Summary of the Thesis

Modulatory role of HSP60 and related key genes in pathogenesis of atherosclerotic inflammation

Cardiovascular diseases (CVDs) is a leading cause of mortality worldwide and has been recognized as a major public health concern by WHO. Atherosclerosis, the underlying cause of major CVDs, is a chronic disease of the arterial wall, where fatty deposition in the vessel wall leads to blockage of the concerned artery. Many compelling theories have been put forwarded to explain the series of events leading to the development of this complex disease. The most accepted explanation of atherosclerotic lesion formation begins with an endothelial injury leading to accumulation and oxidation of LDL molecules in the sub-intimal space followed by recruitment of monocytes, their differentiation into macrophages and subsequent uptake of OxLDL to form foam cells. However, immunological studies into the disease led to the formulation of auto-immune concept of atherosclerosis wherein, ectopic expression of HSP60 in stressed endothelium has been identified as an earliest atherogenic event that activates auto-immune reactions at the site of endothelial injury.

The concept is based on the fact that HSP60 is evolutionarily highly conserved with >55% homology at protein and DNA level between eukaryotic (human) cells and prokaryotic (bacterial) cells. As a result, when autologous HSP60 is expressed on stressed arterial endothelial cell surface, it leads to immune cross-reaction due to the pre-existing immunity against its bacterial counterpart gained by infections or vaccination. Further, emphasis has been laid on the co-expression of HSP60 and adhesion molecules on surface of stressed endothelium for recruitment of mononuclear cells. Supporting evidences in this regard, have been provided by clinical studies of early lesions in aorta, *in vivo* study in

normocholesterolemic rabbits stressed with LPS and a number of *in vitro* studies assessing HSP60 inducing potential of various atherogenic stressors. High fat diet and atherogenic diet induced HSP60 upregulation in atheromatous plaque has been reported in BALB/c mice, but early lesions had not been studied.

In chapter 1, we have evaluated the status of HSP60 during high fat high fructose (HFHF) diet mediated early atherogenic remodeling of thoracic aorta in C57BL/6J mice. Our results showed that HFHF diet induced a characteristic increment in body weight and severe hyperlipidemia, despite lower food consumption. Further, thoracic aorta of HFHF group showed prominent pro-atherogenic remodeling characterized by hypertrophy, lumen dilation, elastin fragmentation and collagen deposition. These early atherogenic changes were accompanied by endothelial dysfunction as evidenced by downregulation of eNOS and endothelial activation coupled with monocyte recruitment as demonstrated by upregulation of MCP-1, VCAM-1 and ICAM-1 along with increased CD68+ staining. At this stage, prominent upregulation of HSP60 in tunica intima and media was observed in thoracic aorta of HFHF diet fed mice. The canonical function of HSP60 as a protein folding machinery requires inevitable support of its co-chaperone, HSP10, which is yet less explored for its role in atherogenesis. Herein, we observed a significant upregulation of HSP10 mRNA and protein in thoracic aorta of mice fed with HFHF, which is the first report linking HSP10 with atherosclerosis. Overall, this study primarily reported HSP60 and HSP10 upregulation in thoracic aorta with signs of endothelial dysfunction, activation and atherogenic remodeling in mice subjected to diet induced hyperlipidemia (Fig. S1).

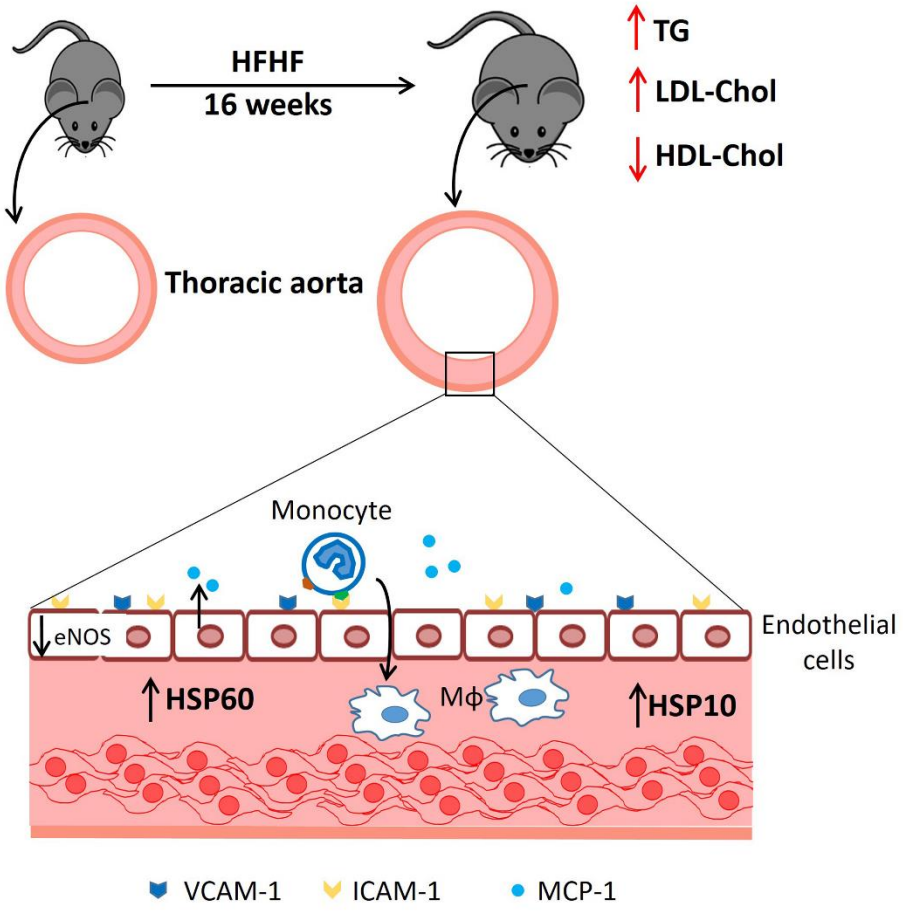

Figure S1: An overview of high fat high fructose diet induced pro-atherogenic changes in thoracic aorta of C57BL/6J mice.

The role of HSP60 as an atherogenic auto-antigen is well-established wherein, the events of its upregulation, surface expression and secretion from stressed endothelium have been emphasized. It is interesting to note that irrespective of the initial stressor, HSP60 upregulation in endothelium is a key atherogenic event that also precedes its surface localization and secretion. Further, intracellular HSP60 upregulation has been reported in various cellular context wherein, it has been identified to have novel intracellular functions including regulation of apoptosis and activation of NF-κB pathway. HSP60 overexpression in VSMCs has also been associated with its proliferation emphasizing the relevance of non-canonical functions of cytosolic HSP60 in vascular cells. Since, HSP60 upregulation is a common endpoint for various endothelial stressors, we hypothesized its involvement in regulation of the atherogenic transformation of endothelium.

Based on our observation of hyperlipidemia induced HSP60 upregulation during vascular dysfunction, we investigated the possible regulatory role of endogenous HSP60 in OxLDL induced atherogenic transformation of HUVEC. We observed HSP60 upregulation in HUVEC exposed to 80 µg/ml OxLDL for 24 h but not at 8 µg/ml, emphasizing the lower potency to OxLDL as inducer of HSP60 that is in agreement with previous reports. Also, OxLDL is known to induce expression of adhesion molecules in endothelial cells at 80 µg/ml dose and not at 8 µg/ml dose. Since, the co-expression of HSP60 and adhesion molecules is essential for their cross-talk, this observation supported our claim of functional relationship between the two.

The surface expression and secretion of HSP60 is known to induce auto-immune response essential for atherogenic initiation. Hence, we assessed HSP60 secretion in our model by ELISA and the underlying sub-cellular dynamics of HSP60 by immunostaining. We

observed prominent secretion and plasma membrane localization of HSP60 in cells treated with OxLDL. Similar study with HUVEC overexpressing HSP60 (HSP60-GFP and pcDNA-HSP60) showed that HSP60 overexpression leads to its extra-mitochondrial accumulation, plasma membrane localization and secretion. Further, we observed HSP60 overexpression induced endothelial dysfunction in HUVEC as evidenced by decreased NO levels and eNOS mRNA expression, similar to OxLDL, whereas HSP60 knockdown (HSP60 KD) inhibited the said changes. HSP60 overexpression also induced endothelial activation as demonstrated by upregulation of MCP-1, VCAM-1 and ICAM-1 along with increased monocyte adhesion. Similar changes were observed in OxLDL treated HUVEC that were prevented in conditions of HSP60 KD.

Since we had observed HSP10 upregulation in hyperlipidemia induced early atherogenesis, we checked its expression in OxLDL treated HUVEC and observed a marked upregulation in the same. We also checked the expression of HSF-1, a transcriptional factor for HSP10 and HSP60, and observed a significant decrement indicating HSF-1-independent mechanism of HSP10 and HSP60 upregulation in OxLDL treated HUVEC that needs further scrutiny. Overall, it can be said HSP60 upregulation is a key atherogenic event that is crucial for its surface expression and secretion, implicating towards immunological processes, and for triggering endothelial dysfunction and activation that marks the initiation of atherogenic lesion formation (Fig. S2).

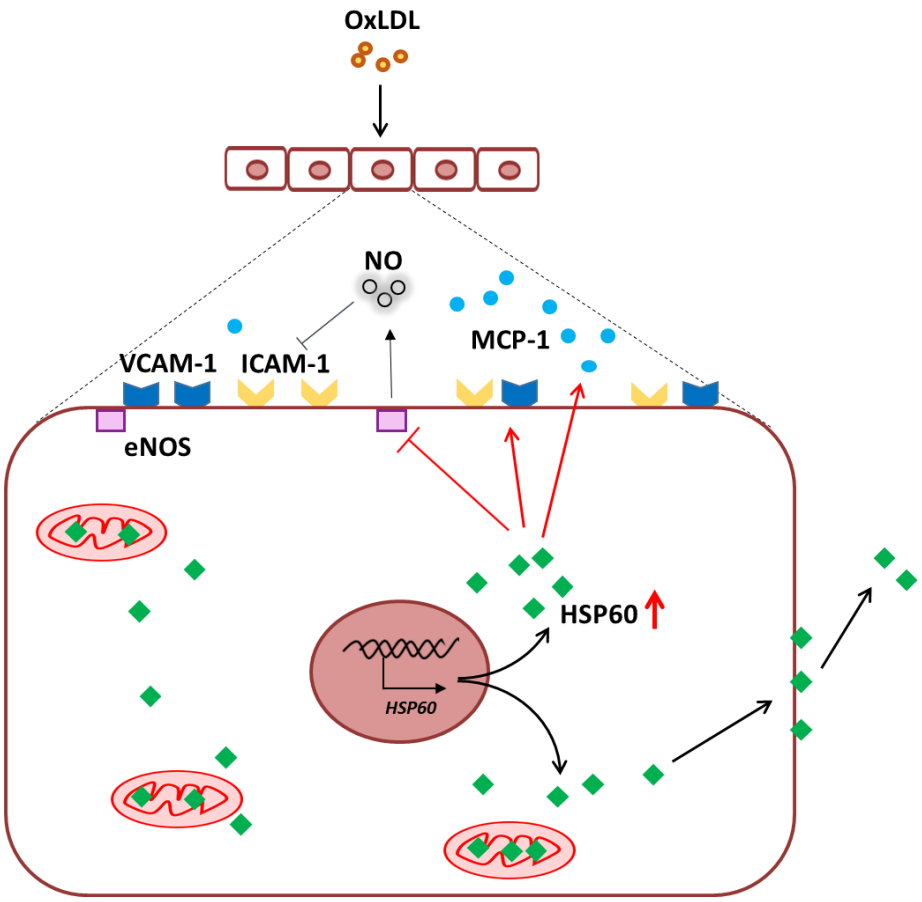

Figure S2: Summary of HSP60 mediated regulation of OxLDL induced atherogenic changes in HUVEC.

Intimal infiltration of monocytes is followed by their differentiation into macrophages that take up OxLDL to form foam cells, marking the formation of fatty lesion. The relevance of HSP60 in pro-inflammatory activation of macrophages have been described but OxLDL induced alterations in endogenous HSP60 in macrophages is lacking. In the next part of the study, we assessed OxLDL induced changes in HSP60 and its regulatory role in foam cell formation using THP-1 monocyte-derived macrophages (MDMs). Our results showed that THP-1 MDMs stressed with OxLDL (8 and 80 µg/ml) secrete HSP60, which might trigger inflammatory pathway in consort with other cytokines. The secretion of HSP60 was preceded by its upregulation at mRNA and protein levels. Further, MDMs deficient in HSP60 were found to be inefficient in restoring mitochondrial membrane potential that was deteriorated in presence of OxLDL, pointing towards subtle regulatory role of HSP60 during OxLDL induced mitochondrial stress. To our surprise, OxLDL accumulation was significantly higher in HSP60 downregulated MDMs, which was attributable to an increase in SR-A1 and CD36 dependent uptake with a simultaneous decline in SR-B1 mediated efflux as evident from results of mRNA expression. Knockdown of HSP60 was also found to aggravate OxLDL induced inflammatory polarization as demonstrated by increased M1 markers and decreased M2 markers. Collectively, this study provided evidence of the non-canonical functions of intracellular HSP60 in modulating transcriptional expression of genes regulating two key aspects of atherogenic manifestations in macrophages viz. intracellular lipid accumulation and subsequent inflammation (Fig. S3).

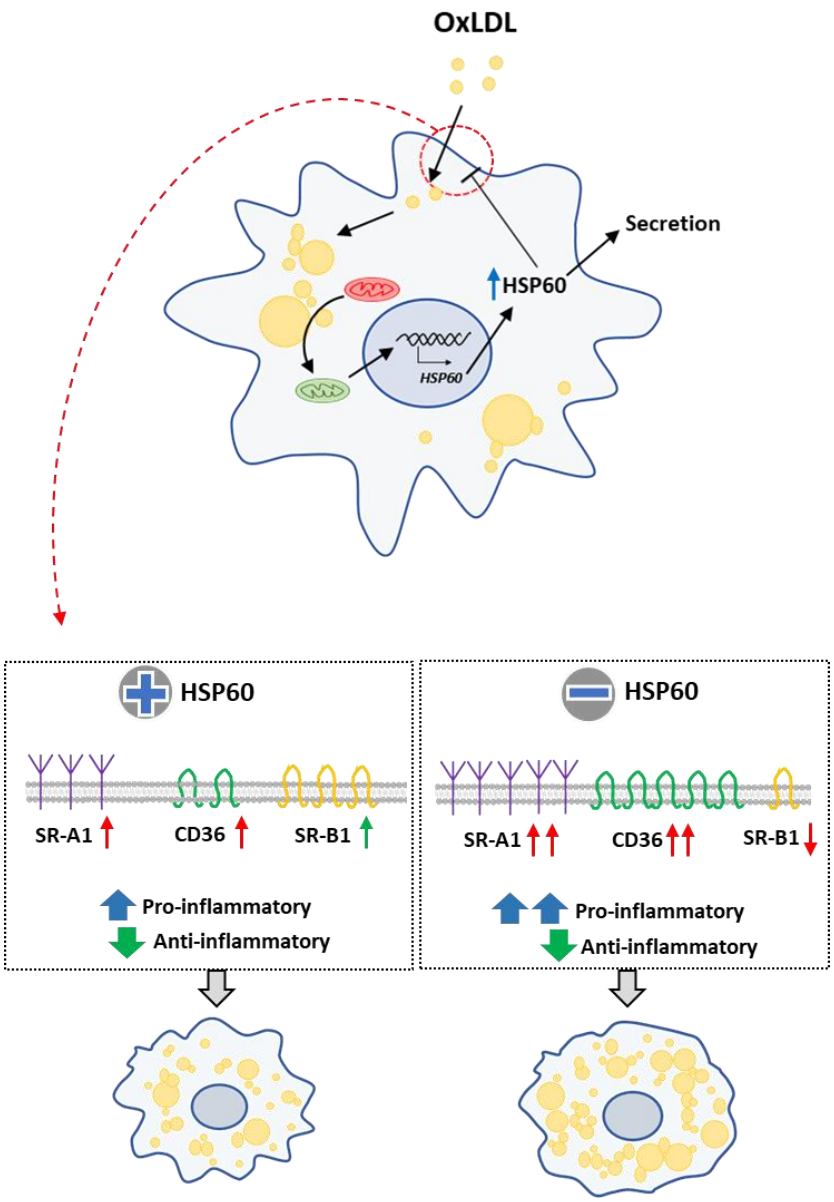

Figure S3: Summary of HSP60 mediated regulation of OxLDL induced atherogenic changes in THP-1 monocyte derived macrophages (MDMs)

HSP60 mediated atherogenic initiation is believed to provide an explanation for the development of atherosclerosis in normocholesterolemic individuals. In this regards, early lesions in humans with no signs of hyperlipidemia have been correlated with endothelial upregulation of HSP60 and presence of HSP60-reactive T cells. In the last part of this study, we investigated if HSP60 has a role to play in circadian disruption induced atherogenic initiation, as the latter has often been associated with development of atherosclerosis. However, till date, the experimental evidences of circadian disruption induced atherosclerosis have been accumulated from studies using hyperlipidemic murine models. In our study, we have subjected C57BL/6J mice fed with laboratory chow to photoperiodic manipulation induced chronodisruption (PMCD) and analyzed thoracic aorta for pro-atherogenic alterations. The results were compared with HFHF diet fed mice that served as hyperlipidemia induced atherogenic model and the combinatorial effect of HFHF and PMCD was also put to scrutiny.

High levels of triglycerides and low levels of HDL-cholesterol were the standout features of PMCD group. Extensive pro-atherogenic structural remodeling was observed in thoracic aorta despite near normal levels of LDL-cholesterol in PMCD group. Further, simultaneous upregulation of HSP60 in the intimal and medial regions and endothelial dysfunction and activation markers were observed in PMCD group. HSP10 upregulation was also observed in thoracic aorta of these mice. A comparative analysis revealed that all the early atherogenic changes in thoracic aorta were more prominent in PMCD group as compared HFHF or HFHF+PMCD groups, both of which had characteristic hyperlipidemic profile. Also, the results showed that HFHF had a dominating presence in HFHF+PMCD group as the atherogenic changes were comparable in these two groups. Thus, it can be said that

HSP60 upregulation plays a central role in the PMCD mediated early atherogenic initiation (Fig. S4).

Taken together, the study reveals that HSP60 upregulation is a crucial event that marks the earliest atherogenic change regardless of the presence of hyperlipidemia. Further mechanistic studies provide evidence for cell-type specific regulatory functions of HSP60 pertaining to atherogenic manifestations. Primary evidence on HSP10 upregulation during early stages of atherosclerosis is also noteworthy.

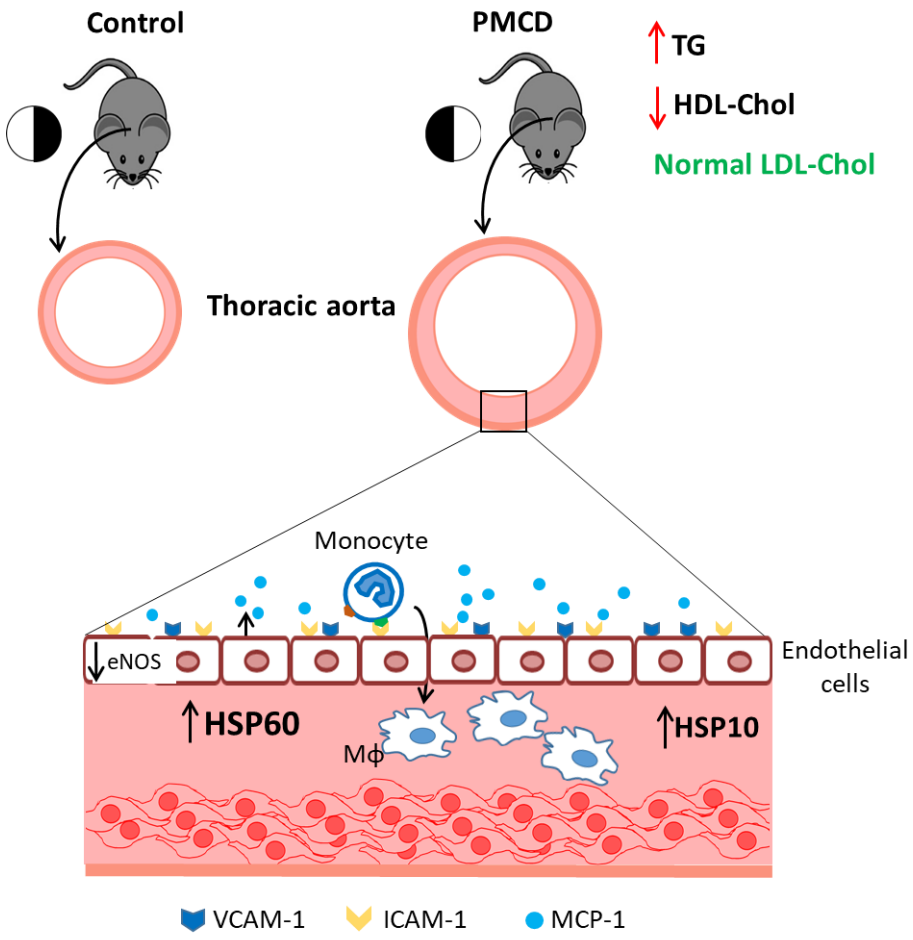

Figure S4: An overview of atherogenic changes in thoracic aorta of C57BL/6J mice fed with laboratory chow and subjected to photoperiodic manipulation induced chronodisruption (PMCD).