**Synopsis of the thesis entitled**

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

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Ph.D student

**Ms. Kavita S. Shirsath**

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Research Guide

**Dr. Ranjitsinh Devkar**

Asst. Professor of Zoology

**Department of Zoology,**

**Faculty of Science,**

**The Maharaja Sayajirao University of Baroda, Gujarat, INDIA.**


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## Introduction

Lifestyle has long been associated with the development of many chronic non-communicable diseases (NCDs). The harmful effects of daily habits likes unhealthy diet, lack of physical inactivity, tobacco and alcohol abuse and abnormal biological rhythms have become increasingly evident. The current global health scenario identifies non-communicable diseases as a major concern. WHO have clearly identified NCDs to be the leading cause of morbidity and mortality with 41 million deaths recorded in 2016, representing 71% of the global mortality. Further, low- and middle-income countries are the worst affected by NCDs (World Health Organization, Geneva, Switzerland). In India, NCDs account for 61% of annual deaths (Body Burden). The four most common NCDs include cardiovascular diseases, cancers, chronic respiratory diseases (such as chronic obstructive pulmonary disease and asthma) and diabetes. Amongst these, cardiovascular diseases (like heart attack and stroke) account for 17.7 million deaths every year, making it the most lethal disease. Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels such as coronary heart disease, cerebrovascular disease, peripheral arterial disease, deep vein thrombosis and pulmonary embolism. These disorders represent acute effects that usually have atherosclerosis as the underlying cause.

Atherosclerosis 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 development of an atheromatous plaque. The most accepted pathophysiological mechanism initiates with damage to endothelial intima caused by various risk factors associated with atherosclerosis. This endothelial dysfunction involves changes in the vascular permeability allowing the seepage of low density lipoprotein (LDL) molecules into the sub-endothelial space, trans-endothelial migration of monocytes and their differentiation to macrophages. This is followed by reactive oxygen species (ROS) mediated oxidation of LDL and uptake of this oxidized LDL (OxLDL) by macrophages to form foam cells. The subsequent series of events include recruitment of smooth muscle cells (SMC) to the lesion, accumulation of connective tissue components, formation of necrotic core and plaque rupture leading to thrombus formation [1]. Apart from these classical hallmarks, a plethora of key players have been

identified which add to the complexity of the disease progression including the interplay of cells of the immune system besides macrophages [2].

For many years, atherosclerosis was believed to be merely a disease of high lipid content in the body, but a much more-complex picture has become apparent in more recent findings. The treatment interventions developed so far (like statins) are mostly based on lowering the underlying risk factors like hyperlipidemia (National Institute of Health, USA). However, these treatments have not been able to completely cure the patients. These failures may be attributed to the lack of complete understanding of the disease. The need of the hour is to gain insight into molecular and cellular modulations resulting in plaque formation, so as to develop a more specific and potentially safer therapeutic strategy against atherosclerosis.

Atherosclerosis has emerged as an autoimmune disease owing to the immunological research into the pathogenic mechanism. Accumulating evidences have shown T cells infiltration to precede lipid deposition and immigration of macrophages during the initiation of atherogenesis. The antigens leading to T-cell activation are not known with certainty, but clearly involve epitopes of OxLDL and possibly heat-shock protein 60/65 (HSP60/65)[3]. Heat shock proteins (HSPs) are a family of proteins whose expression increases at elevated temperatures or other stresses. These proteins facilitate folding of misfolded proteins, thereby preventing their intra-cellular aggregation [4-5]. HSPs have been reported to be associated with CVDs wherein, HSP60 has a direct claim of being atherogenic in both experimental and clinical studies. Because all the humans are exposed to bacterial HSP60 by infection or vaccination, they show a high risk of cross-reactivity with autologous HSP60 expressed by stressed arterial endothelial cells [6]. Thus, the preexisting immunity to HSP60 leads to a humoral and cellular immune response against it, which results in inflammatory cascades and progression of atherosclerosis [7-10]. HSP10 acts an inevitable co-factor for the protein folding activity of HSP60 but is yet less explored for its role in atherogenesis.

Taken together, the literature survey implies towards the existing research lacunae in regards to the role of HSP60 and HSP10 in atherosclerosis. This study highlights focused investigations on 1)

endothelial cells and its dysfunction 2) macrophage polarization. Validation of the hypothesis has been furthered in C57Bl/6J mouse model wherein relevant parameters have been investigated.

## Objectives

The study is divided into following objectives:

**Objective 1**: Assessing the role of HSP60 and HSP10 in manifesting atherogenic changes and endothelial dysfunction.

This objective shall be achieved through following studies:

Study 1: OxLDL mediated atherogenic changes in endothelial cells and subsequent role of HSP60 and HSP10.

Study 2: Overexpression of HSP60 and assessment of endothelial dysfunction in absence of OxLDL.

**Objective 2:** Investigating the role of HSP60 modulations in macrophage polarization and inflammation

This objective shall be achieved through following studies:

Study 1: Impact of OxLDL on HSP60 of macrophages.

Study 2: OxLDL mediated atherogenic changes in HSP60 downregulated macrophages.

**Objective 3:** Atherogenic remodeling in thoracic aorta and role of HSP60.

This objective shall be achieved through following studies:

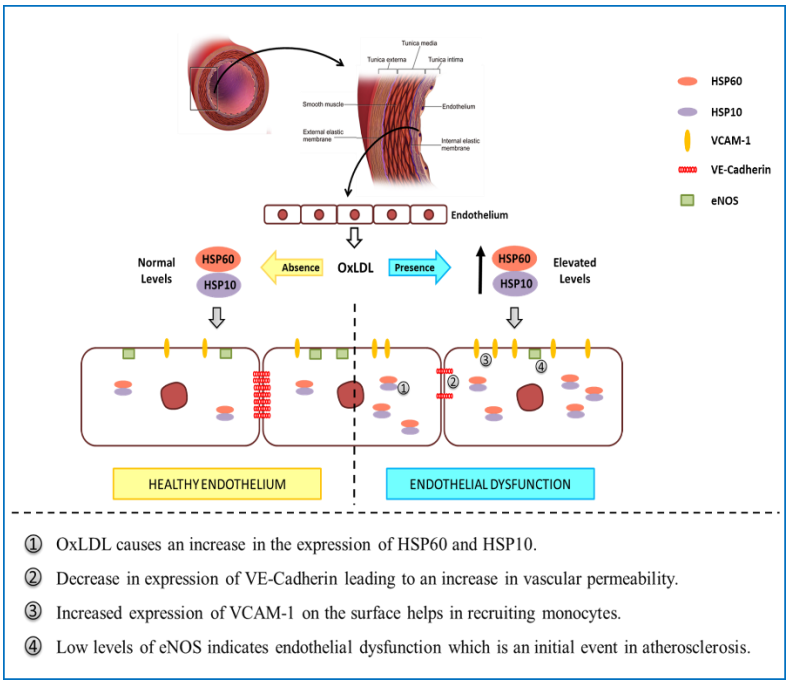
Study 1: Status of HSP60 in diet induced atherogenic mice.

Study 2: Status of HSP60 in photoperiod induced atherogenic changes in mice.

## Observations

**Objective 1: Assessing the role of HSP60 and HSP10 in manifesting atherogenic changes and endothelial dysfunction.**

Study 1: OxLDL mediated atherogenic changes in endothelial cells and subsequent role of HSP60 and HSP10.




(Figure modified from Wikipedia)

The cytotoxicity of OxLDL in HUVEC was assessed by trypan blue exclusion assay. We observed a dose dependent toxicity of OxLDL in HUVEC. The pathologically relevant levels [11] of OxLDL (80 µg/ml) showed ~27% cell death. Further, HUVEC were treated with OxLDL (80µg/ml) and the expression of HSP60 was assessed by RT-PCR and immunofluorescence staining. Also, mRNA expression of HSP10, HSP70, VCAM-1, VE-Cadherin and eNOS was examined by RT-PCR. Cells exposed to 173mM NaCl (mimicking hypertension) [12] and heat shock (42°C) were used as positive control. Interestingly, the results of gene expression study as well as immunocytochemistry showed an increase in the expression of HSP60 in OxLDL treated cells (Fig 1 and 2). Further, the expression of HSP10 (co-factor for HSP60) and HSP70 also increased in OxLDL treated HUVEC. These modulations in the heat shock genes was in sync with endothelial

dysfunction as determined by upregulation of VCAM-1 and downregulation of VE-Cadherin and eNOS in presence of OxLDL. The results of surface staining of HSP60 is under analysis. Overall, the results suggest that the overexpression of HSP60 induced by accumulated OxLDL may be one of the mechanisms by which OxLDL contributes to atherogenesis. However, HSP10 and HSP70 also appear to participate in this pathogenic mechanism.

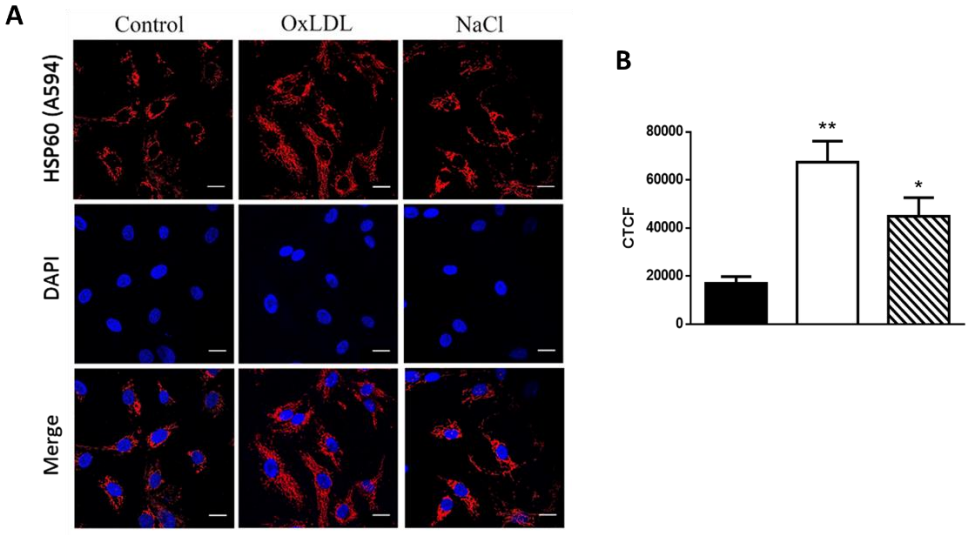



Figure 1: Effect of OxLDL on HSP60 expression in HUVEC. Cells treated with 80µg/ml OxLDL and 173mM NaCl were stained with antibody against HSP60 (A) Representative images of stained cells. Scale bar = 20 µm. (B) Quantification of HSP60 fluorescence intensity. Data Mean ± SEM from three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001 compared to control. CTCF- Corrected Total Cell Fluorescence.

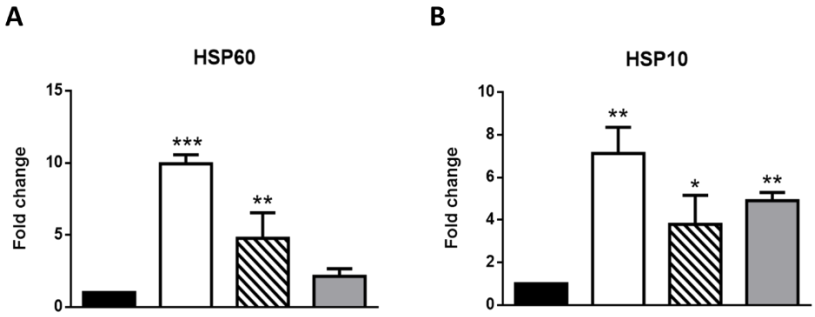
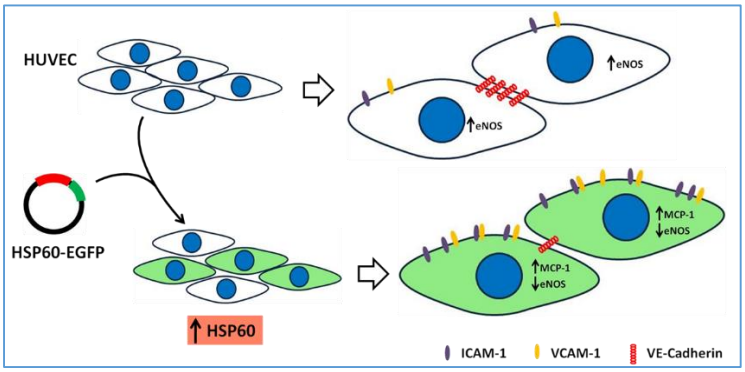



Figure 2: Gene expression in HUVEC: mRNA levels of (A) HSP60 and (B) HSP10 are shown, wherein GAPDH was endogenous control. Data Mean ± SEM from three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001 compared to control.

Study 2: Overexpression of HSP60 and assessment of endothelial dysfunction in absence of OxLDL.




In this study, pCMV3-HSPD1-GFPSpark was transfected in HUVEC and the transient overexpression of HSP60 was confirmed by observing the cells for GFP fluorescence using FLOID imaging station (Thermo Scientific, USA) and RT-qPCR (Fig 3). Further, mRNA expression of genes associated with endothelial dysfunction were checked by RT-PCR. HUVEC overexpressing HSP60 showed increase in VCAM-1 and decrease in VE-Cadherin and eNOS expression indicating similar to OxLDL treated cells. This suggests that HSP60 overexpression in HUVEC induces endothelial dysfunction. Surprisingly, we observed an increment in the expression of HSP10 in cells overexpressing HSP60, suggesting a HSP60 dependent induction of HSP10. However, this needs further experimental evidences. Also, an increase in the expression of inflammatory cytokine IL-6 was recorded in condition of HSP60 overexpression. Thus, the results of this study clearly show that HSP60 upregulation is itself capable of inducing endothelial dysfunction even in absence of OxLDL.

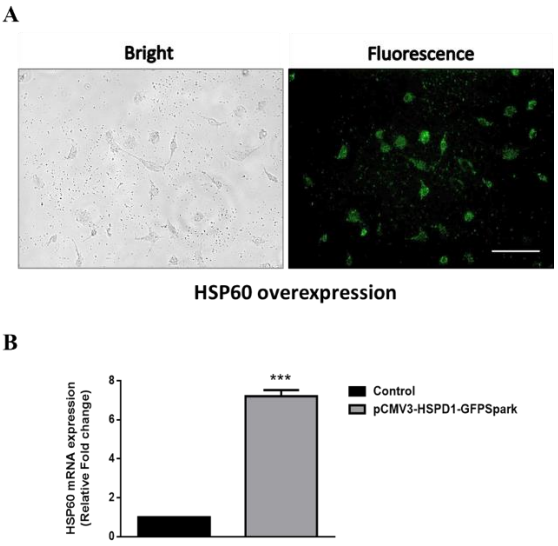
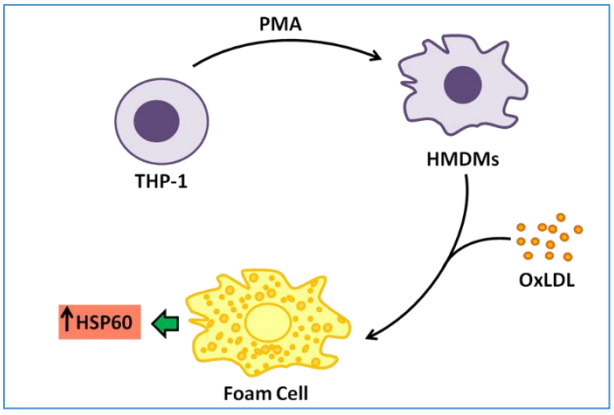



Figure 3: HSP60 overexpression in HUVEC. Cells were transfected with pCMV3-HSPD1-GFPSpark using Lipofectamine 3000 reagent. (A) Representative images of transfected cells. Scale bar = 100 µm. (B) HSP60 mRNA expression in transfected cells. Cells transfected with vector were used as control. GAPDH was used as endogenous control. Data Mean ± SEM from three independent experiments. ***p≤0.001 compared to control.

**Objective 2: Investigating the role of HSP60 modulations in macrophage polarization and inflammation**

Study 1: Impact of OxLDL on HSP60 of macrophages.




THP-1 macrophages were differentiated by exposing the cells to 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24h followed by treatment with 8 (low dose) and 80 µg/ml (high dose) OxLDL. The uptake of OxLDL by THP-1 macrophages was checked by Oil Red O staining and the gene expression were studied by RT-qPCR. Results showed a dose dependent accumulation of

OxLDL in cells indicating foam cell formation. Simultaneously, the expression of SRB-1 (scavenger receptor) was found to increase in OxLDL treated cells. mRNA levels of IL-1β and IL-6 were also upregulated in OxLDL treated cells indicating inflammatory response. All these events represent atherogenic changes caused by OxLDL in macrophages. In addition, we observed a significant increment in the expression of HSP60, HSP10 (Fig 4), HSP70 and HSF-1 (transcription factor for HSPs in presence of OxLDL. Thus, the results suggest that HSP60, along with other HSPs is involved in OxLDL induced atherogenic changes in macrophages.

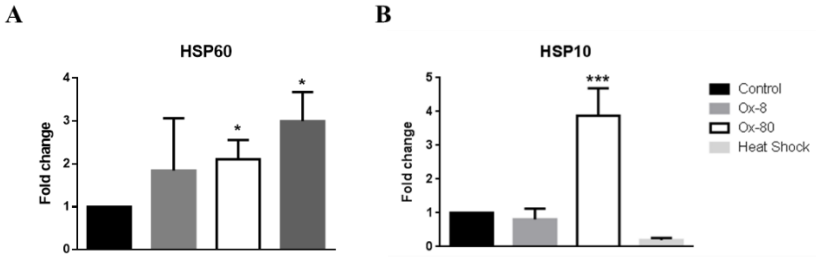



Figure 4: Gene expression in THP-1 macrophages: mRNA levels of (A) HSP60 and (B) HSP10 are shown, wherein GAPDH was endogenous control. Data Mean ± SEM from three independent experiments. *p≤0.05, ***p≤0.001 compared to control.

Study 2: OxLDL mediated atherogenic changes in HSP60 downregulated macrophages.

THP-1 macrophages were differentiated by exposing the cells to 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24h followed by transfection with pshRNA-609 (kind gift by Dr. Thomas Corydon, University of Aarhusm, Denmark) for 48h [13]. The knockdown of HSP60 (by pshRNA-609) was confirmed by RT-PCR and western blotting (Fig 5). Further, the results of uptake of OxLDL by HSP60 knockdown macrophages and the relevant gene expression is under analysis.

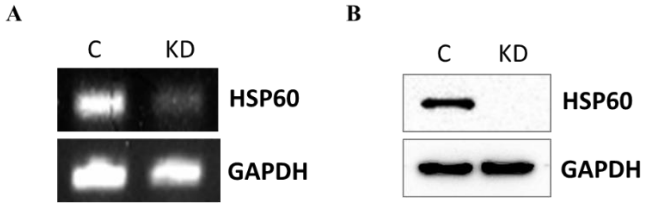



Figure 5: HSP60 knockdown in THP-1 macrophages: Expression of HSP60 checked by (A) RT-PCR and (B) western blotting. C: Control, KD: HSP60 knockdown.

**Objective 3: Atherogenic remodeling in thoracic aorta and role of HSP60.**

Study 1: Status of HSP60 in diet induced atherogenic mice.

C57Bl/6J male mice each weighing 20-22g (6 weeks of age) were obtained from ACTREC, Navi Mumbai, India. The experimental protocol was executed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and approved by the animal ethical committee of the Department of Zoology, The M.S. University of Baroda, Vadodara (Reg No. 827/GO/Re/S/04/CPCSEA). Animals were divided into two groups: Control (fed with standard laboratory chow diet) and HFHF (fed with high fat with high fructose) in rooms maintained in 12hr light/12hr dark cycles (24hr LD cycles) with moderate temperature for 16 weeks after which they were euthanized. The thoracic aorta were collected and assessed for atherogenic changes via histolopathological analysis and gene expression studies. Our results showed an increase in the medial thickness of the aortic wall (Fig 6) and lumen perimeter in HFHF mice as compared to control mice suggesting aortic thickening. Also, the derangement of elastin lamellae was observed in elastin autofluorescence (Fig 6) and a corresponding increase in elastin fragmentation was recorded in HFHF mice. However, mRNA levels of elastin were found to increase indicating initial reparative response. Picrosirius red stained sections showed an increase in collagen content in HFHF mice compared to control (Fig 6). mRNA expression of collagen-I and collagen-III was increased in HFHF mice indicating aortic stiffening. However, the results of RT-qPCR and western blotting did not showed any change in the levels of HSP60 and HSP10. The results of immunohistochemistry is under scrutiny. These results suggest that mice subjected to high fat diet along with high fructose induces atherogenic remodeling of thoracic aorta. However, modulations in HSP60 does not play a role at this stage of the disease progression.

Study2: Status of HSP60 in photoperiod induced atherogenic changes in mice.

C57Bl/6J male mice each weighing 20-22g (6 weeks of age) were obtained from ACTREC, Navi Mumbai, India. The experimental protocol was executed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and approved by the animal ethical committee of the Department of Zoology, The M.S. University

of Baroda, Vadodara (Reg No. 827/GO/Re/S/04/CPCSEA). Animals were divided into four groups: Control (fed with chow diet and kept in normal 12h light/12h dark cycle), CD (fed with chow diet in normal 12h light/12h dark cycle), CD+HFD (fed with HFHF and undergoing photoperiodic manipulation) and CD+HFD+M HFD (fed with HFHF, undergoing photoperiodic manipulation along with timed administration of 10mg/kg melatonin) for 16 weeks after which they were euthanized. The thoracic aorta were collected and assessed for atherogenic changes via histolopathological analysis and gene expression studies. The preliminary observation of the HXE stained slides showed a marked thickening of aorta wall in CD and CD+HFHF group. Surprisingly similar changes were observed in CD+HFHF+M group (Fig 6). The quantitative analysis revealed a significant rise in the medial thickness in all the three groups, with CD showing the maximal effect. However, we observed increment in lumen perimeter only in CD group compared to control. The elastin lamellae distortion and elastin fragmentation was found to be maximum in CD group (Fig 6). The mRNA levels of elastin increased in CD group, however, it was comparable to control in CD+HFD group suggesting that the combined effect of photoperiodic and dietary manipulations stop the reparative changes in elastin expression, which is observed when each of these manipulations were executed separately. Collagen staining showed an increment in collagen content in all the three groups (Fig 6), while mRNA of collagen-I increased in CD and CD+HFD group and collagen-III in CD group. CD+HFHF+M group did not show any change in collagen mRNAs. These results collectively show a prominent effect of CD in atherogenic remodeling either alone or in combination with HFHF. Melatonin, however, did not show much protective effect despite being known for its anti-atherogenic activity. Further, the gene expression study revealed a significant upregulation of HSP60 and HSP10 in CD group as compared to control. Both the proteins decreased in CD+HFHF and CD+HFHF+M group compared to control. This indicates a pathogenic role of HSP60 and HSP10 in photoperiod induced atherogenic changes in thoracic aorta. Further, the results of IHC are currently under analysis.

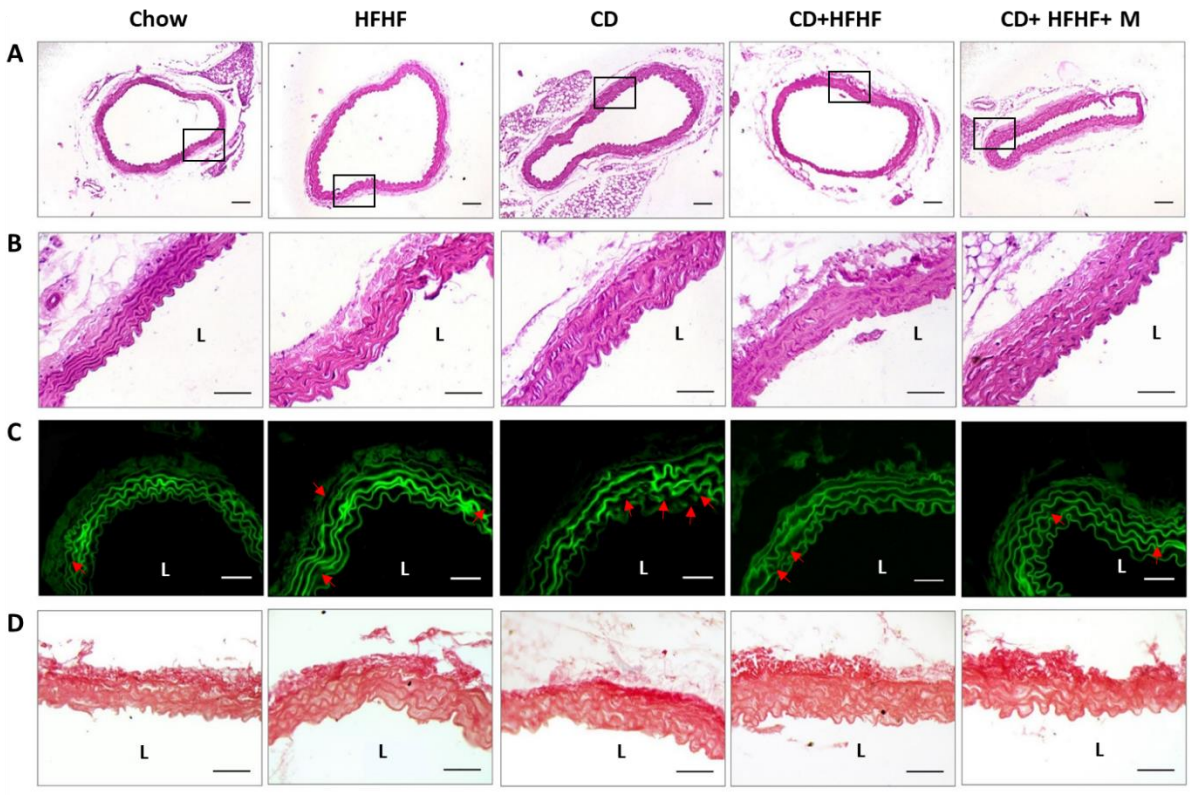



Figure 6: Histological analysis of thoracic aorta section showing (A) HXE staining, (B) its higher maginification showing detailed architechture; Scale bar= 100 µm, (C) elastin autofluorescence; scale bar=50µm and (D) picrosirus red staining for collagen; Scale bar= 50 µm. Red arrows indicate elastin breaks.

## Key Findings

- OxLDL induces atherogenic changes in HUVEC via upregulation of HSP60 and its co-chaperone, HSP10 and modulating the subcellular localization of HSP60. Upregulation in the levels of HSP70 were also recorded, suggesting an interplay of these heat shock proteins being crucial for OxLDL mediated atherogenic changes in endothelial cells.
- HSP60 overexpression is capable of inducing atherogenic changes in HUVEC even in absence of OxLDL, providing an explanation for endothelial dysfunction preceding oxidation of LDL.
- HSP60 was identified as a key player in macrophage polarization and inflammatory events induced by OxLDL. Subtle increment in the expression of HSP10 and HSP70 were also observed.

- The *in vivo* studies showed that dietary and photoperiodic manipulations induce atherogenic remodeling of thoracic aorta. The gene expression analysis showed significant upregulation of HSP60 in thoracic aorta of mice undergoing photoperiodic manipulation. The other heat shock genes (*hsp10, hsp70*) also increased in thoracic aorta of this group indicating an association of biological rhythm and heat shock proteins in atherogenic remodeling of thoracic aorta.

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Ph.D Student — **Ms. Kavita S. Shirsath**

Research Guide — **Dr. Ranjitsinh Devkar**