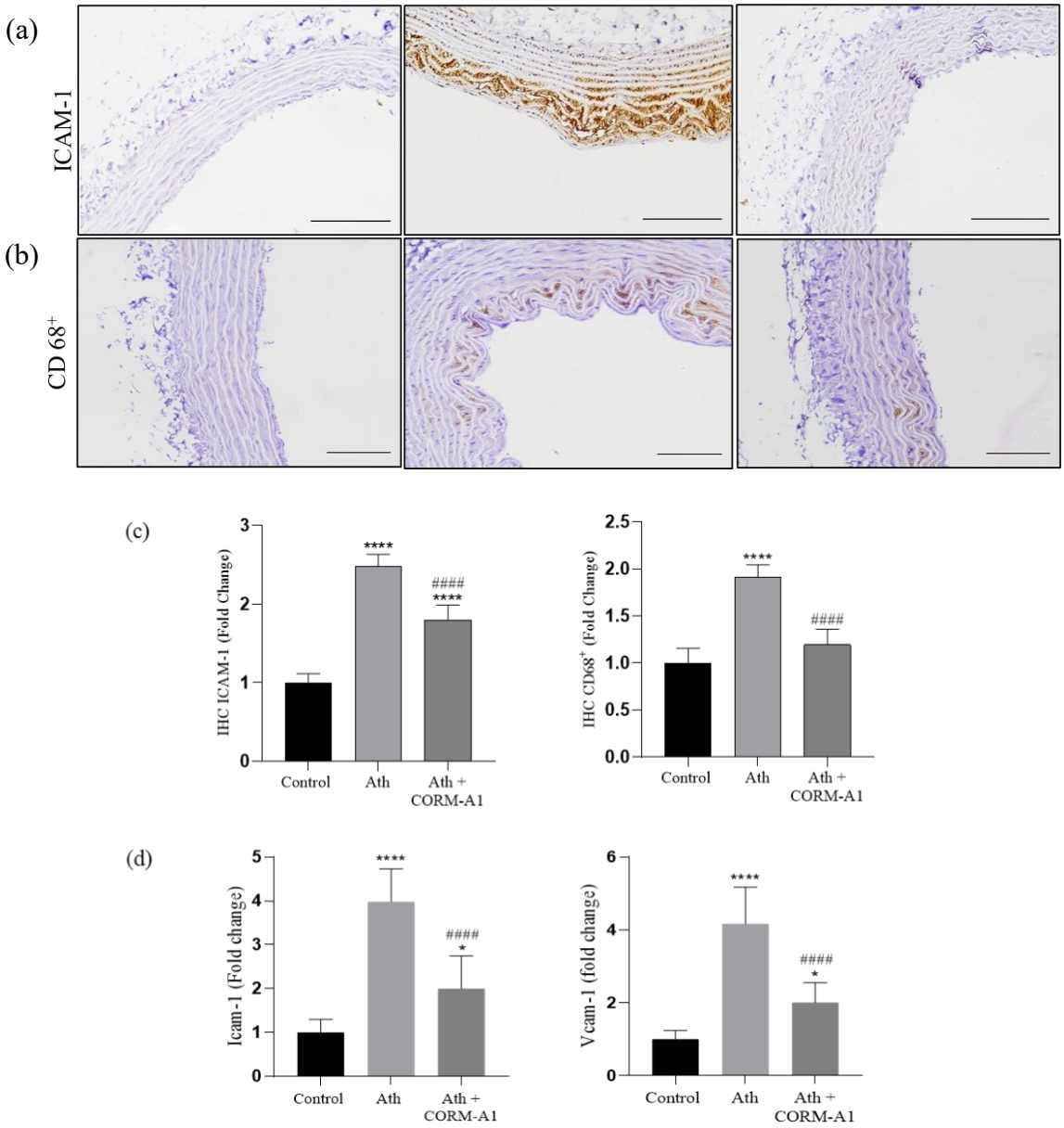


Fig. 4a.4: Thoracic aortae harvested from Sprague Dawley rats assessed for atherogenic changes by Immunohistochemical (IHC) analysis of (a) adhesion molecule (Icam-1*)* and (b) macrophage marker ($CD68^{+}$) (Scale 100µm; n=3). (c) Quantified and represented graphically as fold change. (d) Pro-atherogenic gene expression from thoracic aortae was assessed by qPCR of *Icam-1* and *Vcam-1* normalized with 18s rRNA. Results are expressed as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$ is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}p < 0.0001$ when compared to atherogenic (disease) group.

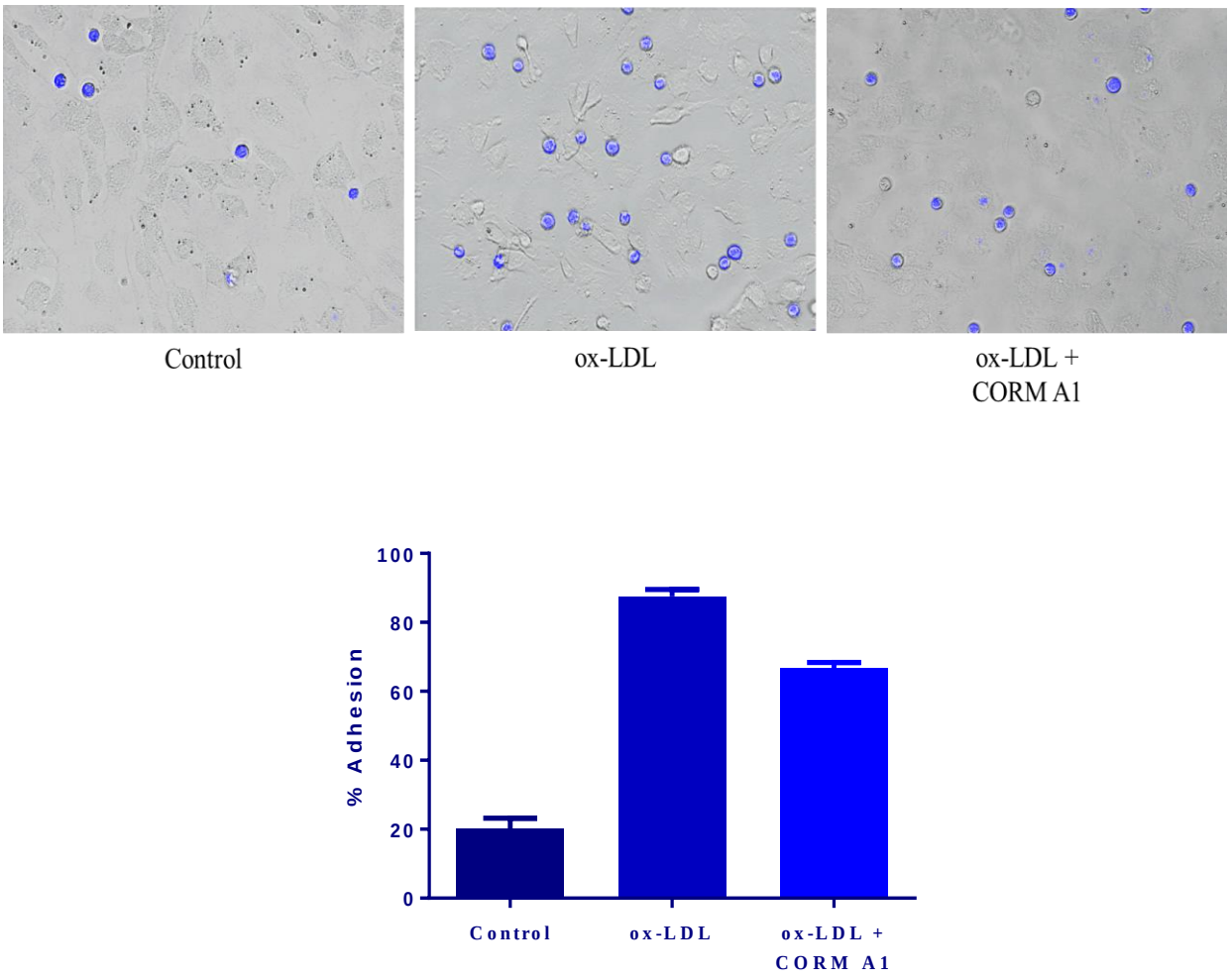


Fig. 4a.5: Endothelial cell activation and monocyte adhesion was assessed by performing adhesion assay. Ox-LDL and/or CORM-A1 treated HUVEC were laid and incubated with Hoechst 33342 stained monocytes and adhesion was assessed microscopically and imaging was done using Floid Imaging station. Collected cells were counted and are represented herein as % adhesion of cells (n=4). Results are expressed as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$ is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}p < 0.0001$ when compared to atherogenic (disease) group.

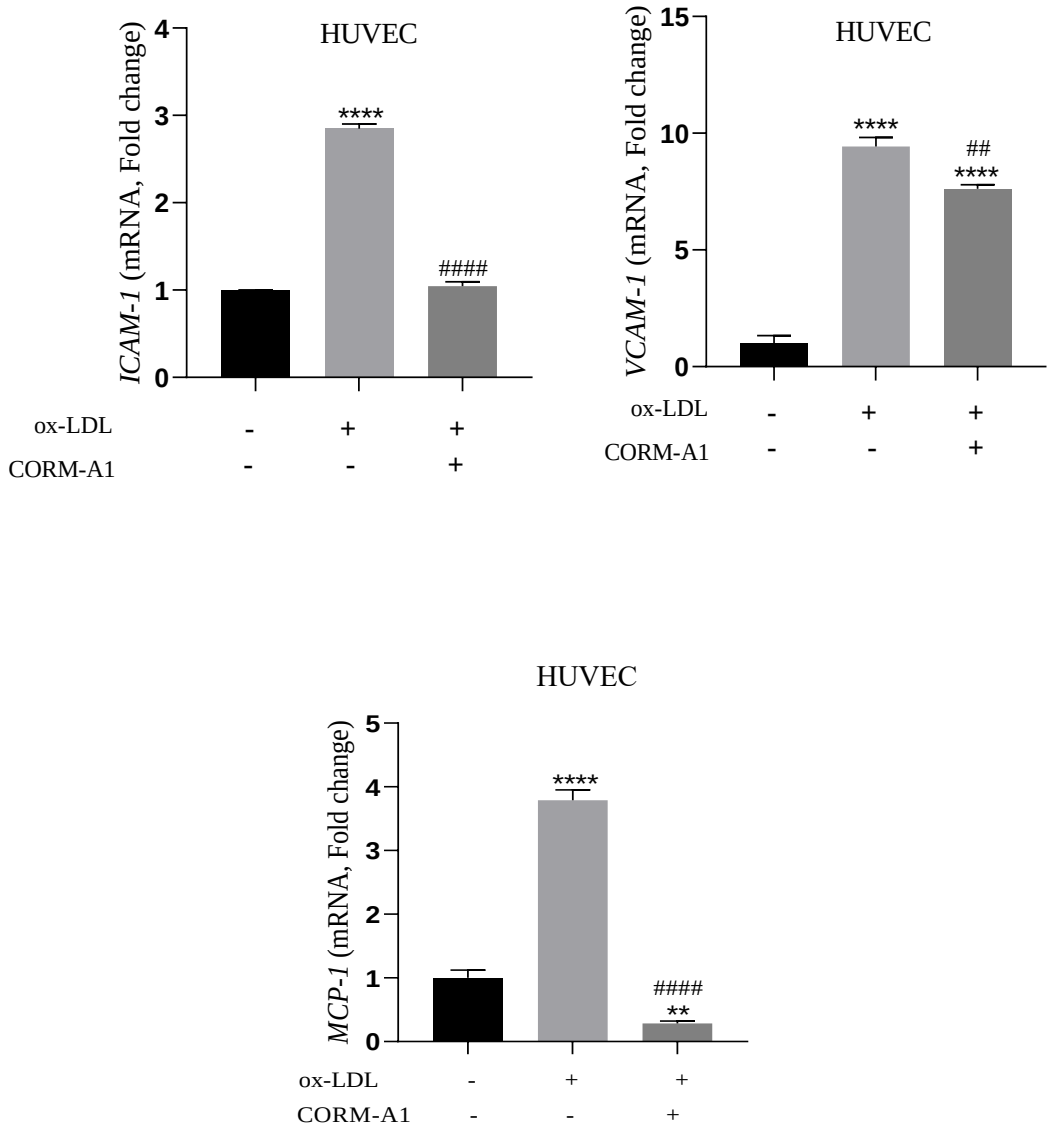


Fig. 4a.6: Transcriptomal quantification of *ICAM1, VCAM1* and *MCP-1* genes in thoracic aortae of SD rats (n=4), HUVEC and MDMs (n=3). Results are expressed as mean ± S.E.M. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 or ****p < 0.0001 is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}$p < 0.0001 when compared to atherogenic group.

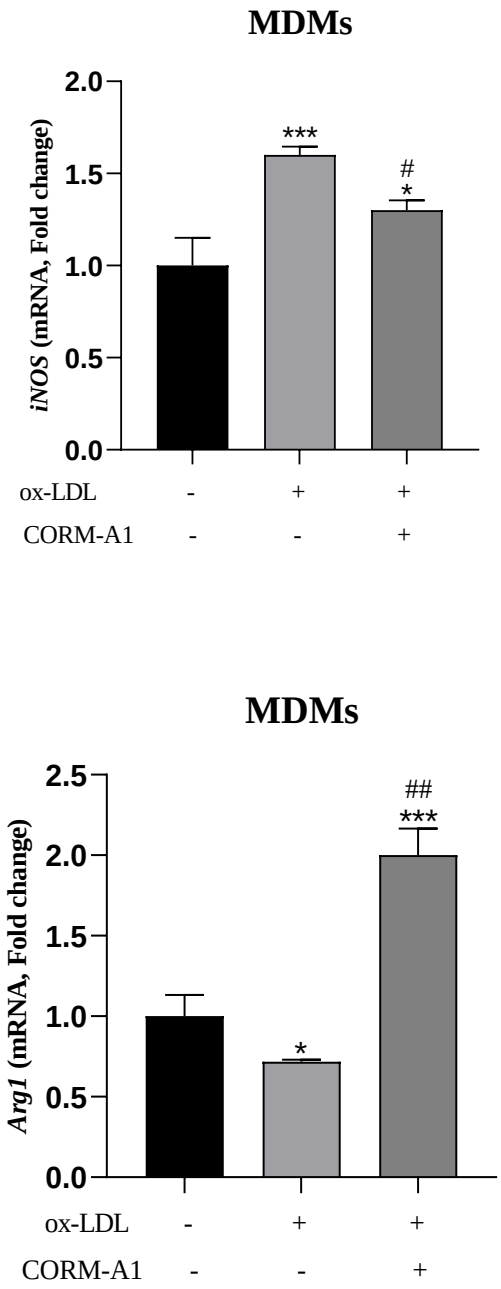


Fig. 4a.7: Transcriptomal quantification of *TNFα*, *iNOS* and *Arg1* genes in thoracic aortae of SD rats (n=4), HUVEC and MDMs (n=3). Results are expressed as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$ is when compared to control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}p < 0.0001$ when compared to atherogenic group.

Discussion

Arterial bifurcations are prime locations for shear stress responses and lesion formation. miR34a-5p is recorded not only for its oxidative stress responsive expression but also as a sheer stress responsive miRNA. miR34a-5p, as observed in earlier chapters, elevates in atherogenic conditions, partly as result of elevated transcription factors P53 and NF-κB. KLF4 is a stress responsive gene that is lowered in atherogenic conditions promoting detrimental effects (Sharma *et al.*, 2012). 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 (Atkins and Simon, 2013). 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. The cells were 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.

Atherogenic lesion formation initially witness inflammation as a primary response to the stressors. 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 no M1 polarization.

Taken together this compile of work demonstrates miR43a-5p mediated inactivation of 3'UTR functioning of KLF4 in atherogenic conditions that promotes proinflammatory changes. Further cell specific response to KLF4 was documented with miR34a-5p mediated endothelial cell activation in HUVECs and progressive M1 polarization in MDMs. CORM-A1 mediated lowering of miR34a-5p and KLF4 elevation can be used as a potential therapeutic strategy combating primary inflammatory response to pro-atherogenic manifestation.