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RESTORATION OF AUTOPHAGIC FLUX IN VASCULAR ENDOTHELIAL CELLS TO PREVENT ATHEROSCLEROSIS LESION DEVELOPMENT

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ABSTRACT

Atherosclerotic lesions preferentially develop in arterial areas exposed to low shear stress where endothelial cells express a pro-inflammatory and pro-senescent phenotype. Defective endothelial autophagy also favours an atheroprone phenotype in low shear stress areas. Autophagy is a cellular mechanism in-charge of recycling damaged organelles and proteins. Fusion of autophagosomes and lysosomes is a critical step in autophagy that is regulated by stabilization of microtubules by post-translational acetylation. This study tests the hypothesis that inhibition of endothelial deacetylase HDAC6 stabilizes α-tubulin and increases endothelial autophagy flux in low shear stress conditions, promoting an atheroprotective phenotype despite unfavourable shear stress conditions. Inhibiting endothelial HDAC6 activity in low shear stress using Tubastatin-A raised levels of acetylated α-tubulin about 50-fold significantly enhanced the LC3-II/I ratio, LC3 punctae and the autophagy flux. Tubastatin-A significanty reduced the expression of endothelial pro-inflammatory markers like ICAM-1, VCAM-1 and MCP-1, in cells exposed to low shear stress and TNF-α. Similar observations were made in endothelial cells where HDAC6 expression had been reduced by 50% using shRNA. Lack of HDAC6 led to increased acetylation of tubulin, conversion of LC3-I to LC3-II and reduced expression of inflammatory markers, ICAM-1 and MCP-1. In order to link the effect of stabilization of the microtubule network via acetylation, the pro-autophagic and the anti-inflammatory phenotype we observed, we used cells that were lacking a key autophagy protein ATG5, using shATG5. Expression of ATG5 was reduced approximately by 50-55%. We observed a pro-inflammatory phenotype in ATG5-deficient cells, which was not rescued by Tubastatin-A treatment. Atherosclerotic plaque size was significantly decreased in the aortic arch of chimeric HDAC6$^{-/-}$/ApoE$^{-/-}$mice transplanted with HDAC6$^{+/+}$/ApoE$^{-/-}$ bone marrow, when compared to chimeric HDAC6$^{+/+}$/ApoE$^{-/-}$ control mice. Taken altogether, these results indicate that HDAC6 inhibition may be an interesting strategy to restore endothelial autophagic flux by stabilising microtubules and to promote an atheroprotective endothelial phenotype despite unfavorable shear stress conditions.

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ABBREVIATIONS

4-HNE	4-Hydroxynonenal
5SLC7A5	Solute Carrier Family 7 Member
ABCA1	ATP-Binding Membrane Transport Proteins
ACE	Angiotensin Convertinng Enzyme
ADAM-17	A Sheddase, Disintegrin And Metalloproteinase Domain-Containing Protein 17
AGEs	Advanced Glycation End Products
AMPK	5'-AMP-Activated Protein Kinase
BAG1	Bcl2-Associated Athanogene 1
Bif-1	Bax-Interacting Factor-1
CALCRL/RAMP2	Calcitonin Reeptor Like/ Receptor Activity Modifying Protein 2
cAMP	Cyclic Adenosine Monophosphate
CHIP	Carboxy-Terminal HSC70-Interacting Protein
CVDs	Cardiovascular Diseases
DAPK	Death Associated Protein Kinases
EETs	Epoxyeicosatrienoic acids
EGFR	Epidermal Growth Factor Receptor
EDHF	Endothelium-Derived Hyperpolarizing Factor
ENO2	Enolase 2
eNOS	Endothelial Nitric Oxide Synthase
EPG5	Ectopic P Granules Protein 5
ESCRT	Endosomal Sorting Complexes Required for Transport
ET-1	Endothelin-1
FYCO1	FYVE And Coiled-Coil Domain-Containing 1

GEFs	Guanine Nucleotide Exchange Factors
GSK-3β	Glycogen synthase kinase-3β
GLUT1	Glucose Transporter 1
H_2S	Hydrogen Sulphide
HIF-1α	Hypoxia-Inducible Factor 1α
Hsp70	Heat-Shock Protein70
HUAECs	Human Umbilical Aortic Endothelial Cells
HUVECs	Human Umbilical Venous Endotheial Cells
ICAM-1	Intercellular Adhesion Molecule-1
IFN- γ	Interferon- γ
IL-1	Interleukin-1
IL-8	Interleukin-8
INPP5E	Inositol Polyphosphate-5 Phosphatase E
JAM-1	Junctional Adhesion Molecule-1
JNK	c-Jun N-terminal Kinases
LAMP2A	Lysosomal Associated Membrane Protein Type 2A
LIR	LC3-interaction Region
LBRC	Lateral Border Recycling Compartment
LDLR	LDL Receptor
LFA1	Lymphocyte Function-Associated Antigen-1
MAPK	Mitogen-Activated Protein Kinase
MCP-1,	Monocyte Chemoattractant Protein
MIPA	Micropexophagy-Specific Membrane Apparatus
MMP	Matrix Metalloprotease

NF-Kb	Nuclear Factor Kappa Light Chain Enhancer Of Activated B Cells
NFAT1	Nuclear Factor of Activated T-Cells 1
NO	Nitric Oxide
NOX1	NADPH Oxidase 1
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
ORP1L	Oxysterol-Binding Protein-Related Protein 1
Ox-LDL	Oxidized-LDL
PAK	p21 Activated Kinase
PKC	Protein kinase C
PDGF	Platelet Derived Growth Factor
PECAM-1	Platelet Endothelial Cell Adhesion Molecule
PGI2	Prostacyclin
PI3KC2α	Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2- A
PI3P	Phosphatidylinositol 3-Phosphate Ptdins 3P
PINK1	PTEN-Induced Kinase 1
PKA	Protein Kinase A
PLEKHM1	Pleckstrin Homology And RUN Domain Containing M1
PPAR	Peroxisome Proliferator Activated Receptor
Ptdins3k	Phosphatidylinositol 3-Kinase
PTEN	Phosphatase And Tensin Homolog
Rab	Ras-Related Protein In Brain
RAGE	AGE Receptor
RANTES	Regulated On Activation, Normal T Cell Expressed And Secreted

RILP	Rab Interacting Lysosomal Protein
ROS	Reactive Oxygen Species
Ser/Thr	Serine/Threonine
sGC	Soluble Guanylyl Cyclase
SR	Scavenger Receptors
SSRE	Shear Stress Responsive Elements
STK11	Serine/Threonine Protein Kinase
TAZ	Transcriptional Co-Activator With PDZ-Binding Motif
TECPR1	Tectonin Beta-Propeller Repeat- Containing 1
TNF-A	Tissue Necrosis Factor- A
TOR	Target Of Rapamycin
TRP	Transient Receptor Potential
TRPV4	Transient Receptor Potential 4
TSC2	Tuberous Sclerosis 2
UVRAG	Ultraviolet Irradiation Resistant-Associated Gene
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VSMCs	Vascular Smooth Muscle Cells
VLA4	Very Late Antigen-4
vWF	von Willebrand Factor
WHO	World Health Organization
XBP-1	X-Box Binding Protein 1
YAP	Yes-Associated Protein

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INTRODUCTION

A. ENDOTHELIUM

A.1. THE VASCULAR NETWORK

A.1.1 Definition

Arteries, along with veins, capillaries and the heart make up the circulatory system (Figure 1). This highly hierarchical network of vessels is responsible for the transport of nutrients, oxygen and removes waste from the peripheral tissue via blood (Fang and Hirschi 2019). Due to the gradient in their levels, transport and delivery of these circulating components occurs through the high surface are of capillaries.

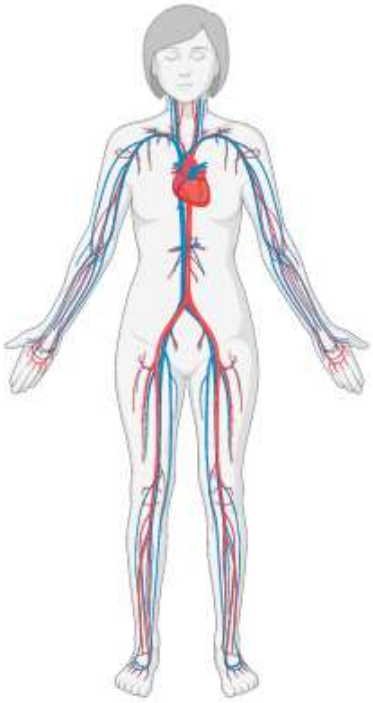


Figure 1: Schematic representation of the human circulatory system (Servier Medical ART)

William Harvey described the closed circulatory network in 1628, as comprising of arteries and veins but could not validate the existence of the connecting vessels, capillaries. Employing a double convex lens, in 1661, Marcello Malphigi was able to visualize the capillaries, as fine blood vessels connecting the arterial and venous systems. Although, several investigators closely studied the circulatory system, the concept of a cellular lining in the walls of the blood vessels remained elusive. About two centuries later, with the use of microscopic tools and silver nitrate staining techniques, Swiss investigator Wilhelm His, identified the lining of the capillary wall. In 1865, he coined the term "endothelium" to differentiate this lining of vessels from the lining of body cavities. The initial definition was as follows: "*cell lining of blood vessels, lymphatics, and mesothelial-lined cavities*" (Aird 2007). Over the following years, with the help of advancements in microscopic techniques, key features of endothelial cells were identified. Electron Microscopy images revealed the presence of specialized organelles in these cells, like caveolae then called "plasmalemmal vesicles", Weibel-Palade bodies, which are storage granules specific to endothelial cells, and junctional complexes (Aird 2007).

Structurally, blood vessels consist of three main layers: the innermost intima, made up of a smooth muscle layer lined by endothelial cells; the second media layer, and the outermost adventitia (Figure 2) (Zaromitidou et al. 2016). The *tunica intima* forms the tube-like space for the blood to flow through. It is lined by a monolayer of endothelial cells, supported by a basement membrane or *basal lamina*. The subendothelial layer is an elastic connective tissue layer meant to provide endothelial cells with stability and flexibility. Surrounding the endothelial cells is a layer of pericytes. These are mesenchymal, pluripotent cells, capable of differentiating into many different types of cell populations like fibroblasts, vascular smooth cells or adipocytes (Ivanova and Orekhov 2016). The *tunica media* is made up of layers of smooth muscle cell and elastin fibers, responsible for maintaining blood pressure exerted on the vessel wall by regulating the contraction or dilation (Mercadante and Raja 2020). Finally, the *tunica adventitia* is the outermost layer of the blood vessel and is composed entirely of fibro-elastic connective tissue. The adventitia layer consists of the nerve plexi, which help to control the contraction and relaxation of the

larger blood vessels (Majesky 2018). To facilitate the distribution of nutrients across the entire vessel wall, a branched network of smaller vessels is formed called the *vasa vasorum* (Billaud et al. 2017).

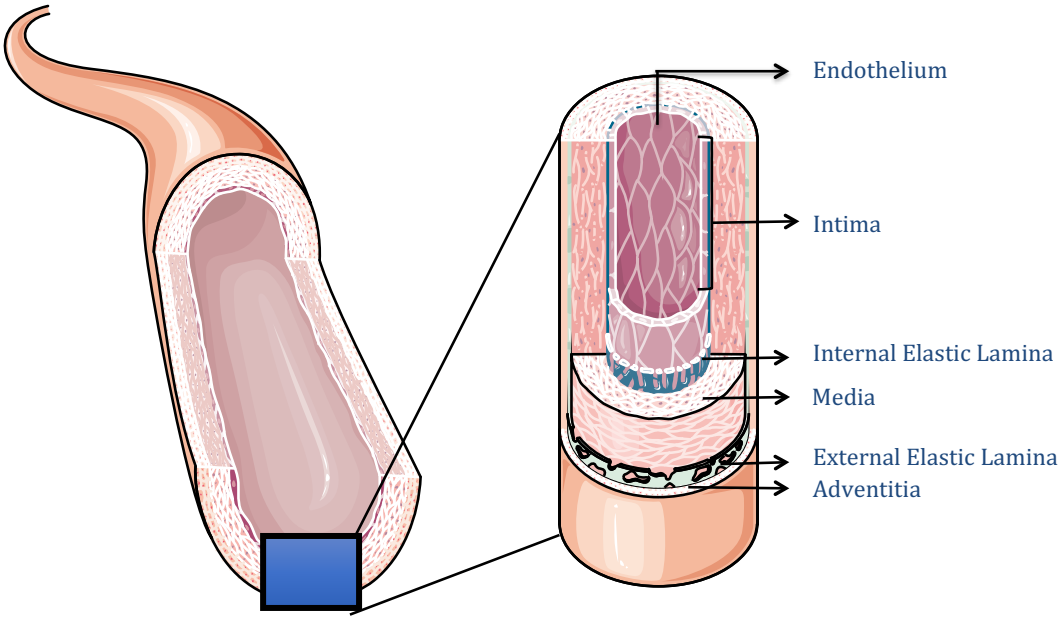


Figure 2: Schematic representation of the aorta and the different layers of the vessel wall (Servier Medical ART)

Blood from the left ventricle is pumped into the aorta, which divides into four different sections: ascending aorta, aortic arch, descending thoracic aorta and abdominal aorta. The ascending aorta divides into carotid arteries which supply blood to the brain (Furuta et al. 2019), the aortic arch carries blood to the back and neck, the descending aorta to the chest and ribs, and the abdominal aorta to the iliac arteries

Endothelial cells are flat, cuboidal cells which can take on different phenotypes like arterial, venous or form capillaries (Majesky 2018). Their dimensions are on an average, 30-50µm in length, 0.1-10µm in thickness and 10-30µm in width (Krüger-Genge et al. 2019). They are polarized cells, aligned in the direction of the blood flow. They are anchored to a basement membrane constituted of glycoproteins secreted by endothelial cells themselves (Krüger-Genge et al. 2019a).

Interestingly, endothelial cells also exhibit heterogeneity across the vascular network ranging from tight junctions underlined by a continuous basement membrane, to a fenestrated lining or a discontinuous endothelium, wherein the endothelium is interspersed with gaps in the lining and a poorly developed basement membrane (Aird 2012). Endothelial cells lining the venous vessels show differences in regulation of vascular permeability and immune cell trafficking when compared to endothelial cells in arteries (Aird 2007).

A.1.2 Structural and Functional Heterogeneity of Endothelial Cells

Endothelial cells have a variety of different functions depending on their location, which contributes to the phenotypic heterogeneity found throughout the vascular network. Arterial endothelial cells are exposed to different blood pressures, blood flow, oxygen and pH levels, than vein endothelial cells. Response to environmental stimuli dictates the morphological modifications (Reiterer and Branco 2020). The concept of heterogeneity in endothelial cells was first described by William Aird. Structurally, endothelial cells exhibit variations in their morphology (size, polarization, thickness), gene expression, release of extracellular matrix and expression of junctional and adhesion proteins (Aird 2012). Endothelial cells differ in size, from aortic endothelial cells being up to 1µm thick to vein endothelial cells at 0.1-0.2µm (Krüger-Genge et al. 2019b). Thickness of the endothelial cells varies based on their localization. The glycocalyx layer has been observed to be thicker in the common carotid artery compared to carotid sinus (Tarbell and Cancel 2016).

Types of endothelium can also be categorized based on their intercellular junctions: continuous, fenestrated or discontinuous. A continuous endothelium is found in the vasculature of the brain, retina, skin, skeletal muscle and lungs; while a fenestrated endothelium is present in the inner lining of endocrine glands, intestinal villi, glomerulus, and renal tubules (Wisse 1970; Aird 2012). Fenestrae can be as wide as 70nm, and are underlined by a 5-6nm thick basement membrane (Félétou 2011). The sinusoidal endothelium in the liver, spleen and bone marrow are discontinuous in nature, and lack any underlining membrane holding the endothelial cells together (Aird 2007).

Barrier function is one of the main defining characteristsics of endothelial cells across the entire vascular network. It depends on the role of tight junctions and adherent proteins in the afore mentioned continuous endothelium (Aird 2007; 2012; Félétou 2011). For example, the barrier properties of endothelial cells, like the transcytosis function, are key in maintaining the blood brain barrier. On the contrary, in tissues which are involved in absorption or filtration, the endothelial barrier is very different. In liver sinusoidal endothelial cells, the discontinuous nature of the endothelium allows the transfer of nutrients and small molecules from the blood to the hepatocytes. The fenestrated characteristic of endothelial cells aids in the rapid uptake or secretion of fluids, solutes and other biomolecules in organs like the kidney or small intestine (Aird 2012; Yazdani et al. 2019).

Endothelial cells can also adapt according to the metabolic needs of the surrounding tissue. Cardiomyocytes, for example, require breakdown of fatty acids to generate ATP, for their contractile function (Umbarawan et al. 2018). In the heart, CD36, a scavenger receptor involved in the uptake of long chain fatty acids, is highly expressed not only in cardiomyocytes, but also endothelial cells of the microvessels (Abumrad and Goldberg 2016). Neurons, on the other hand rely on glucose metabolism for energy. Hence, endothelial cells in the blood brain barrier express the glucose transporter 1 (GLUT1) and solute carrier family 7 member 5 (SLC7A5) (Yazdani et al. 2019).

Variations also occur in the intracellular organization. For example, pulmonary endothelial cells carry a high number of Weibel Palade bodies, which are storage organelles involved in inflammation and permeability (Kaufman and Costanza 2020). These organelles are rare in myocardial capillaries and absent in the thoracic aorta. Endothelial cells in the brain capillaries carry a higher number of mitochondria, present more tight junctions and less pinocytosis, when compared to blood capillaries in the peripheral organs (Kluge et al. 2013). Additionally, while endothelial cells release various factors regulating homeostasis, expression profiles of these proteins differ between arteries, veins and the microvasculature. Arterial endothelial cells express about 5 times more Angiotensin Convertinng Enzyme (ACE) than venous

enodthelial cells. Another distinct difference was observed in the expression of anti-coagulant, tissue-type plasminogen activator (t-PA) (Aird 2001). Smaller *vena cava* vessels expressed higher levels of t-PA than aortic endothelial cells or Human Umbilical Venous Endothelial Cells/ Human Umbilical Aortic Endothelial Cells (HUVEC/HUAECs) (Krüger-Genge et al. 2019b; Snopko et al. 1987). Integrin α1β1, is specifically expressed on endothelial cell surfaces in micro vessels, along with leukocyte trafficking protein, plasma-lemma vesicle-associated protein-1. On the other hand, large vessels have higher expression levels of the Protein kinase C receptor. Pro thrombotic, von Willebrand factor (vWF) is predominantly expressed by venous endothelial cells. Tissue factor pathway inhibitor, is differentially expressed in different organs, for example, its expression is highest in placenta and lungs and lowest in the brain (Aird 2012; Krüger-Genge et al. 2019).

A.1.3 Physiological Role of the Endothelium

i) Vascular Tone

Endothelial cells regulate vascular tone by releasing a combination of several vasoconstrictors like endothelin-1, or vasodilators like nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), prostacyclin, and recently discovered, hydrogen sulphide (H_2S) (Kanagy et al. 2017; Mallat et al. 2017), and epoxyeicosatrienoic acids (EETs) (IMIG 2019).

ET-1 is one of the most potent endogenous vasoconstrictors (Cahill and Redmond 2016). It is produced mainly by endothelial cells, but also by vascular smooth muscle cells, leukocytes and macrophages (Cahill and Redmond 2016). ET-1 and NO are functionally closely interdependent (Lüscher et al. 1990); NO strongly inhibits the release of ET-1 from the endothelium and vice versa (Anggrahini et al. 2009). Elevated levels of ET-1 have been implicated in the pathogenesis of hypertension, vasospasm and heart failure, in addition to atherosclerosis (Ihling Christian et al. 2001). Increased expression of ET-1 and ECE is seen in human arteries at different stages of atherosclerosis. The biological effects of ET-1 are mediated through activation of two receptors, ET_A and ET_B (Davenport et al. 2016). The ET_B receptor is also found on endothelial cells, where its

activation results in vasodilation mediated by PGI_2 and NO release (de Nucci et al. 1988; Cahill and Redmond 2016).

The EETs have actions on both endothelial cells and smooth muscle cells (Imig 2016). Specifically, the four regioisomeric EETs function as endothelium- derived hyperpolarizing factors (Park et al. 2018). In response to acetylcholine, bradykinin, or shear stress, endothelium-derived EETs act on smooth muscle cells to activate large conductance, calcium-activated potassium (BK_{Ca}) channels causing relaxation (Park et al. 2018).

Furthermore, the results of Duflot, et al, demonstrated for the first time in humans, that diabetes modulates EET -mediated pathways and promotes endothelial dysfunction in arteries. They demonstrated that a decrease in EETs production along with perturbed NO bioavailability are associated with endothelial dysfunction in type 2 diabetic patients (Duflot et al. 2019). Acute hyperglycemia or hyperinsulinemia dilates arteries by a NO-dependent mechanism involving endothelial NO- synthase phosphorylation ((Baron et al. 1995; Steinberg et al. 1994).

Vasodilators, released by the endothelium are also responsible for preventing thrombus formation, vascular stenosis (Ghimire et al. 2017) and via the NO/cGMP pathway, slow down cardiac hypertrophy (Park et al. 2018; Nakata Sei et al. 2008). These vasoactive messenger molecules work synergistically with other vasoconstrictors like catecholamines and vasodilators like angiotensin II. Defects in the formation or release of these regulatory molecules can result in endothelial dysfunction, which is also exacerbated by oxidative stress (Ahmad et al. 2018).

The renin–angiotensin system (RAS) is a circulating hormonal system that regulates blood pressure, blood flow, fluid volume and electrolyte balance. RAS is activated locally in the heart and vessel wall have expanded that view and it is now appreciated that it plays a critical role in the initiation and progression of atherosclerosis (Fukuda and Sata 2008). Except renin, all other components of RAS are produced in the vasculature. Endothelial cells express angiotensin converting enzyme (ACE), the dipep-tidyl carboxypeptidase that converts angiotensin-1 to the physiologically active angiotensin-II (Ang-II). Both local and circulating Ang-II exert their activities through the binding to

Ang-II type 1 (AT1) or type 2 (AT2) receptors. the physiological and pathophysiological actions of Ang-II (Cahill and Redmond 2016; Tirapelli et al. 2009)

Endothelial nitric oxide synthase (eNOS) uncoupling, soluble guanylyl cyclase (sGC) desensitization, inactivation of prostacyclin synthase, oxidative activation of the endothelin-1 pathway, and direct inactivation of NO by super-oxide, are some of the mechanisms involved in regulating vascular tone (Daiber et al. 2020). Endothelial cells release NO, hydrogen peroxide, and prostacyclin, in response to mechanical stimuli, shear stress and chemical stimuli in the form of acetylcholine or bradykinin (Ghimire et al. 2017).

Cytosolic Ca^{2+} is a secondary messenger involved in the regulation of vascular tone. Vasodilators elevate cytosolic Ca^{2+}, which in turn opens calcium-activated K+ ion channels like K_{Ca} 2.3 and K_{Ca} 3.1 (Hasenau et al. 2011; John et al. 2018). The elevation in Ca^{2+} levels is due to the sudden release of ions from the ER mediated by Inositol triphosphate, followed by lowered influx of Ca^{2+}. Entry of Ca^{2+} is also facilitated by GPCR signaling via transient receptor potential (TRP) channels. The transient receptor potential 4 (TRPV4) channel helps increase endothelial Ca^{2+} levels and activation of K_{Ca} 3.1. This leads to endothelial polarization, NO release and, when transferred to vascular smooth muscle cells can cause relaxation (Guerra et al. 2018; John et al. 2018; Mallat et al. 2017; Félétou 2009).

Shear stress exerted by blood flow also regulates vascular tone by mediating NO production (Iring et al. 2019). One of the phosphorylation sites regulated by shear stress is serine 1177, which can be phosphorylated by AKT (Iring et al. 2019; Dimmeler et al. 1999). NO causes vasodilation by activating soluble guanylyl cycles in vascular smooth muscle cells (VSMCs) (R. K. Mallat et al. 2017) followed by production of cyclic-GMP, activation of cGMP-dependent protein kinase and serine/threonine (Ser/Thr) kinase dependent phosphorylation, like Ca^{2+} ion channels (Boo et al. 2002; Sriram et al. 2016; Holland et al. 2018).

Hence, the various factors released by endothelium play a criticial role in maintaining the vascular tone of blood vessels, and prolonged endothelial dysfunction has deleterious effects on the pathophysiology of the arterial wall.

ii) Haemostasis

Haemostasis is the mechanism to prevent and stop bleeding from an injured blood vessel (LaPelusa and Dave 2020). Under physiological conditions, circulating platelets do not interact or adhere to endothelial cells (Hamilos et al. 2018). Endothelial cell activation, due to wounds or disease conditions, leads to platelet activation. This is mediated by the release of von Willebrand factor, the appearance of P-selectin on the plasma membrane, the platelet-activating factor and chemokines like platelet factor 4, regulated on activation, normal T cell expressed and secreted (RANTES) and Neutrophil activating factor 2 (Rossaint et al. 2018). Endothelial cells secrete various factors and mediators, which are important for the regulation of blood coagulation and prevent platelet adherence and aggregation. Prostacyclin (PGI2) and NO are the major anti-thrombotic factors secreted by endothelial cells (Mitchell et al. 2008). In addition, endothelial cells also maintain blood fluidity. This occurs by promoting the activity of numerous anticoagulant pathways, whereas the protein C/protein S pathway is the most important. Factors VIIIa and Va, two cofactors which are essential for blood coagulation are inactivated by activated protein C (Stavenuiter et al. 2014).

Tissue plasminogen activator (tPA) is one of the factors released by endothelial cells to convert plasminogen to plasmin. tPA has relatively low levels of expression under basal conditions but increases during injury or lesion formation. The plasmin thus produced destroys the fibrin and dissociates the extracellular matrix allowing degradation of a clot (Tjärnlund-Wolf et al. 2012).

iii) Leukocyte Migration

In physiological conditions, circulating leukocytes rarely interact with endothelial cells. However, in case of injury or lesion, this interaction is facilitated by a cascade of signaling pathways which cause the release of pro-inflammatory chemokines and expression of adhesion molecules (Figure 3). Leukocyte extravasation or diapedesis is initiated by the activation of leukocytes and endothelial cells in response to various cytokines Interleukin-1 (IL-1) and Tissue necrosis factor- α (TNF-α) and chemokines Interleukin-8 (IL-8) (Yeh et al. 2018; McGettrick et al. 2006).

Upon activation by the different mediators, exocytosis of Weibel-Palade bodies from endothelial cells occurs, bringing the selectin molecules to the luminal surface. Endothelial (E)- and platelet (P)-selectin that are expressed on the endothelial apical surface help capture leukocytes and mediate their rolling onto the endothelium via leukocyte (L)-selectin (Filippi 2016). Firm adhesion is regulated by adhesion receptors of the immunoglobulin family, namely leukocyte integrins ((LFA-1: lymphocyte function-associated antigen-1 or $\alpha_L\beta_2$ integrin), Mac-1: macrophage-1 antigen or $\alpha_M\beta_2$ integrin), and VLA-4 : very late antigen-4 or $\alpha_4\beta_1$ integrin)), which bind to their endothelial ligands, including intercellular adhesion molecule-1 and 2 (ICAM-1 and 2) and vascular cell adhesion molecule 1 (VCAM-1), respectively (Muller 2016). Arrest of leukocytes on the endothelium is mediated by a shift towards binding with high-affinity β2-integrins (Shaw et al. 2004).

Following firm adhesion, leukocytes polarize themselves and crawl onto the endothelial apical surface in search for a permissive site of extravasation (Phillipson et al. 2006). Leukocyte motility or crawling depends on asymmetric rearrangement of the leukocyte cytoskeleton in response to chemokines (Ridley et al. 2003). During this process, filamentous actin (F-actin) polymerizes asymmetrically forming the leading edge of the cell, and providing the forces required to propel the cell forward, whereas a network of actin-myosin assembles along the cell sides to prevent lateral membrane protrusions to occur (Williams et al. 2011).

Adhesion of leukocytes to the endothelial cells activates signaling pathways that induce VE-cadherin to be cleared from the site of transmigration to cause gaps along

the inter-cellular junctions (Muller 2016). Engagement of endothelial ICAM-1 and VCAM-1 by leukocyte integrins transduces signals that phosphorylate VE-cadherin which causes weakening of the junctions as the cytoplasmic tail is uncoupled from the actin cytoskeleton (Allport et al. 2000; Muller 2016). Subsequently, the leukocytes engage a sequence of interactions to cross the endothelial barrier, which involve junctional adhesion molecule-1 (JAM-1), platelet endothelial cell adhesion molecule (PECAM-1) and CD99. Leukocyte extravasation can occur across the vascular endothelium either at endothelial cell junctions, called paracellular migration, or through individual endothelial cells, termed as transcellular migration (Ley et al. 2007) via a transcellular channel between the apical and basal membrane facilitating leukocyte transmigration while leaving the endothelial cell junctions intact (Carman et al. 2007).

In both paracellular and transcellular diapedesis, clusters of endothelial ICAM-1 accumulate around adherent leukocytes allowing them to laterally move or "crawl" over the endothelial layer with the help cellular protrusions; the formation of a transmigratory cup made of ICAM-1 clusters and of docking structures as well as the recruitment of PECAM-1, CD99, and JAM-A to leukocyte–endothelial cell contact via the lateral border recycling compartment (LBRC) are also essential (Carman et al. 2007; Mamdouh et al. 2009). These ICAM-1 projections are actin-rich and have been associated with the opening and resealing of junctional gaps that control endothelial permeability during diapedesis (Carman and Springer 2008; Woodfin et al. 2011; Mamdouh, Mikhailov, and Muller 2009). In addition to ICAM-1, other molecules normally thought of to be restricted to the cell borders, including PECAM, JAM-1, and CD99 are seen around the leukocyte migrating transcellularly (Muller 2016). Transmigrating leukocytes extended active protrusions and were surrounded by rings enriched in LFA-1 (Carman et al. 2007). ICAM-1 and VCAM-1 on endothelial cells localize to F-actin-rich docking structures around adherent leukocytes (Carman et al. 2007; Millán et al. 2006). Intermediate filaments, such as vimentin, participate in forming a robust docking structure at the interface between the leukocyte and endothelial cells (Nieminen et al. 2006). As the lymphocytes extend dynamic protrusions into endothelial cells, ICAM-1 clusters then internalized into caveolin-1 and F-actin-rich membrane invaginations and vesicles and translocated to the basal

plasma membrane, such that the cells seemed to glide through the LFA-1 ring leaving a small cluster of LFA-1 on the endothelial apical surface (Millán et al. 2006; Muller 2016; Filippi 2016). Interestingly, caveolin-1 localized more frequently around lymphocytes taking the transcellular route than paracellular (Filippi 2016).

Once the leukocyte has migrated across the endothelial barrier, it still must cross the subendothelial layer. Monocytes move between the abluminal surface of the endothelial cell and the basal lamina searching for areas where collagen IV and laminin are deposited at low density (Voisin, et. al. 2009). These correspond to areas where there is a gap in the layer of pericyte cells. Migration to these sites is aided by interactions between LFA-1 on the leukocyte and ICAM-1 on pericytes (Proebstl et al. 2012)

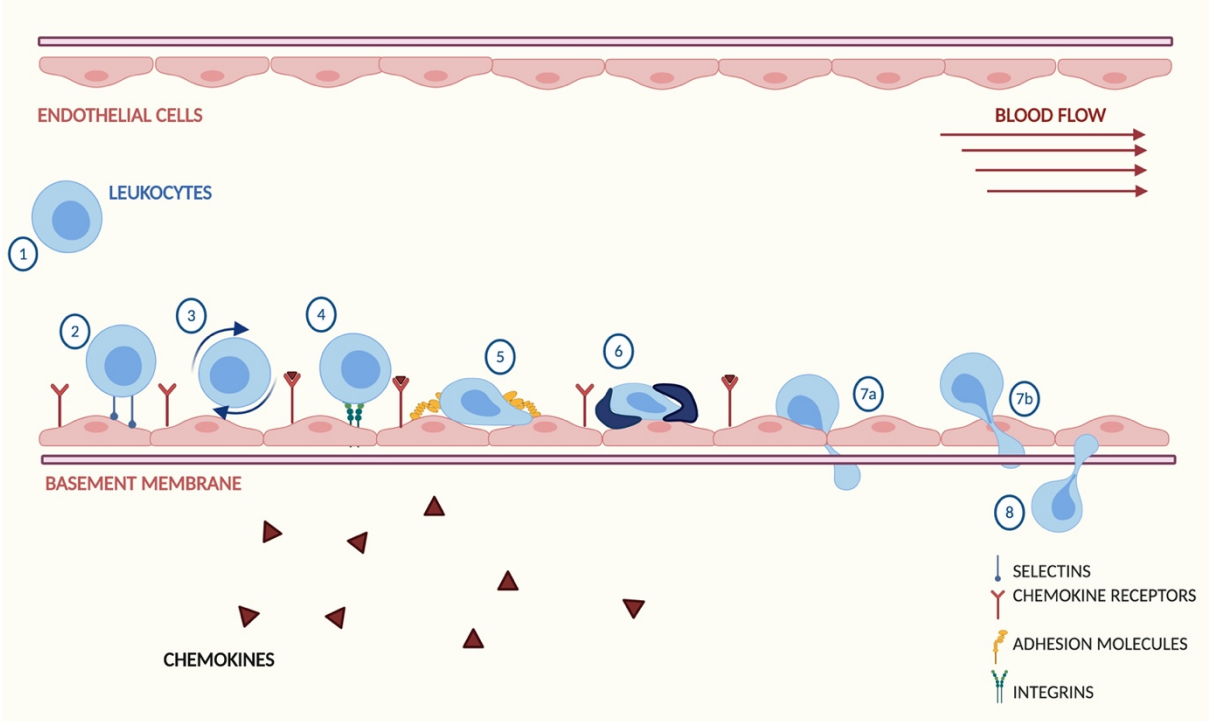


Figure 3: Schematic representation of the mechanism of leukocyte extravasation through the endothelial barrier.

Leukocytes are recruited from the bloodstream by a sequential multi-step cascade. ***(1)*** *Patrolling leukocytes present in the bloodstream are* ***(2)*** *recruited by activated endothelial cells. Leukocytes bind to the luminal surface of the endothelial layer via selectins (P/E/L- selectins). This step is called 'tethering of leukocytes.* ***(3)*** *Following tethering to the endothelial cells, leukocytes start rolling across the endothelial layer by forming stronger interactions with selectins, ICAM-1, VLA-4, LFA-1 (lymphocyte function associated antigen-1).* ***(4)*** *Subsequently, the release of chemokines (CXCL-CXCR) triggers leukocytes and endothelial cells to express integrins on their surface. Interactions via integrins cause stronger bonds to form between the two types of cells, causing 'arrest' of leukocytes.* ***(5)*** *Following this, the leukocytes start crawling.* ***(6)*** *As they crawl along the endothelial barrier, leukocytes form a structure called the transmigratory cup. This helps leukocytes to cross the endothelial barrier either via* ***(7a)*** *paracellular route or* ***(7b)*** *transcellular route.* ***(8)*** *Once they have crossed the single-cell layer, they must cross the basement membrane and the pericyte layer, which is facilitated by the different integrins, adhesion molecules and laminins. Here they continue to participate in the feedback mechanism loop by aggravating the inflammation process*

A.2 ENDOTHELIUM AND SHEAR STRESS

Endothelial cells are lined by a matrix on their luminal and basolateral side, called the glycocalyx, It is the apical or lumen-facing side of the glycocalyx layer that has been implicated in mechanotransduction (Zhang et al. 2018; Zeng et al. 2018). This layer is composed of glycoproteins, proteoglycans and glycosaminoglycans, along with several soluble proteins, either of endothelial origin or derived from the plasma, incorporated into this network (Reitsma et al. 2007). On average, the thickness of the glycocalyx layer is 1-5 μm (Tarbell and Cancel 2016). Although it is largely viewed as an intact layer serving as a vascular barrier, the composition and structure are altered with changes in shear stress arising from different blood flow patterns (Chatterjee 2018), and chemical factors, like the pH, or mechanical forces which contribute to the continuous dynamics of formation and shedding of the layer (Cruz-Chu et al. 2014). It functions as a macromolecular sieve, which allows only certain plasma proteins, up to the size of 70kDa, to permeate. The negative charge repels all negatively charged biomolecules, erythrocytes, leukocytes and platelets (Reitsma et al. 2007). The negative charge is attributed to the presence of glycosaminoglycans, bound to proteoglycan cores (Reitsma et al. 2007; Zeng et al. 2018).

Glycosaminoglycans are polysaccharide chains of varying lengths and levels of sulphation. like heparan sulphate, dermatan, keratan, and chondroitin sulphates, and long chains of hyaluronic acid (Zhang et al. 2018); which are linked to the endothelial cell surface by proteoglycans and glycoproteins, like CD44 (Reitsma et al. 2007). The proteoglycans are mainly comprised of two families of proteins: glypicans and syndecans; play an important role in integrating the glycocalyx layer with the endothelial cell body Cruz-Chu et al. 2014). Glypicans are glycosyl phosphatidylinositolated proteins and are responsible for binding to the caveolae in the cell membrane. Glypican-1 via GPI anchors interacts with heparan sulphate. Syndecans- 1,2 and 4 interact with heparan sulphate, and Syndecan-1 also harbors sites for linkage with hyaluronic acid (Fu and Tarbell 2013). The absence of syndecans or heparan sulphate is considered as a clinical marker for glycocalyx degradation (Johansson et al. 2011). Glycoproteins, like selectins, integrins, and immunoglobulins (Zeng et al. 2018) add to the composition and integrity of the

glycocalyx layer. E-selectin and P-selectin are the most commonly expressed selectins on endothelial cells, and they are responsible for interactions of endothelial cells with leukocytes (Tarbell and Pahakis 2006; Sperandio 2006). Integrins are heterodimeric protein complexes made of a and b-sub-units. They are expressed on several cell types including endothelial cells, leukocytes, platelets. Integrin $\alpha_v\beta_3$ is expressed on the luminal side of endothelial cells and helps form endothelial-platelet interactions, while $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, help bind to extracellular matrix components like fibronectin and collagen (Reitsma et al. 2007).

The glycocalyx, via its intracellular domain, acts a mechanosensor for shear stress. Under laminar blood flow, exerting high shear stress, the glycocalyx layer is robust and protects the endothelium. It has been mainly implicated in the regulation of NO production in endothelial cells in response to blood flow induced shear stress (Tarbell and Cancel 2016; Dragovich et al. 2016; Xiaohui Zhang et al. 2018a; Florian et al. 2003). The two proposed mechanisms as per Davies, for mechanotransduction by glycocalyx to regulate NO production are: centralized and decentralized mechanism (Davies 2009; Davies 1995). The centralized pathway involves the signal transduction via glypicans linked to hyaluronic acid which stimulates eNOS, residing in the caveolae along with other signaling molecules (Tarbell and Pahakis 2006). Some studies suggest the involvement of CD44 receptors present in the caveolae, to bind to heparan sulphate and participate in the signaling pathway. The decentralized mechanism to initiate signaling pathways involves syndecans linked to heparan sulphate and chondroitin sulphate, which associate with the cytoskeleton in order to disseminate the signal to multiple sites in the cell, like focal adhesions, nucleus, different organelles or intercellular junctions; (Tkachenko et al 2005; Fu and Tarbell 2013).

Another mechanism which could possibly regulate glycocalyx mediated NO production is enzymatic degradation of the glycocalyx (Florian et al. 2003). Several known enzymes like A sheddase, disintegrin and metalloproteinase (MMP) domain-containing protein 17 (ADAM-17) and heparanase, have been shown to exacerbate the degradation process due to reduced threshold for oxidative stress, in the absence of deacetylase enzyme Sirtuin 1 (Zhang et al. 2018).

ii) Caveolae

Caveolae, are “flask-shaped” non-coated, mechanotransducing invaginations of the cell membrane that play a critical role in lipid homeostasis and signal transduction (Figure 4). They are rich in cholesterol, sphingolipids, and a variety of signaling molecules, such as G-protein-coupled receptors, heterotrimeric G proteins, Transient receptor protein (TRP) cation channel subfamily V number 4, receptor tyrosine kinase and Transforming Growth Factor β I/II(TGFβRI/II) (Shihata et al. 2016).

Caveolins are integral proteins embedded as a hairpin loop in the inner leaflet of the lipid bilayer with both the N- and C-termini facing the cytoplasm (Gu et al. 2017). There are three main isoforms of Caveolin, Caveolin-1, 2, and 3. Caveolin-1 and -2 are highly expressed in non-muscle and smooth muscle cells (Murata et al. 2007) . Endothelial cells express high levels of Caveolin-1, which is involved in endothelial inflammation, adhesion and phagocytosis. Various studies have investigated the complex role of Caveolin-1 in atherogenesis in different cell types. While some studies have reported that in endothelial cells, caveolae and caveolin-1 favors atherogenesis by promoting transcytosis of low density lipoproteins (LDL) from bloodstream to the sub-endothelial layer and disrupts the vascular tone by inhibiting the release of eNOS, others have reported that in vascular smooth cells, Caveolin-1 plays an anti-atherogenic role by suppressing cell proliferation and migration. On the other hand, Caveolin-1 expression in macrophages exacerbates atherosclerosis development by increasing phagocytosis and modulating cholesterol metabolism. Overall, it has been viewed that Caveolin-1 enhances LDL transport, reduced eNOS release and macrophage infiltration and accumulation in order to influence plaque development.

Atheroprotective laminar flow, in comparison to static conditions, increases the number of caveolae and expression of Caveolin-1, thereby increasing polarization of caveolae/cav-1 towards the upstream portion of endothelial cells in the direction of the flow (Harding et al, 2018). Caveolae formation at the membrane enables endothelial cells to receive extracellular stimuli and induces intracellular responses, making them essential for endothelial cells. However, other studies have reported the

opposite, wherein the absence of Caveolin-1 provides atheroprotection (Xinbo Zhang et al. 2020). Caveolin-1 has also been shown to be involved in autophagy activation in adipocytes and cardiomyocytes (Sun et al. 2010; Wu et al. 2017). Knocking down of Caveolin-1 in bovine aortic endothelial cells *in vitro* increases the expression of LC3-II, implying that Caveolin-1 is a key player of autophagy (Shiroto et al. 2014). The absence of Caveolin-1 attenuates the initiation of atherosclerosis by promoting autophagic flux in vascular endothelial cells exposed to autophagy stimulation in vitro and in early atherosclerotic lesions in vivo. Caveolin is involved in the autophagy mechanism by interacting with the ATG5-ATG12 protein complex. The absence of Caveolin-1 influences the distribution of ATG5 and enhances the release of ATG5 from other lipid fractions in the plasma membrane, which could possibly lead to increased formation of autophagosomes, and autophagic flux overall (Xinbo Zhang et al. 2020). Caveolin-1 deficient mice have also exhibited lowered endothelial cell activation and cholesteol influx into the arterial wall (Fernández-Hernando et al. 2009; 2010).

Caveolin-1 is able to form homodimers and heterodimers with G protein-coupled receptors, tyrosine kinases, eNOS and some components of the MAPK pathway. Studies have demonstrated that loss of caveolae in mice impairs endothelium-dependent regulation of vascular tone. Caveolae are also implicated in the regulation nitric oxide (NO) production. Activated eNOS is then enabled to produce and release NO, after uncoupling from the Caveolin-1 scaffold protein (J. Yu 2006)

iii) The Primary Cilium

Primary cilia allow cells to sense fluid flow in the form of blood along the blood vessels, urine flow through kidneys, bile acid in the liver, and interstitial fluid flow within the bone canaliculi, amongst many others (Figure 4) (Abou Alaiwi, et. al. 2009). In the vascular system, the primary cilia are present in arteries, veins, and the corneal endothelium (Pala et al. 2018)

Ciliary length is also positively correlated with mechanosensory action. Blood vessels with a relatively low fluid force have longer cilia while blood vessels with a high fluid force are devoid of cilia or have very short cilia. This is because of the inability of

primary cilia to stand against high levels of fluid flow. It results in ciliary disassembly and loss of intraflagellar transport (IFT), which is necessary for ciliary reassembly (Sherpa et al. 2017)

The role of primary cilia in the development of atherosclerosis was revealed in Apoe$^{-/-}$ mice fed a high fat and cholesterol diet. *Tek-Cre Ift88/* Apoe$^{-/-}$ animals, in which endothelial ITF88 (intraflagellar transport 88) was specifically ablated, displayed a significantly greater increase in plaque formation compared to their wild type littermates (Dinsmore and Reiter 2016). The lack of endothelial cilia in vascular branches resulted in significant upregulation of pro-inflammatory cytokines, such as IL-1β, TNF-α, TGF-β, and IL-6, along with a higher lymphocytes and macrophages infiltration. Hence, it was proposed that vascular endothelial cilia possess an anti-atherosclerotic role. The protective effect is likely independent of circulating lipids, as endothelial cilia don't seem to influence lipid parameters (Luu et al. 2018; Dinsmore and Reiter 2016).

iv) Integrins

The integrin family of receptors plays an important role in cellular crosstalk with the microenvironment. These transmembrane proteins are responsible for the interactions between the intracellular cytoskeleton and extracellular matrix proteins, the immunoglobulin family of receptors, or adhesion proteins such as ICAM-1 and/or VCAM-1 (Figure 4) (Hynes 2002; Ross et al. 2013). Integrins expressed by endothelial cells also contribute to mechanotransduction and activation of signaling cascades. In their study, Xanthis, et al., demonstrated that integrin β1, converts from its inactive form to active conformation in the presence of unidirectional flow, and helps endothelial cells to maintain their alignment in the direction of flow, a phenotype that is lost in the presence of bidirectional flow or deletion of β1 (Hynes 2002; Ross et al. 2013). It is well-established that integrins are localized to the basal side of the cell and transduce mechanical stimuli outside-in (Orr et al. 2006). However, β1 integrin is shown to localize to the apical side of endothelial cells and transduce signals without ligand binding under unidirectional flow, a phenomenon called "sea-anchoring" (Matthews et al. 2010; Xanthis et al. 2019). Shear stress induces ligation of matrix-binding integrins which leads to activation of proteins like p21 activated

kinase (PAK). PAK is implicated in the activation of proinflammatory signaling pathways involving Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinases (JNK); expression of pro-inflammatory genes like VCAM-1 and increasing endothelial permeability (Orr et al. 2005). Fibronectin, in the matrix, also plays a key role in the activation of this molecular mechanism. Deletion of fibronectin in a murine model resulted in lowered levels of PAK and NF-κB at atheroprone regions. One of the most common matrix-binding integrins activated by shear stress is the α5 group. Blocking α5β3 was shown to inhibit the PAK pro-inflammatory signaling pathway (M. Chen et al. 2016). *In vivo* studies testing the effect of integrin inhibitors, have reported that mice treated with α5 integrin inhibitors or endothelial-specific deletions of α5, exhibit a reduced pro-inflammatory response at areas of disturbed flow and induced-disturbed flow in carotid ligation models. Sun et al. also demonstrated that inhibiting α5 using blocking antibodies or siRNA, reduced the turbulent flow-induced expression of ICAM-1 and VCAM-1, inflammasome expression and release of IL-1β. Sun, et al reported that inhibition of formation of heterodimers containing α5 or α2, also reduced NF-κB activation (Sun, et. al. 2016). Integrins also regulate endothelial function by regulating the shear stress-induced phosphorylation of eNOS mediated by Akt and PKA (Boo et al. 2002).

v) Piezo1

Piezo ion channels are activated by mechanical stimuli arising from blood flow, such as stretch or shear stress (Figure 4) (Douguet et al. 2019; Murthy et al. 2017). Piezo1 is present in endothelial cells, vascular smooth muscle cells, cardiac fibroblasts, cardiac myocytes, adipocytes, platelets, and T cells (Beech and Kalli 2019), while Piezo2 is highly expressed in mechanosensory neurons and responsible for light touch, reflex regulation of breathing, and baroreception (Murthy et al. 2017).

Piezo1 monomers come together to form one functional unit (Beech and Kalli 2019). The N-terminal domain of Piezo1 is assumed to be the mechanosensor. This domain consists of nine repeat sequences which form the propeller-like blades, each blade containing four helix transmembrane segments (Douguet et al. 2019). The C-terminal domain is constituted of the C-terminal extracellular domain, and the intracellular domain (CTD) (Zhao et al. 2018; Ge et al. 2015). An inverted dome-like indentation of

the membrane is formed, suggesting its role in the regulation of gating in response to membrane tension (Guo and MacKinnon 2017; Haselwandter and MacKinnon 2018). Piezo1 is a key endothelial mechanosensor required for early vascular development. It is expressed in the developing cardiovascular system, notably in the endothelium and endocardium (Guo and MacKinnon 2017; Haselwandter and MacKinnon 2018). It is also present in human umbilical vein and fetoplacental endothelial cells (Li et al. 2014; Morley et al. 2018). Stimulation of Piezo1 in endothelial cells by blood flow causes the ion channels to remain open longer, also known as slow inactivation, and leads to a steady increase in intracellular Ca^{2+} (Li et al. 2014).

The opening of endothelial Piezo1 channels by shear stress activates proteolytic enzymes, downstream Ca^{2+}-dependent calpains. This in turn causes focal adhesion turnover, proteolytic cleavage of actin cytoskeletal elements, and alignment of ECs to blood flow (Douguet et al. 2019). It has been reported that genetic deletion of Piezo1 or its inhibition with GsMTx4 in endothelial cells suppresses shear stress- induced Ca^{2+} influx and impairs cell alignment in response to flow (Li et al. 2014; Ranade et al. 2014). Thus, Piezo1 is vital during early development for the alignment and migration of endothelial cells to blood flow.

Shear stress activation of Piezo1 in arteries releases ATP from ECs, partly through pannexin channels, which in turn stimulates P2Y2 purinergic receptors in an autocrine manner. The downstream G_q/G_{11}-mediated activation of PECAM-1, VE-cadherin, and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) enhances phosphorylation of eNOS at Ser 1176 via I3K/AKT, resulting in NO production (S. Wang et al. 2016). Laminar flow induces, Piezo1-dependent release of adrenomedullin from the endothelium, causing phosphorylation of eNOS at Ser 632 by protein kinase A (PKA) downstream of G_S-coupled Calcitonin Reeptor like/ Receptor Activaty Modifying Protein 2 (CALCRL/RAMP2) receptors, thus further enhancing NO levels (Iring et al. 2019). Increased NO levels in turn mediate vasodilatation and exerting an athero- protective function.

When endothelial cells are subjected to turbulent flow, Piezo1 activation induces endothelial inflammation and atherosclerosis (Douguet et al. 2019; Chiu and Chien

2011; Gimbrone and García-Cardeña 2016). It was observed that in endothelial cells exposed to low shear stress, accumulation of inflammatory cells decerases upon endothelial Piezo1 deletion. Moreover, endothelial deletion of Piezo1 or G_q/G_{11} in mice lacking low-density lipoprotein (LDL) receptors and fed a high-fat diet attenuated endothelial inflammation, as well as neointima formation after partial carotid ligation, causing atherosclerosis (Albarrán-Juárez et al. 2018). In turbulent flow conditions, Piezo1-/ G_q/G_{11} complex activates Integrin α5 and focal adhesion kinases, which stimulates the NF-κβ pathway downstream and promotes the expression of pro-atherogenic genes.

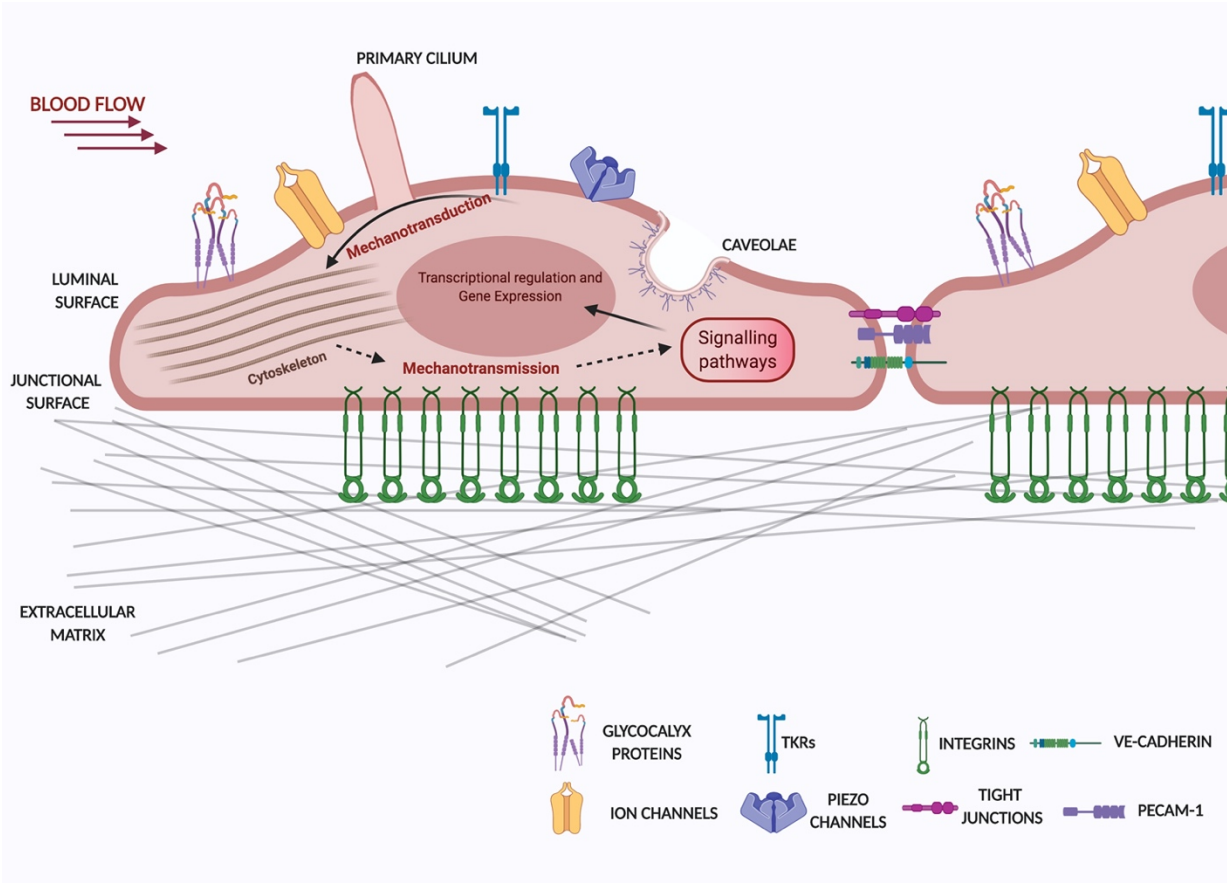


Figure 4: : Schematic representation of different mechanosensors.

Mechanosensors like the primary cilium, caveolae, integrins, Piezo-1, ion channels and junctional proteins, are responsible for receiving the mechanical stimuli exerted by blood flow on endothelial cells. The transduced signal is then transmitted throughout the cytosol to various destinations like focal adhesion, microtubule organizing centre, via the cytoskeleton, to activat several signalling and transcriptional pathways.

A.2.2 ROLE OF SHEAR STRESS

i) Alignment and Migration

Exposure of endothelial cells to shear stress elicits different directional cellular patterns. Endothelial cells become elongated and polarized in the direction of parallel flow (McCue et al. 2006). When a lesion is formed perpendicular to the flow direction, the cells upstream of the lesion tend to migrate to the lesion site faster than those downstream. Endothelial cells exposed to laminar flow or high shear stress have been known to impart an atheroprotective phenotype (Chiu and Chien 2011). The alignment of endothelial cells occurs in 3 phases: the first being formation of actin stress fibers, reorganization of the microfilaments and microtubules and formation of tight cell junctions; second, the cells tend to migrate along the microtubule axis; and lastly, the elongation and polarization of cells with the help of intracellular stress fibers and intercellular junctions.

Activated integrins help endothelial cells to adhere to the sub-cellular matrix. The adhesion mechanism modulates the activity of Rho GTPases, which causes the disassembly of cytoskeletal filaments, specifically Rac1 GTPase. The rapid reorganization of the cytoskeleton and downstream signaling during endothelial cell alignment in response to flow predominantly involves sequential and coordinated activation of the small GTPases (Galbraith et al. 1998; Mack and Iruela-Arispe 2018).

Migrating endothelial cells exhibit high expression Rac1 regulated by α4 integrins, that, when bound to paxillin, suppresses Rac1 activation. However, phosphorylation of α4 integrin by protein kinase A blocks its binding to paxillin, thereby altering the localization and activation of Rac1 (Alpha et al 2020).

Integrins also regulate cell adhesion and migration by associating with focal adhesions. The Arg-Gly-Asp (RGD) integrin-binding motif of the purinergic receptor, $P2Y_2$ receptor, co-localizes with integrins: a_vb3 and a_vb5 (Liao et al. 2014; Bagchi et al. 2005).

The interaction of $P2Y_2$ receptors with integrins transduces physical stimuli into chemical signals, thereby altering the cytoskeleton. $P2Y_2$ receptors were initially designated the role of mechanosensors, however a recent study has shown that shear stress induced $P2Y_2$ activation is also responsible for stress fiber formation, endothelial cell alignment and migration. Actin shear stress fibers are aligned in the direction of flow, which causes the endothelial cell remodeling. $P2Y_2$ receptors also transiently interact with VE-Cadherin, activating Rac-1 via VEGFR2 (Sathanoori et al. 2017).

ii) Apoptosis

Laminar flow is known to decrease apoptosis in the presence of several pro-atherogenic factors like TNF-α, oxidized-LDL (ox-LDL) or reactive oxygen species (ROS). One of the main pro-apoptotic mediators are death associated protein kinases (DAPK). Cell survival can be mediated by the inhibition of IL-1b, DAPK and Caspase-3 (Alpha et al. 2020). Under physiological conditions, high shear stress plays an important role in mediating anti-apoptotic phenotype by inhibiting the TNF-α induced expression of JNK, increased NO production and inhibitors of apoptosis like IAP-1 and IAP-2 (Jin et al. 2005).

A.2.3 Regulation Of Molecular Pathways By Shear Stress

Endothelial cell function is majorly regulated by shear stress induced transcription factors, and defect in their activity could facilitate the pathogenesis of atherosclerosis. These factors modulated by mechanical stimuli are defined as shear stress responsive elements (SSRE) and they undergo different adaptations in response to the shear stress exerted (Bondareva et al. 2019). The most common epigenetic mechanism involved in the regulation of the transcriptional changes of these response elements is hypermethylation (Evrard et al. 2016). A recent mechanism reported has been that of acetylation of histone 3 at lysine 27 (H3K27). Gene ontology analysis revealed that involvement of response elements participating in pro-atherogenic endothelial modifications in response to disturbed flow, like the Wingless and Int-1 (WNT) pathway, interleukin-mediated pathways, endothelial-mesenchymal transition,

apoptotic processes and regulation of phospholipid biosynthesis (Bondareva et al. 2019).

Low and high shear stress modulate different aspects of endothelial cells via these mechanosensitive transcription factors like Kruppel-like factor 2 and 4 (KLF-2, KLF-4), nuclear factor (erythroid-derived 2)-like 2 (NRF2), and YAP/TAZ/TEAD (Niu et al. 2019). These transcription factors are upregulated in endothelial cells exposed to laminar flow. Laminar flow downregulates other transcription factors like activator protein 1 (AP-1), nuclear factor kappa B (NF-κB) (Noguchi and Jo 2011; Hajra et al. 2000), WW domain-containing transcription regulator 1 (WWTR1) (Wang et al. 2016; Wang et al. 2016) and hypoxia inducible factor 1 alpha (HIF-1α) (Feng et al. 2017; Min and Schwartz 2019; Wu et al. 2017).

i) The KLF2 and KLF4 pathways

KLF2 and KLF4 regulate the expression of anti-inflammatory, antioxidant, and anti-thrombotic genes in endothelial cells (Nayak, Lin, and Jain 2011) like *eNOS* and thrombomodulin (THBD) (Simmons, Kumar, and Jo 2016). Myeloid cell specific depletion of *KLF2* has been reported to promote the development of atherosclerosis in mice deficient in LDL receptor (LDLR) by increasing the recruitment of neutrophils and macrophages, and raising the nitroxidative stress (Emini Veseli et al. 2017).

In the presence of disturbed flow, *KLF2* and *KLF4* upregulate multiple pro-inflammatory, pro-thrombotic, and vasoconstrictive genes, such as *VCAM-1,* monocyte chemoattractant protein (MCP-1), E-selectin, endothelin 1, and plasminogen activator inhibitor-1 (Simmons et al. 2016; Niu et al. 2019). When exposed to laminar or unidirectional flow, KLF2 inhibits the expression of pro-inflammatory genes by recruiting transcriptional co-activator p300 to block the hyperactivation of NF-κB (Sweet et al. 2018; Sen-Banerjee Sucharita et al. 2005). Recent investigations looking into the potential role of miRs in atherogenesis has revealed that KLF2 is implicated in the expression of miR-126 in response to blood flow (Sweet et al. 2018; Santovito et al. 2020). miR-126-5p is anti-atherogenic and is up-regulated by laminar flow in arterial endothelial cells (Schober et al. 2014).

Nuclear miR-126-5p inhibits proapoptotic caspase-3 activity through a direct miRNA-protein interaction. This noncanonical mechanism involves a specific Mex3a-guided nuclear transfer of miR-126-5p. In this compartment, the interaction with miR-126-5p may prevent catalysis of nuclear components to preserve cell viability.

Another transcriptional target of KLF2, as reported by Fang, et al phosphatidic acid phosphatase type 2B (*PPAP2B*), a flow sensitive gene which has also been associated with coronary artery disease and stroke (Fang and Davies 2012). Specifically, *PPAP2B* is essential for laminar flow induced endothelial cell alignment and anti-inflammatory effects. Laminar flow also regulates metabolic pathways like glycolysis and mitochondrial respiration by activating KLF2. It was shown that 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), involved in glycolysis, is a direct target of KLF2 (Niu et al. 2019). A novel role of KLF4 has been reported stating its role in the maintenance of endothelial quiescence by interacting with the promoters of Connexin proteins present in the gap junctions of endothelial cells. KLF4 specifically binds to the promoter of connexin-40, which is responsible for endothelial cell proliferation (Denis et al. 2019).

Other than transcriptional regulation, in response to flow induced shear stress, KLF2 and 4, are also responsible for regulating lipid uptake and contributing to the initial lesion formation. Double knockout murine models, *KLF2*$^{-/-}$ *ApoE*$^{-/-}$ and *KLF4*$^{-/-}$ *ApoE*$^{-/-}$ showed an increase in lipid uptake, foam cell formation and lesions.

ii) NRF2 pathway

Nuclear factor erythroid 2-related factor 2 (NRF2) controls the expression of several genes responsible for cytoprotection, inflammation, and metabolism in response to oxidative stress (Niu et al. 2019). NRF2 functions by binding to a conserved NRF2 promoter sequence, 5'-A/GTGAC/GTCAGCA-3' (Hayes and Dinkova-Kostova 2014). Target genes of NRF2 include heme oxygenase-1 (HO1) and NADPH dehydrogenase quinone (*NQO1*) (Hayes and Dinkova-Kostova 2014; Suzuki, et al. 2013). NRF2 activation in response to laminar flow is important for endothelial cell adaptation to oxidative stress. In endothelial cells, laminar flow increases eNOS-dependent nitric

oxide (NO) production and oscillatory flow promotes oxidative stress via NADPH oxidase, xanthine oxidase, eNOS uncoupling, and mitochondrial-derived ROS (Simmons et al. 2016; Takabe et al. 2011). NRF2 binds to the proximal promoter region of *IL-6* and *IL-1β* genes and suppresses the expression of IL-6 and IL-1β. NRF2 activation also protects against TNFα-induced activation of *VCAM1* gene expression in endothelial cells (Niu et al. 2019; Xl et al. 2002).

iii) The YAP/TAZ pathway

Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are effectors of the Hippo pathway. YAP/TAZ in the Hippo pathway emerged as a master regulator of multiple cellular processes, such as cell proliferation, migration, and inflammation (Totaro et al. 2018; Hansen et al. 2015). Accumulating studies have indicated that YAP/TAZ/ TEAD signaling represents a promising therapeutic target for the prevention and treatment of atherosclerosis (Niu et al. 2019) When Hippo signaling is activated, the upstream kinases MST1/2 are activated, which in turn phosphorylate and activate LATS1/2; LATS1/2, which then phosphorylate YAP and TAZ. Disturbed flow upregulates YAP/TAZ by phosphorylation at serine 127, or endothelial VCAM1-dependent inflammation by phosphorylation of YAP at tyrosine 357 (Li et al., 2019) Phosphorylation of YAP at Ser127 facilitates the cytosolic retention of YAP and promotes its degradation via the ubiquitin-proteasome system. When the Hippo pathway is deactivated, YAP and TAZ are dephosphorylated and imported into the nucleus, where they form active transcriptional complexes with DNA- binding transcription factors including TEA-domain (TEAD) transcription factors (Totaro et al. 2018). Increased nuclear YAP and decreased phosphorylated YAP were also found in athero-prone regions of arteries in mice (Yuan et al. 2020).

These transcriptional complexes induce the expression of a wide range of genes (e.g., cytoskeletal proteins, cell–cell junctions, inflammatory molecules, Connective tissue growth factor (CTGF, or CCN2), cysteine rich angiogenic inducer 61 (Cyr61, or CCN1) and secreted proteins) that are involved in cell growth, apoptosis, migration, and communication (Dobrokhotov et al. 2018; Totaro et al. 2018).

iv) HIF pathway

Activation of the Hypoxia-inducible Factor 1α (HIF-1α) pathway drives vascular permeability and leukocyte recruitment at lesion sites. Endothelial HIF-1α aggravates low shear stress-induced atherosclerosis development by triggering the miR-19a/chemokine (C-X-C motif) ligand-1 (miR-19a/CXCL1) axis and stimulating leukocyte adhesion to activated endothelial cells (Akhtar et al. 2015). In disturbed flow conditions, expression of HIF-1α increases via transcriptional activation of NF-κB and HIF-1α protein stabilization by the deubiquitinating enzyme Cezanne. NADPH oxidase 1 (NOX1)- dependent generation of ROS is also caused by HIF-1α stabilization. Activation of HIF-1α favours the expression of HIF-1α increases via transcriptional activation of NF-κB and HIF-1α protein stabilization by the deubiquitinating enzyme Cezanne. NOX4-dependent generation of ROS is also caused by HIF-1α stabilization. Activation of HIF-1α favours the expression of MCP1, SELE, and VCAM-1; glycolytic enzymes like enolase 2 (ENO2); PFKFB3, and pyruvate dehydrogenase kinase-1, and suppression of mitochondria respiration (Feng et al. 2017; Wu et al. 2017).

A.3 ROLE OF ENDOTHELIUM IN ATHEROGENESIS

A.3.1 Definition

Cardiovascular diseases (CVDs) are the leading cause of death, globally. World Health Organization (WHO) has recorded up to 17.9 million deaths each year, which accounts for 31% of all causes of global mortality. CVDs are disorders affecting the heart and blood vessels, which include coronary heart disease, cerebrovascular disease, rheumatic heart disease, deep vein thrombosis and pulmonary embolism, to name a few (WHO Report, 2017).

Atherosclerosis is a chronic inflammatory disease of large and medium arteries characterized by stenosis or narrowing of the arteries. Stenosis is caused by lipid retention in the intima of the arterial wall, its oxidation, followed by chronic inflammation. Other than its lipid-rich constituents, plaques also consist of necrotic

tissue from the arterial walls, inflammatory cells like macrophages, lymphocytes, dendritic cells, and VSMC.

A.3.2 Risk factors

Risk factors of CVD are usually attributed to various metabolic disorders like obesity, diabetes mellitus, hypertension, and hypercholesterolemia (Figure 5). Other known risk factors are tobacco smoking, high levels of triglycerides and C-reactive protein, lack of physical activity, age, genetic predisposition, stress and/or alcohol.

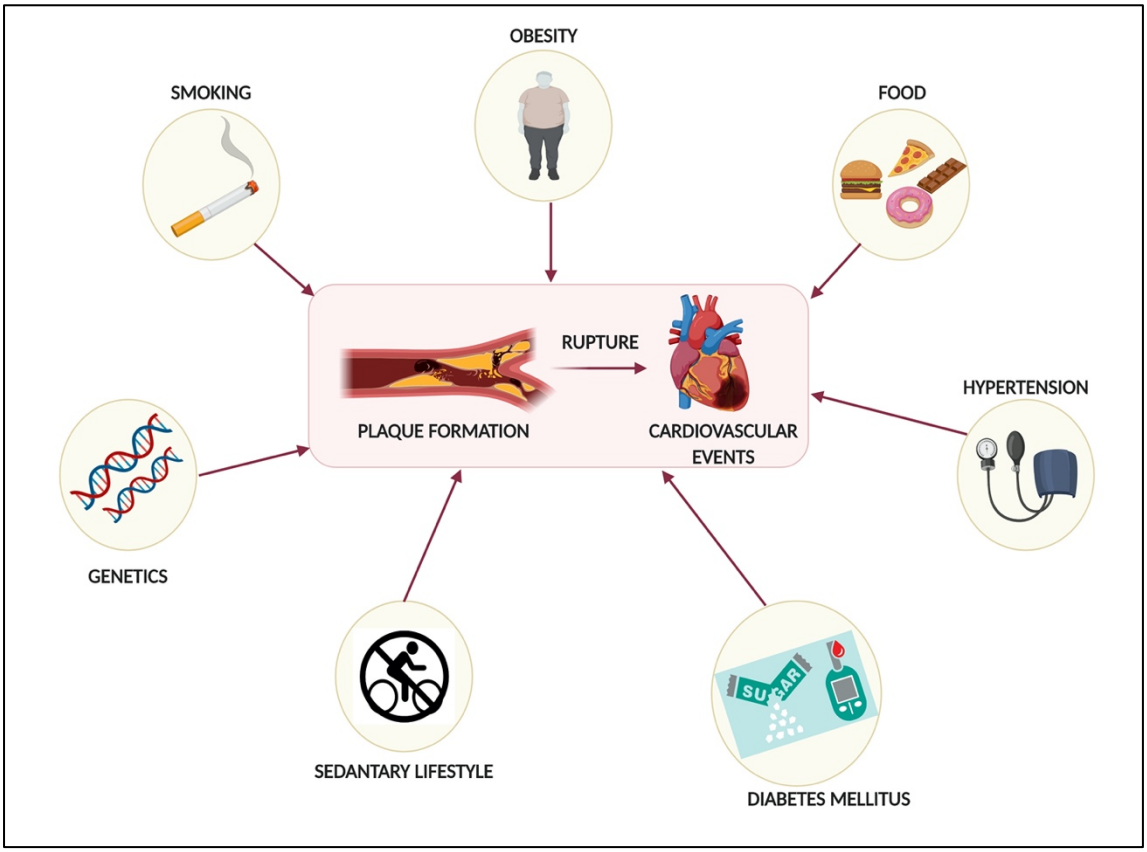


Figure 5: Different risk fators associated with atherosclerosis developement and progression (Created with BioRender.com)

i) Diabetes Mellitus

Diabetes raises the risk of cardiovascular events by 2-4 times. Diabetes mellitus is a group of metabolic disorders caused by a defect in the carbohydrate metabolism. It is

characterized by hyperglycemia due to abnormalities in insulin secretion or insulin resistance in cells (Bertoluci and Rocha 2017). Various studies have reported the effect of diabetes on the microvasculature in CVDs, renal diseases and defective retinal system. One of the interlinking mechanisms between diabetes and cardiovascular diseases is the formation of advanced glycation end products (AGEs) (Bornfeldt 2016). AGEs contribute to atherosclerosis by initiating endothelium activation and expression of adhesion molecules. Another mechanism that is upregulated by high blood glucose levels is glycation of LDL particles, which is regarded as one of the atherogenic modifications of LDL (Poznyak et al. 2020). AGEs also inhibit reverse LDL transport in monocytes by decreasing the expression of ATP-binding membrane transport proteins like ABCA1 and ABCG1. This leads to vasoconstriction, which is attributed to the increased release of endothelin 1. Glycation end products also interact with the constituent proteins of the extracellular matrix to advance atherosclerosis. The extracellular matrix gets modified by glycation, which enhances its interaction with AGE receptor (RAGE) on endothelial cells, macrophages, and VSMCs (Katakami et al. 2009; Chistiakov et al. 2014).

Elevated glucose levels alter other cellular metabolic pathways, such as the mitochondrial electron transport pathway, which in turn leads to over production of ROS (Tabit et al. 2010). *In vivo* studies have shown that elevated levels of NADPH oxidase protein, NoX1, are involved in ROS production. *Nox1*$^{-/-}$ murine models exhibited attenuated levels of plaque formation. Another key player identified in the oxidative stress pathway, related to diabetes-associated atherosclerosis is the Glutathione peroxidase (Gpx1). When the insulin secretion process is hampered in *Gpx1*-deficient mice, via pharmacological treatments, the mice have shown a pro-atherogenic phenotype with increased monocyte infiltration, expression of inflammatory markers and plaque size (Chew et al. 2010).

Studies have demonstrated that hyperglycemia alters endothelial functions, by activating protein kinase C (PKC) and increases vascular permeability, monocyte adhesion, the expression of cell adhesion molecules, the generation of ROS, and the activation of NF-kβ (Poznyak et al. 2020). Glucose metabolites can also function

upstream of pro-inflammatory pathways to activate cytokines like protein kinase C. Protein kinase C is a key mediator in cellular signaling pathways corresponding to the expression and activation of cytokines, growth factors and other messenger proteins. High glucose uptake by vascular endothelial cells initiates the synthesis of diacylglycerol, which is involved in PKC activation (Zeadin et al 2013). PKC is also involved in response to oxidative stress. In the context of atherosclerosis, elevated PKC levels (via diacylglycerol) causes reduced NO synthesis, leading to defective vasodilation, endothelial dysfunction, increased release of cytokines and extracellular matrix (Nishikawa et al. 2000). Activation of PKC-b is known to promote atherosclerosis via CD11c or Integrin αX, which is a transmembrane protein expressed on the surface of various immune cells like dendritic cells, monocytes neutrophils and some forms of B-cells. PKC-b is involved in the induction of CD11c expression and activation of macrophages, to favor the progression of the disease (Kong et al. 2013).

Changes in chromatin are important regulatory mechanisms interlinking hyperglycemia and complications in the vasculature. For example, with the help of genome-wide analysis, it was shown that high glucose levels led to elevation in acetylation of histone H3K9/14 in aortic endothelial cells. This was also correlated to the down regulation of DNA methylation. These changes in transcription are correlated to the modifications in signaling pathways implicated in atherogenesis (Pirola et al. 2011). Hyperglycemia also initiates the methylation of histone H3 and other lysine modifications. Set7 lysine methyltransferase is known to be responsible for the methylation at lysine 4 of histone H3 (Keating and El-Osta 2013). This led to changes in the promoter of *RELA* gene, which encodes for the NF-kβ-p65 subunit. Downstream, this upregulates the NF-kβ mediated expression of MCP-1 and VCAM1 (Simmonds and Foxwell 2008).

VE-cadherin is involved in membrane permeability and binds with its cytoplasmic domain to β-catenin, plakoglobin, and catenin-p. This protein complex interacts with receptors of VEGFR2 growth factors, FGFR1, and TGFb-R (Gavard 2014). Treatment of endothelial cells with high glucose also leads to tyrosine phosphorylation of VE-

cadherin, with a consequent dissociation from β -catenin, barrier disruption, and increased trans-endothelial migration of monocytes.

In type 1 diabetic patients, plasma hyaluronic acid, a main constituent of the glycocalyx, and hyaluronidase are increased, resulting in increased synthesis and shedding of hyaluronic acid. Therefore, type 1 diabetes is often characterized by damage to the endothelial glycocalyx layer. Hyperglycemia attenuates the shear stress-dependent dilatation in distal arteries, due to loss of mechanotransducing properties of the endothelial glycocalyx by hyperglycaemia (Noble and Drake-Holland 2010). All these physiological changes could be the initial steps in atherogenesis in the presence of diabetes.

ii) Hypercholesterolemia and Obesity

Hypercholesterolemia is defined as the elevation in the levels of low-density lipoprotein cholesterol in the blood. Elevated levels of LDL can increase transcytosis, which combined with endothelial dysfunction, cause LDL flux into the arterial wall (Mundi et al. 2018). Obesity is a result of imbalance in the metabolism and homeostasis of fat mass. Adipose tissue can act as endocrine organs by secreting molecules, which have various systemic and local targets, one being the endothelium and causing defects in endothelial permeability. Exposure to LDL and apolipoprotein can cause disruption of gap junctions, which could possibly, be one of the factors responsible for LDL flux. LDL weakens the endothelial cell barrier by activating Rho-A (Ras homolog) (Beckers, van Hinsbergh, and van Nieuw Amerongen 2010).

Obesity also results in the increased release of pro-inflammatory cytokines and growth factors in the circulation, like TNF-α, IL-6, IL-12, leptin, VEGF, free fatty acids. On the other hand, excess adipose tissue can affect the expression of anti-inflammatory molecules (Coelho et al. 2013; Mundi et al. 2018). TNF-α was one of the first molecules to be identified, released by adipose tissue. TNF-α is also responsible for stimulating pro-inflammatory signaling pathways, like Rho A/ROCK (Coelho et al. 2013; Kant et al. 2011). ROCK inhibits the activity of MLC phosphatase, and phosphorylated MLC phosphatase causes changes in the actin-myosin contraction

pattern. This increases the endothelial permeability allowing LDL to cross the barrier and initiate atherogenesis (Beckers, van Hinsbergh, and van Nieuw Amerongen 2010). TNF-α also promotes retention of LDL in the sub-endothelial layer by activating NK-kβ and peroxisome proliferator activated receptor (PPAR) (Bian et al. 2014). TNF-α mediated activation of these proteins further causes enhanced expression of Caveolin-1, Caveolin-2 and LDL receptors. Another one of the many downstream targets of TNF-α is NADPH oxidase signaling pathway. Changes in the oxidative pathway leads to modifications in the endothelial cell junctions, thereby impacting the endothelial barrier (Marcos-Ramiro et al. 2014).

Indirectly, adipose tissue secreted cytokines influence atherosclerosis development, by causing insulin resistance and mediating diabetes. Adipocyte-secreted cytokines also negatively regulate the expression of junction proteins like ZO-1, via the ROS production and activation of mitogen-activated protein kinase (MAPK), p38 (Ebnet 2008; Mundi et al. 2018). Adiponectin has the reverse effects than TNF-α mediate inflammation, endothelial permeability, defects in intercellular gap junctions, and tubulin disassembly; via PKA and cyclic adenosine monophosphate (cAMP) signaling pathways (Xu et al. 2008). In physiological conditions, Adiponectin is responsible for the production of AMP-activated kinase and NO secretion. The sharp reduction in Adiponectin production in atherosclerotic conditions is indicative of its role in protecting the endothelium (Mundi et al. 2018).

Another mechanism, by which VEGF, specifically via VEGFR1, influences the transport of LDL, is by undergoing autophosphorylation and internalization of the LDLr bound to LDL (Usui et al. 2007). Free fatty acids release by adipose tissue, like palmitate, disrupts the endothelium by activating the Nucleotide-Binding Oligomerization Domain, Leucine-Rich Repeats and Pyrin Domain Containing 3 (Nlrp3) inflammasome complex. This complex is involved in raising the levels of mitochondrial ROS and reducing the expression of ZO-1 and ZO-2 junctional proteins (Zihni et al. 2016).

iii) Hypertension

Hypertension is the most common risk factors associated with increased cardiovascular deaths. Hypertension promotes the development and progression of atherosclerosis, targeting endothelial dysfunction, oxidative stress, and remodeling. It is characterized by modifications in the arterial endothelium and hypertrophy of VSMCs in the *tunica media*. Studies in rat aortas have shown that hypertension could possibly lead to an increase in LDL entry by altering the endothelial permeability associated transient formation of leaky junctions (Dharmashankar and Widlansky 2010; Dinh et al. 2014; Mundi et al. 2018). LDL fluxes across the leaky junction; the intima and the media are affected by the transmural pressure, which, in turn, affects the compaction of the intima. A damaged glycocalyx layer augments the flux of plasma contents, which are increased during hypertension (Facchini, Bellin, and Francisco Toro 2016).

Angiotensin II, the main effector of renin angiotensin system (RAS), the activation of which is key in regulating blood pressure and cardiovascular function, has been implicated in LDL transcytosis and increase LDL retention in the subendothelial space (Bian et al. 2014) Proteins like LDLR, Caveolin-1, and cavin-1 are, which are involved in caveolae-mediated transcytosis, are also associated with angiotensin II-induced LDL transport.

Another mechanism mediated by hypertension which could potentially explain the changes in endothelial permeability involves calcium. Shear stress alters the cellular calcium levels, which in turn regulates the calcium dependent activation of MLC kinase, and RhoA kinase dependent. The activation of these kinases leads to the MLC phosphorylation and contraction of cells, ultimately causing disruption of the endothelium.

iv) Smoking

Smoking has been strongly associated with atherosclerosis at various stages of the disease progression, from lesion formation to acute occlusion. Smoking injures the

blood vessel wall by damaging endothelial cells, thus potentially increasing permeability to lipids and other plasma components. Tobacco smoke facilitates alterations in β-catenin trafficking in the endothelium by IL-b, thus inducing the expression of cyclooxygenase-2 and eventually causing hyperpermeability. Unsaturated aldehyde sacrolein and crotonaldehyde can activate NADPH oxidase and the subsequent production of superoxide anion, reasing oxidative stress (Jaimes et al. 2004)

Tobacco smoke interacts with IL-1b to suppress the activity of phosphatase and tensin homolog (PTEN), leading to a disassembly of VE-cadherin/b-catenin membrane complexes (Barbieri et al. 2012). Cigarette smoke contains metals that catalyze the oxidation of cellular proteins, causing a loss of microtubule function resulting in microtubule depolymerization, proteasomal degradation of α-tubulin, and eventually endothelial leakiness. Long-term exposure to nicotine increases aortic endothelial cell death and promotes the transport of macromolecules across the endothelium, thereby disrupting the monolayer barrier and giving way for entry of inflammatory cells to aggravate the condition at lesion site (Bernhard et al. 2005).

A.3.3 Pathogenesis of Atherosclerosis

Atherosclerosis is caused by disturbed lipid homeostasis and the downstream inflammatory response it invokes, leading to the retention cholesterol-rich lipids, andimmune cells in the arterial wall ((Barbieri et al. 2012). Atherogenesis is described in three main steps: 1) initiation of lesion formation and development of plaque, 2) progression and 3) plaque rupture (Figure 6) (Rafieian-Kopaei et al. 2014).

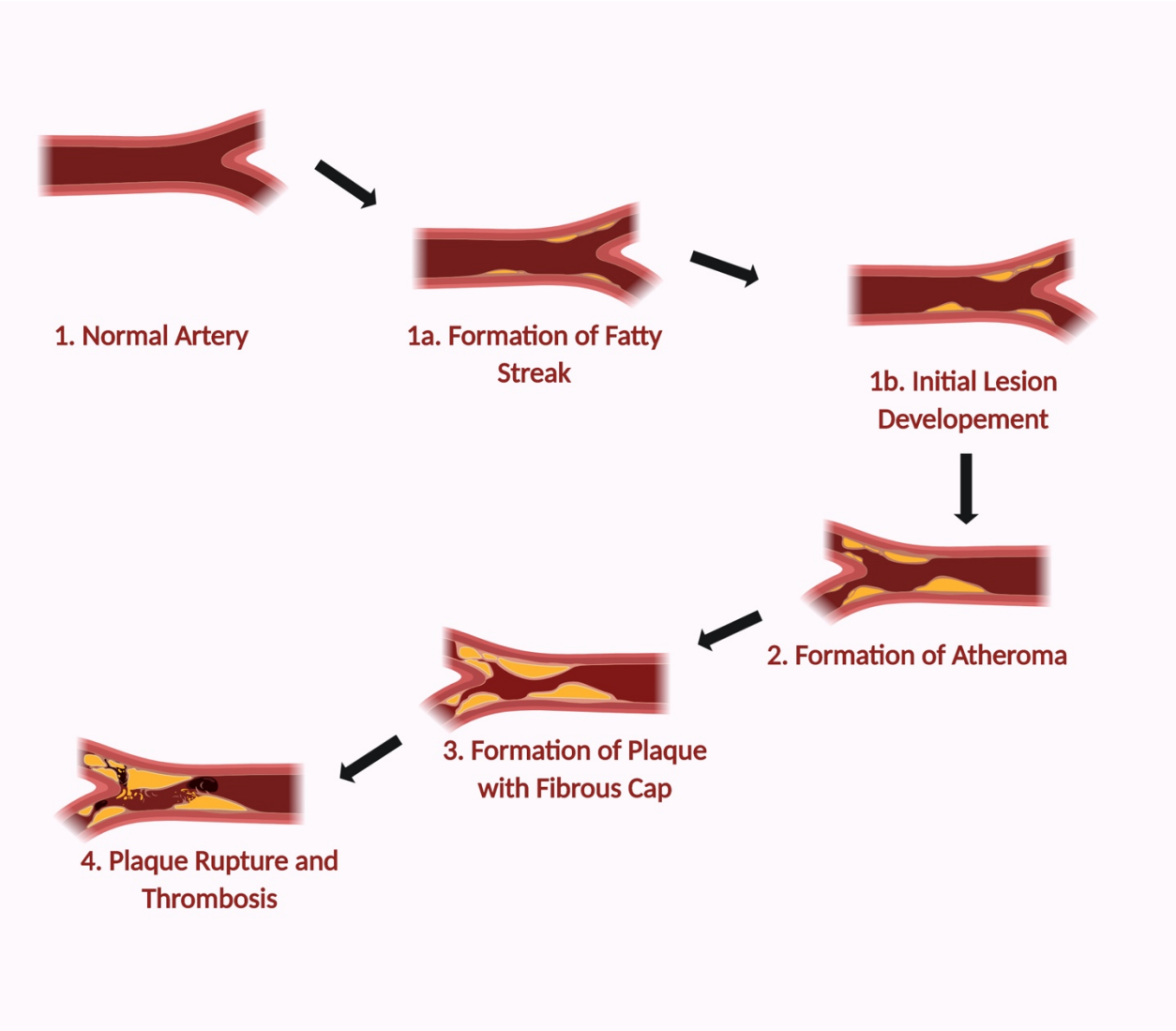
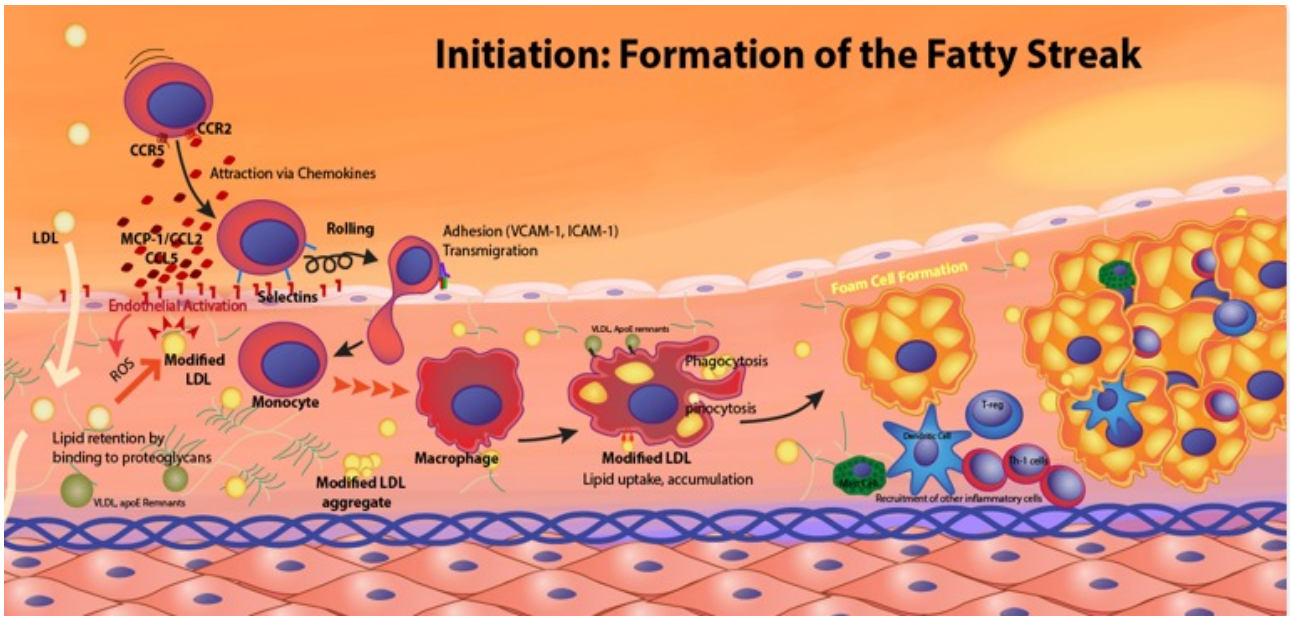


Figure 6: Schematic representation of the different stages of atherosclerotic lesion formation (Created with BioRender.com)

i) Initiation of Lesion Formation and Development of Plaque

Atherogenesis begins with the development of fatty streaks along the wall of the blood vessel (Figure 7) (Lusis 2000). Accumulation of extracellular lipids in the intima

is attributed to endothelial dysfunction, which allows the lipids to cross the monolayer and migrate into the sub-intimal layer. This leads to necrosis of the cells



in the vessel wall, which progressively distorts the intima layer. Disruption of the endothelial layer or endothelial dysfunction is caused by different physical stimuli exerted due to blood flow, or chemical stimuli lipids, high glucose or reactive oxygen species (Poznyak et al. 2020).

Figure 7: Initition of the atherosclerotic lesion: The fatty streak formation begins with endothelial dysfunction followed by retention of LDL (Linton et al. 2000)

LDL become sequestered in the sub-endothelial space by adhering to extracellular matrix proteins like proteoglycans (Raggi et al. 2018). Hypertension causes stiffening of the vessel wall, which makes smooth muscle cells produce more proteoglycans and further allows binding of lipid particles (Wight 2018). LDL cross the endothelial barrier due to the increased endothelial permeability which favors paracellular transport between leaky cells or, active receptor-mediated transcytosis across the cell membrane by transporters like scavenger receptors (SR)- SRA group, SR-BI, SR-E1 and Alk1 (Armstrong et al. 2015; Kraehling et al. 2016).

Once in the sub-intimal space, LDL can undergo modification and become aggregated and/or oxidized. Aggregates of LDL particles form large complexes that can range in

size from 0.1µm to 1µm. These aggregates can be taken up by immune cells by pinocytosis or phagocytosis (Kruth 2002). As they enter the intima, they are acted upon by reactive oxygen species and enzymes, like lipoxygenases (Le 2014) and myeloperoxidases (Carr et al. 2000; Delporte et al. 2013), which convert them into oxidized lipid particles. These oxidized lipid particles are pro-inflammatory in nature and serve as ligands for scavenger receptors present on immune cells. This causes the inflammation to aggravate at the lesion site (Wu et al. 2017). Uptake mediated by scavenger receptors, unlike LDL receptors, is not influenced by feedback inhibition loop, and therefore contribute to the continuous, unrestricted uptake of oxidized lipid complexes (Shen et al. 2018; Abdul Zani et al. 2015).

Recruitment and migration of leukocytes are a key step in atherogenesis. The endothelium responds to the damaging stimuli by upregulating the transcriptional messengers like NF-kB and releasing a series of chemokines that enhance recruitment and adhesion of leukocytes on the endothelium, along with other pro-coagulant factors and vasoconstrictors like endothelin and angiotensin-II. Leukocytes bind on endothelium with the help of E-selectin, adhesion molecules like VCAM-1 and ICAM-1 . Oxidized lipoproteins are first taken up by dendritic cells residing in the arterial intima. Foam cells induce cytokine and chemokine production and the additional recruitment of circulating immune cells, setting off the cascade of the inflammatory response. Cholesterol droplets induce the activation of the inflammasomes in the cytoplasm of the macrophages in the arterial intima. Inflammasomes are protein complexes that respond to exogenous danger stimuli by cleaving pro-interleukin-1β and IL-18 to be secreted as activated cytokines (Duewell et al. 2010; Sheedy et al. 2013). IL-1α is also secreted in response to scavenger receptor activation by modified cholesterol (Freigang et al. 2013). In the extracellular space, IL-1β, IL-1α and IL-18 interact with their corresponding receptors and stimulate the release of reactive oxygen species, matrix metalloprotease- (MMP) activation and proliferation of T-cells, like T-helper-1 and the further production of cytokines (Yue, et al. 2011). T-cells play an essential role in maintaining the proinflammatory phenotype. These cells, trapped in the necrotic core or initial lesion site, are responsible for the continuous release of cytokines, which further contribute to atherogenesis. Interestingly, regulatory T-lymphocytes (TREGs) and T-helper 17 stem the progression of

atherogenesis by secreting TGF-beta and IL-10, and IL-17 , respectively (Gisterå et al. 2013).

ii) Plaque Progression

Prior to this stage, the atheroma was constituted of a necrotic core and continuous accumulation of extracellular lipids, monocyte adhesion, infiltration, migration of VSMCs and secretion of extracellular matrix. Following disease progression, the plaque develops into a fibroatheroma (figure 8). The two main features of a fibroatheroma are a necrotic core and fibrous cap. Stable plaques or lesions in the blood vessels are characterized by the presence of a thick, fibrous cap. At this stage, the plaque does not cause luminal restriction of blood flow, as the arterial wall undergoes remodeling and the diameter is preserved (Otsuka, et al. 2014; Otsuka et al. 2012). Angiogenesis in the vasa vasorum of the adventitia gives rise to new vessels. These new vessels aid the entry of immune cells into the necrotic core. This network of vessels is usually fragile, allowing leakage of plasma contents and erythrocytes. Intra-plaque hemorrhages are common at this stage and they contribute to the expansion of the necrotic core (Bentzon et al. 2014).

Smooth muscle cells play a key role in the formation of a plaque from the initial lesion stage. VSMCs, present in the media layer of the vessel wall, migrate into the intima layer at the site of lesion (Figure 8). Here they undergo proliferation and secrete extracellular matrix, which form the bulk of the plaque. VSMCs also undergo metaplasia to resemble macrophages. These cells are able to engulf ox-LDL and form foam cells. VSMCs are activated and recruited to the lesion site by activated endothelial cells and foam cell-induced platelets. Platelets release platelet derived growth factor (PDGF) and other cytokines which direct the migration and proliferation of VSMCs in the intima. The extracellular matrix surrounding the plaques consist of interstitial collagen, elastin, proteoglycans and glyco-saminoglycans. Some of these macromolecules can trap lipoproteins and promote lipid accumulation within the intima. Infiltrating leukocytes can also add to the lesion by proliferating at the lesion site. Retention factors such as semaphorins can retard the exit of these leukocytes and contribute to their persistent presence in the

atherosclerotic plaque (Jackson 2019). Impaired clearance of dead cells, known as defective efferocytosis, can also add to the necrotic core (Boada-Romero et al. 2020).

The extracellular matrix secreted by the VSMCs is responsible for both, plaque progression and plaque rupture. As mentioned above the fibrous cap surrounding the necrotic core maintains the stability of the plaque. But due to continuous recruitment and accumulation of lipids, the different cell populations and extracellular matrix, the plaque size increases, and the fibrous cap becomes thinner and weakens. The balance maintained between factors, which synthesize or degrade the matrix, also regulates the process. Factors like PDGF and TGF-β stimulate collagen production, while MMPs and Interferon- γ (IFN- γ), inhibit the collagen synthesis. Cytokines released from the lesion site activate the foam cells to secrete MMPs, which degrade collagen and elastin in the fibrous cap (Newby 2007).

Initially, the plaque grows inwards, towards the adventitia, but during the later stages, the plaque continues to grow towards the lumen of the vessel. The plaque undergoes calcification and causes aggravated ulcerations on the lumen of the arterial wall. Over time, plaques can grow in size, big enough to restrict blood flow (Figure 8) (Lusis 2000).

iii) Plaque Rupture

One of the severe complications associated with atherosclerosis is rupture or dislodgement of the plaque, caused by thinning of the fibrous cap. A ruptured plaque can cause occlusion of the arteries resulting from thrombus formation (Figure 8), which ultimately leads to myocardial infarction or strokes. The rupture of the plaque is influenced by a combination of various factors described below.

With accelerated inflammation and apoptosis, the necrotic core grows due to deposition of cellular debris and lipids. This alters the stability of the plaque, whilst altering the biomechanical factors around the plaque. As the plaque grows into the lumen and obstructs the blood flow, it increases the hemodynamic forces at the “shoulder region” or the region adjacent to the plaque. This makes it the weakest

point and most susceptible site for the rupture of the fibrous cap. Vulnerable plaques or zones are composed of low VSMCs, T-lymphocytes and high number of macrophages. These small, yet fragile parts of the plaque can also cause rupture and trigger thrombosis (Loftus 2011).

Indirectly influencing the rupture of the fibrous cap is intraplaque hemorrhage. This occurs when a rupture occurs in the *vasa vasorum* (Zhonghua Sun 2014). This zone is fragile as it is a fairly recent site of angiogenesis and microvascularization. The intraplaque hemorrhage aggravates the instability of the fibrous cap by causing intramural hematoma and subsequently leads to its rupture. Another event that occurs in the later stages of the disease pathology is plaque calcification. The calcification mainly occurs at the necrotic area and surrounding the plaque and causes the arterial wall to become rigid. The balance maintained between the rate of deposition and resorption, by the osteoblast-like cells influences calcification.

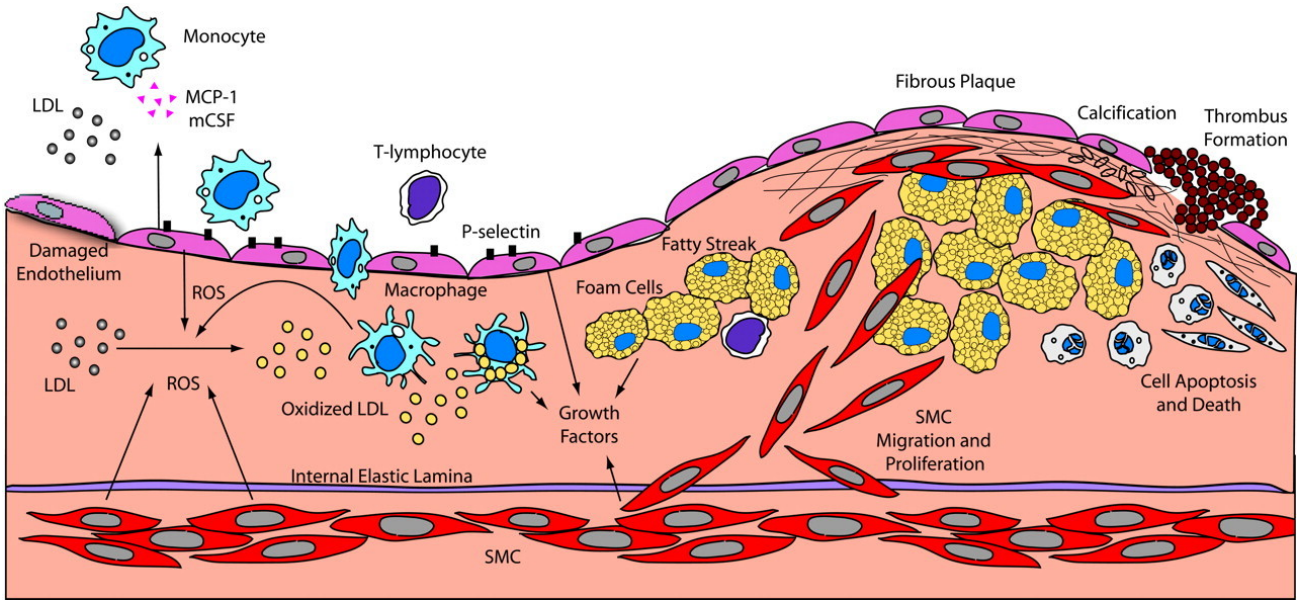


Figure 8: Schematic representation of the overall mechanism of atherosclerosis: Intitiatoin to plaque rupture (Nageswara R et al. 2005).

B. AUTOPHAGY

Autophagy is an evolutionarily conserved, catabolic cellular pathway, defined as "the process by which intracellular waste is delivered to lysosomes for degradation" (Mizushima 2018). The term autophagy came into use after the discovery of lysosomes by Christian de Duve in 1955. There is a wide range of substrates that can be degraded by autophagy, from small proteins to double-membrane organelles, like the mitochondria (Mizushima 2018). Initially the concept of autophagy was introduced as a 'self-eating' process to degrade mis-folded proteins and damaged organelles, however, it was later inferred as an adaptive response to cellular stress, like depletion of nutrients or growth factors, hypoxia or infection (Yorimitsu and Klionsky 2005). In case of stress, the cell disintegrates and recycles non-essential components to provide nutrients and energy (Z. Yang and Klionsky 2010). Over the years, research has implicated autophagy in the pathophysiology of several diseases like neurodegenerative diseases, autoimmune, cancer, and metabolic disorders (Yin et al. 2016; Deretic et al. 2013; Yang and Klionsky 2010).

B.1 HISTORY OF AUTOPHAGY

Lysosomes were discovered in rat liver homogenates, which displayed a latent acid phosphatase activity (Novikoff et al. 1956) . Further investigations of the organelles found in these homogenates, led to the discovery of various hydrolytic enzymes, which are highly active at low pH. Duve termed this organelle, as "lysosome" based on its lysis activity to degrade cellular components (de Duve et al. 1955). Clark, in mice renal cells, not only observed structures resembling lysosomes, but also vacuoles occasionally carrying cargo like damaged mitochondria (de Duve et al. 1955). The results of Novikoff supported his findings. They had found similar hydrolytic enzyme-positive structures containing structures like enoplasmic reticulum (ER), ribosomes and mitochondria (Novikoff et al. 1956). Following studies observed a double-membrane structure, containing organelles, but lacking lytic enzymes. This structure was termed as "autophagosome". Subsequently, this organelle was observed as a single membrane structure, supporting an acidic

environment and consisting of damaged cellular components undergoing different stages of degradation by the lytic enzymes. It was termed as “autolysosome” (Arstila and Trump 1968). The findings, reported over the years, broadly described the steps of a pathway described by Duve as autophagy, in 1963. Over the following decades, autophagy was mainly deciphered morphologically based on electron microscopy findings.

Genetic approaches to unravel the mechanism of autophagy were first carried out in yeast, S*. cerevisiae.* Yoshinori Ohsumi and his team discovered about 15 autophagy-related genes (*ATGs)* (Tsukada and Ohsumi 1993; Ohsumi 1999), for which he was awarded the Nobel Prize in 2016. These genes were deemed essential for the sequestration and delivery of cargo to the lysosomes for degradation.

B.2 TYPES OF AUTOPHAGY

There are 3 types of autophagy mechanisms decribed over the years: chaperon-mediated autophagy, microautophagy and macroautophagy.

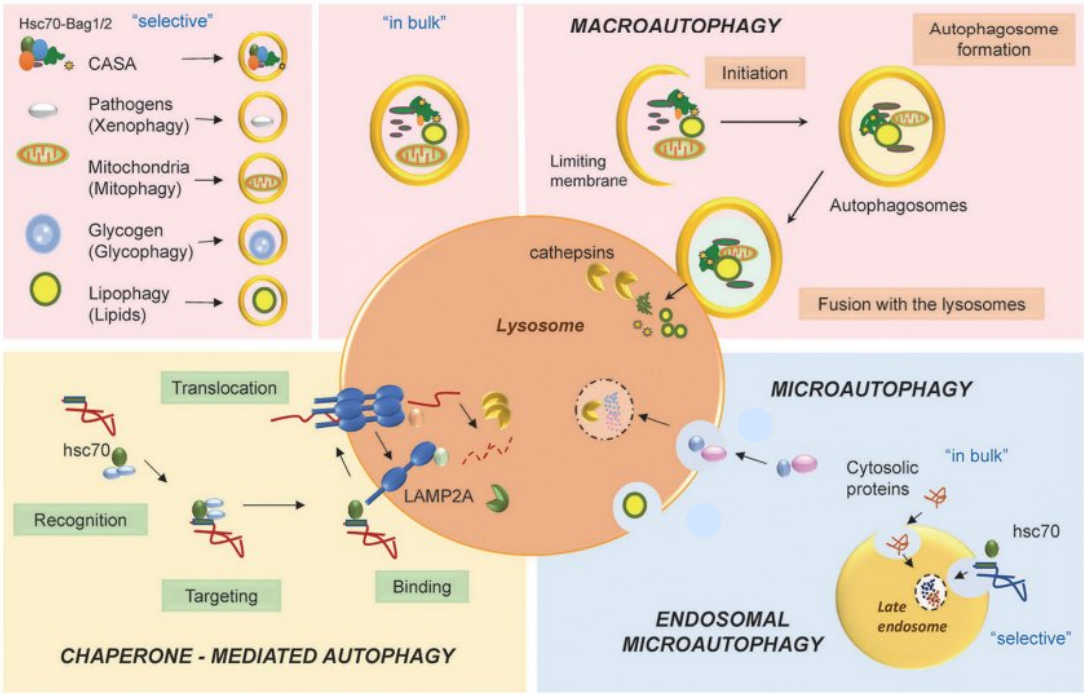


Figure 9: Schematic of the mechanisms of different types of autophagy pathway: Chaperon-mediated auophagy, Microautophagy and Macroautophagy (Tekirdag and Cuervo 2018)

B.2.1 Chaperone Mediated Autophagy

Chaperone-mediated autophagy was the first selective autophagy pathway to be described. Proteins are the only substrates that can be degraded by this mechanism, and the characteristic "selectivity" associated with it, comes from the mechanism of delivery of these protein substrates to the lysosomes (Kaushik and Cuervo 2018). Mis-folded or dysfunctional proteins are identified by a specific motif in their sequence. This allows them to interact with the chaperone proteins, which are responsible for the trafficking and entry of the proteins into the lysosomes. Most commonly known chaperone proteins are Heat-shock Protein70 (Hsp70) (Figure 9). Others include Carboxy-terminal HSC70-interacting protein (CHIP), Hsp40, and Hsp90 (Ferreira et al. 2013).

Chaperone-mediated autophagy was first investigated and described by Fred Dice (Dice 1982), using the protein Ribonuclease A. They identified an 11-amino acid peptide sequence as the motif that renders the selective property of this pathway. This sequence has been narrowed down to a 5-amino acid sequence: « KFERQ » (Dice 1982). This sequence may vary among different mammalian proteins; however, the general construct remains the same. A glutamine residue on either side must flank the sequence, 1 or 2 positively charged residues (lysine or arginine), 1 or 2 hydrophobic residues (Leucine, valine or Phenylalanine), and 1 negatively charged residue (Kaushik, et al, 2018). Heat shock cognate 71 kDa protein (HSC70) identifies and binds to the KFERQ sequence and facilitates its trafficking to the lysosomes. HSC70 is also known to participate in chaperone-assisted selective autophagy, wherein; it interacts with chaperone proteins like BCL2-associated athanogene 1 (BAG1), and aids in the delivery of ubiquitin-labeled protein substrates to the autophagosomes for degradation (Arndt et al. 2010).

A key step in the lysosomal degradation of proteins is dependent on lysosomal associated membrane protein type 2A (LAMP2A). The interaction of HSC70 with the membrane receptor is imperative in the docking of the proteins. LAPM2A, one of the 3 splice variants of the LAMP2 protein, is the only isoform essential for chaperone-mediated autophagy (Eskelinen et al. 2005). The cytosolic end of LAMP2A carries a 12-amino acid motif, which binds to the substrate complex via HSC70 (Cuervo et al. 2000; Cuervo, et al. 1997). The docking step is a coupled mechanism, wherein; the

protein substrate and HSC70 bind to LAMP2A at the same time. The substrate binds to the translocation complex via the 3 positive amino acid residues of LAMP2A cytosolic motif, with 3-negatively charged residues present on the unfolded configuration of the target protein (Cuervo et al. 2000). LAMP2A forms a trimeric structure to increase the binding affinity and internalization of the substrate (Bandyopadhyay et al. 2010). Cytosolic HSC70 binds to the monomers of LAMP2A and aids in the formation of the translocation complexes, however, it dissociates before the complex is completely formed. Luminal HSC70 is responsible for maintaining the stability of the LAMP2A trimer as it undergoes conformational changes, and certain domains may be exposed to degradation by proteases (Bandyopadhyay et al. 2008).

Even though HSC70 plays a central role in the triad involved in chaperone-mediated autophagy, LAMP2A is the rate-limiting variable in the process. Transcription of LAMP2A is upregulated during oxidative stress or DNA damage, to facilitate *de novo* synthesis and is regulated by the calcineurin / nuclear factor of activated T-cells 1 (NFAT1) pathway. Oxidative stress induces the translocation of NFAT1 to the nucleus, where it directly binds to the LAMP2A promoter site and causes its upregulation (Valdor et al. 2014).

B.2.2 Microautophagy

Microautophagy was described in 1966 by de Duve and Wattiaux in rat liver samples (Valdor et al. 2014). Microautophagy is defined as the process by which intracellular proteins or damaged cytosolic components are directly engulfed by lysosomes for degradation (W. Li et al. 2012). The lysosomal membrane undergoes several conformational changes, like tubular invaginations or protrusions, to allow the uptake of the targeted cellular content (Figure 9) (Mijaljica, et al. 2011).

Non-selective microautophagy relies on the activity of an ATPase and a proton gradient across the vacuolar membrane, along with a supply of lipids and excised membranes to form the microautophagy vesicle (Sattler and Mayer 2000). On a molecular level, non-selective microautophagy is partially regulated by target of Rapamycin (TOR) and EGO protein complex. EGO complex is involved in mitophagy

and helps with the exit from rapamycin-induced growth arrest. The protein complex is comprised of Ego1, Ego 3 and Gtr2 (Dubouloz et al. 2005). Another protein complex involved in the regulation of microautophay is vacuolar transport chaperone. Upon starvation, the tubular invaginations are enriched in Vacuolar Transport Chaperon (VTC) complex. Mutated versions of VTC showed impaired scission of the microautophagy vesicles from the vacuoles (Dubouloz et al. 2005; Mijaljica, et al. 2011).

Studies carried out in yeast, have shown that microautophagy is able to identify and discriminate amongst the different types of cargo, giving rise to pathways like micropexophagy (Cho et al. 2018), micromitophagy (Lemasters 2014), macrolipophagy (Seo et al. 2017). In micropexophagy, the peroxisomes are sequestrated by vacuolar membranes, and the micropexophagy-specific membrane apparatus (MIPA) is responsible for the fusion of the tips of the tubular invaginations. The MIPA complex consists of a core of ATG proteins, ATG11, 20, 24-26, 28 and 30. As the vacuolar membrane fuses on the lumen-facing side, the peroxisomes are degraded in the acidic environment (Sakai et al. 2006; Manjithaya et al. 2010)). Goldfarb first reported Micronucleophagy in 2003, in *Saccharomyces cerevisea* (Kvam and Goldfarb 2004). The first step in this process is the formation of junctions between the nucleus and the vacuolar invaginations, regulated by protein interactions between Vac8 and Nvj1. Recent studies also suggest the role of V-ATPase in the formation of these junctions (Roberts et al. 2003; Farré et al. 2008). The molecular machinery for this process employs a number of autophagy proteins, ATG1-18 and 22. The fusion step involves several classical proteins involved in homotypic membrane fusions, like, Sec17 and 18, Vam 3 and 7, Vps33, 39 and 41. Lipid biosynthesis is crucial for this type of autophagy. This was proved by the involvement of proteins like Osh1 and Tsc13, which are responsible for sterol homeostasis and synthesis of fatty acids, respectively. Nucleolus and nucleoplasm proteins seem to be degraded by the hydrolytic enzymes involved in micronucleophagy (Kvam and Goldfarb 2004; R. Krick et al. 2008; Roswitha Krick et al. 2009; D. Mijaljica and Devenish 2013).

Endosomal microautophagy was first studied to elucidate the role of autophagy in antigen presentation in dendritic cells (Tekirdag and Cuervo 2018). Cytosolic

proteins are processed for presentation in the multivesicular bodies or late endosomes. The endosomal sorting complexes required for transport *(*ESCRT*)* complex is responsible for the formation of the multivesicular body, and when proteins are internalized via an ESCRT-dependent pathway, they undergo complete degradation (Sahu et al. 2011). Other studies have now reported endosomal microautophagy to occur in other cell types, like in the liver and brain (Caballero et al. 2018).

Previously, proteins carrying the KFERQ-like motif were known to interact with HSC70 and be degraded by chaperone mediated autophagy. However, analyses have shown that the multivesicular bodies are also enriched in cytosolic proteins carrying this peptide sequence, like GAPDH and RNases. A hypothesis states that the switch between the different autophagic pathways could be due to the different intrinsic modifications that the proteins undergo, like mutations and post-translational modifications, that render them susceptible to be degraded by one pathway rather than the other (Sahu et al. 2011). HSC70 binds to a substrate protein via the KFERQ-like motif, along with co-chaperone, Sgt (Uytterhoeven et al. 2015). Unlike in chaperone mediated autophagy, HSC70 is internalized and degraded, in the endosomal pathway, (Sahu et al. 2011; Uytterhoeen et al. 2015).

B.2.3 Macroautophagy

The unique feature that makes macroautophagy different from chaperone mediated autophagy or microautophagy, is the *de novo* formation of a cytosolic vacuole, carrying the sequestered cargo (Yorimitsu and Klionsky 2005). The site for formation of autophagosomes is the phagophore assembly site. In yeasts, it is called the perivacuolar site, where most of the machinery proteins localize and assemble (Reggiori and Klionsky 2013). In mammalian cells, there are several phagophore assembly sites scattered across the cytoplasm (Itakura and Mizushima 2010; Y. Chen and Klionsky 2011). Following initiation, the membrane surrounding the target cargo begins to extend and forms a structure called the phagophore, which is the first double membrane structure identified in this pathway (He and Klionsky 2009).

Various sources of the membrane have been reported, like the plasma membrane (Ravikumar et al. 2009), mitochondria (Hailey et al. 2010), Golgi body (Takahashi et al. 2011) or the ER (Ylä-Anttila et al. 2009) (Figure 10). The phagophore continues to extend, eventually enveloping the target substrate (Figure 10).. Once the substrate is entirely engulfed, the double membrane envelope closes and forms a vacuolar structure called the autophagosome (Parzych and Klionsky 2018) (Figure 10). The size of this structure depends in the cargo and can range from 0.5μm to 1.5μm in mammalian cells (Mizushima 2007). Once the autophagosome is formed, it must be trafficked to the lysosomes (Figure 10). Here, they must fuse so that the autophagy substrate can be degraded. In the presence of the low pH and hydrolytic enzymes, both, the components of the autophagosome membrane and the cargo are degraded. Some contents of the vacuole, including the membrane, are recycled and released back into the cytosol by permease proteins, and can be utilized for other biosynthetic pathways or production of energy (Yorimitsu and Klionsky 2005). The five major protein complexes required for the formation of autophagosomes and their delivery to the lysosomes are:

1) ULK1 complex (Unc-51-like kinase): ULK1, RB1-inducible coiled protein (RB1CC1/FIP200), ATG13, ATG10 (Y. Chen and Klionsky 2011).
2) Class III PI3K complex: Vacuolar protein sorting 34 (Vps34), Beclin-1, Vesicular Transport factor p115, ATG14,
3) WIPI, ATG2.
4) ATG9
5) Ubiquitin-like proteins: ATG12 which forms a complex ATG12-ATG5-ATG16L1, and ATG8 (includes LC3 and GABARAP) (Mizushima et al., 2011; Nakatogawa et al., 2009).

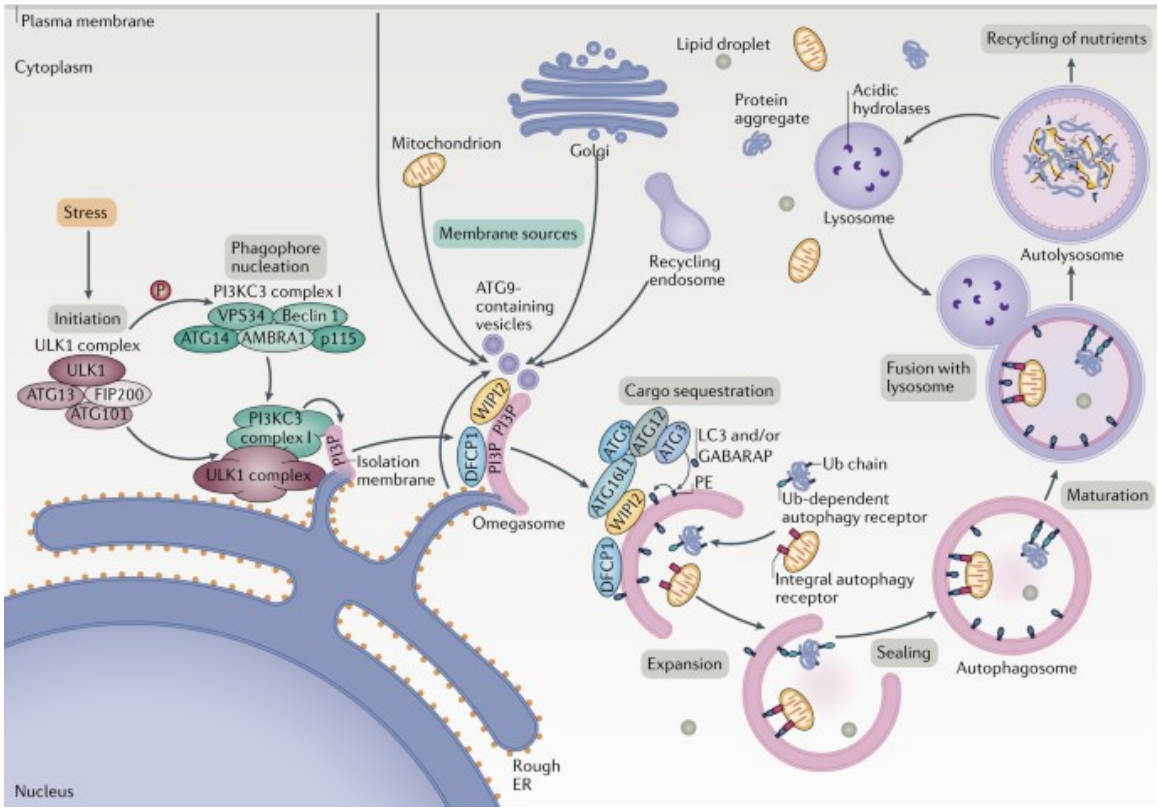


Figure 10: Overall mechanism of autophagy and the protein complexes involved at the different stages of the pathway ((Dikic and Elazar 2018)

i) Initiation

Initiation is regulated by two autophagy protein complexes: Unc-51-like kinase (ULK) 1/2 and Class III PI3K. The ULK1/2 complex consists of Unc 51-like protein 1/gaia2; ATG13; ATG17, retinoblastoma protein-1 inducible coiled 1 (RB1CC1/FIP200) (Hara et al. 2008). One of the most common triggers for autophagy is depletion of amino acids. This inhibits the activity of a key molecular regulator, serine/threonine kinase protein mammalian target of rapamycin (mTOR) (Neufeld 2010). mTOR is the catalytic subunit of the mammalian target of rapamycin complex 1 and 2 (mTORC1 and 2) (Jung et al., 2010). Under nutrient-rich conditions, mTORC1 is bound to the complex, where it inactivates ULK-1/2 and ATG13 by phosphorylation (Jung et al. 2009). When cells are starved, mTORC1 is released from the complex. Thus ULK1/2 and ATG13, are dephosphorylated, and thereby activated, which leads to autophagy initiation. After dissociation from mTOR, ULK1 undergoes autophosphorylation, and

subsequently phosphorylates ATG13 and FIP200 (Zachari and Ganley 2017). mTOR also regulates autophagy by targeting another protein, Transcription Factor EB (TFEB). TFEB participates in a feedback loop to activate mTORC, where it upregulates the expression of mTOR-activating Ras-related GTP-binding protein D (RagD) (Malta et al. 2017).

Another cell energy sensor molecule is 5'-AMP-activated protein kinase (AMPK). Serine/threonine protein kinase (STK11/LKB1) induces autophagy via AMPK activation. Raptor is a known substrate of AMPK, and undergoes phosphorylation at serine residues and consequentially, downregulates mTOR. Another substrate which undergoes phosphorylation, and inhibits mTOR activity, to boost autophagy, is Tuberous Sclerosis 2 (TSC2) (Tripathi et al. 2013). TSC2 interactions with WIPI3 and FIP200, favored by AMPK, leads to inhition of mTOR (Parzych and Klionsky 2018) . This signaling axis is part of the feedback circuit, involving CREB-regulated transcription coactivator 1. AMPK can also activate autophagy by directly phosphorylating ULK1, Vps34 and Beclin-1 (Kim et al. 2013)

The Class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, is another important protein complex recruited at the phagophore assembly site.. It includes Vps34, Vps15, ATG6 and ATG14, and is part of the autophagy induction machinery. Vps34 is required for the formation of phosphatidylinositol (3)-phosphate (PtdIns (3)P), which is responsible for recruiting other ATG proteins. Vps34, Vps15, ATG6 and Vps38 form the second complex, which mainly plays a role in later stages of autophagy. In mammalian cells, PtdIns3K I includes Vps34, Beclin-1 and p150. The orthologs of ATG14 and Vps38 are ATG14L and Ultraviolet irradiation resistant-associated gene (UVRAG) respectively (Gottlieb, 2013). Atg14L recruits a subset of class III PI3-kinase to the ER, and this Atg14L-mediated delivery of PI3P at the ER makes the role of endoplasmic reticulum in the autophagosome formation essential (Matsunaga et al. 2010).

Beclin-1 is a key regulator in this complex. Beclin-1 has three main domains: BH3 (Bcl-2 homology 3), CCD (coiled coil domain) and ECD (evolutionary conserved domain) (Zhu and He 2015). A negative regulator is Bcl2 which binds to Beclin-1 and

inhibits its interaction with Vps34, thereby blocking autophagy (Pattingre et al. 2005). Phosphorylation by ULK-1, MAPKAPK and DAPK promotes Beclin-1 activity. Positive regulators of Beclin-1 are AMBRA1 and Bif-1. AMBRA1 is phosphorylated by ULK1, which makes it dissociate from the microtubules, and allows binding to Beclin-1 (Fimia et al. 2007).

UVRAG is known to participate and regulate autophagy at four different stages of the pathway (Itakura and Mizushima 2009). First, UVRAG binds to Beclin-1, in the Beclin-1-Vps34-p150 complex. This binding has been correlated to the increase in Vps34 activity, and therefore regulation of autophagy (Funderburk., Wang, and Yue 2010).. Second, it binds with Bax-interacting factor-1 (Bif-1). Bif-1 colocalizes with LC3, ATG9 and ATG5. Third, it helps in the fusion of autophagosomes with lysosomes by interacting with homotypic fusion and vacuole protein sorting (HOPS) proteins. It was also reported that it forms a complex with Beclin-1-Vps34-p150 which binds with Rubicon protein. However, this interaction inhibits maturation of autophagosomes by reducing the activity of Vps34 (Itakura and Mizushima 2010) (Figure 12).

ii) Nucleation and Elongation

Formation of phagophores begins by their nucleation on ER-originating membrane domains called omegasomes (Figure 11). They are double-membraned structures, enriched in phosphatidylinositol 3-phosphate (PI3P), which is produced by the PtdIns3K. This allows the recruitment to the phagophore membrane of PI3P-binding proteins: WIPI2 and the zinc finger FYVE domain-carrying protein or ZFYVE1 (Axe et al. 2008).

The first conjugation system is the ATG12-ATG6-ATG16L1 complex. The covalent bond between ATG5 and the ubiquitin-like protein ATG12, depends on the activity of E1 activating enzyme, ATG7 and E1 activating enzyme, ATG10. This is an irreversible conjugation, independent of E3 ligase activity. ATG16 binds in a non-covalent manner to ATG5 to form a complex. At the site of autophagosome formation, ATG16 is also known to polymerize and form multimeric complexes. This conjugation is important

for the trafficking of ATG12-ATG5 to the assembly site, which depends on the interaction of ATG16L1 with WIPI2. This complex is bound to the vacuolar membrane until the complete formation of the autophagosome, following which it dissociates from the membrane. It is negatively regulated by a Golgi protein, RAB33A, which interacts with ATG16L1 and inhibits its binding to ATG5. Other members of this complex can be inhibited via acetylation by the enzyme KAT2B/p300.

The major protein complex implicated in the elongation step of the pathway is ATG8. LC3 and GABARAP are two major subfamilies of ATG8 isoforms (Hemelaar et al. 2003; Weidberg et al. 2010). ATG8 is processed by ATG4, a cysteine protease which exposes a glycine residue at the C-terminus end ZFYVE1 of ATG8. The specificity of the four isoforms of ATG4 has not been completely elucidated, however some studies reported that ATG4B is able to interact with all members of the ATG8 family, but ATG4A shows affinity for GABARAP sub-family (Kauffman et al. 2018).

The E1-like enzyme, ATG7 then activates ATG8 and transfers it to ATG3, which helps the binding of phosphatidylethanolamine (PE) at the free glycine residue site. This conversion of a free, diffuse form of ATG8 (LC3-I/GABARAP-I) to a lipidated, membrane bound form (LC3-II) is an important step in autophagy (Kirisako et al. 2000).
The conjugation of ATG8 and PE occurs on the ER-exit site, due to the translocation of SEC12 to the ER-Golgi intermediate compartment, by FIP200. Phospholipidated ATG8 is also involved in selective autophagy, wherein it helps in the identification and sequestration of cargo. ATG8 acts as adaptors for the recruitment of LC3-interactng region (LIR)-motif carrying proteins to the growing surface of phagophore membrane. While there seems to be some redundancy in the roles of GAPABAP and LC3, pulldown assays have revealed clear differences in their interactomes, which suggest diverse roles of these families of proteins in the sequestration of specific cargo (Schaaf et al. 2019). ATG4 can also unconjugate PE from AT8, which is inhibited by phosphorylation of ATG4 by ULK1.

Expansion of the phagophore requires recruitment of lipid membranes. One proposed mechanism is the ATG9-mediated delivery of recycled lipids from a source site to the phagophore assembly site . ATG9 is the only transmembrane, core

machinery protein, with 6 transmembrane domains. ATG9 protein complex is required for the localization of the Ubiquitin-like proteins to the phagophore assembly site . The trafficking of ATG9 to the phagophore assembly site depends on ULK1, ATG2 and ATG18. ATG9 recruits the ATG12-5-16L1 and ATG8-PE complexes to the phagophore assembly site. The ATG1-13 complex is also involved in the retrieval of ATG9 from the phagophore assembly site (Köfinger et al. 2015). Another mechanism is ATG16-mediated. ATG16L1 associates with clathrin-coated pits, followed by internalization and uncoating, the ATG16L1-associated plasma membrane becomes associated with phagophore precursors, which mature into phagophores and subsequently autophagosomes (Xie and Klionsky 2007).

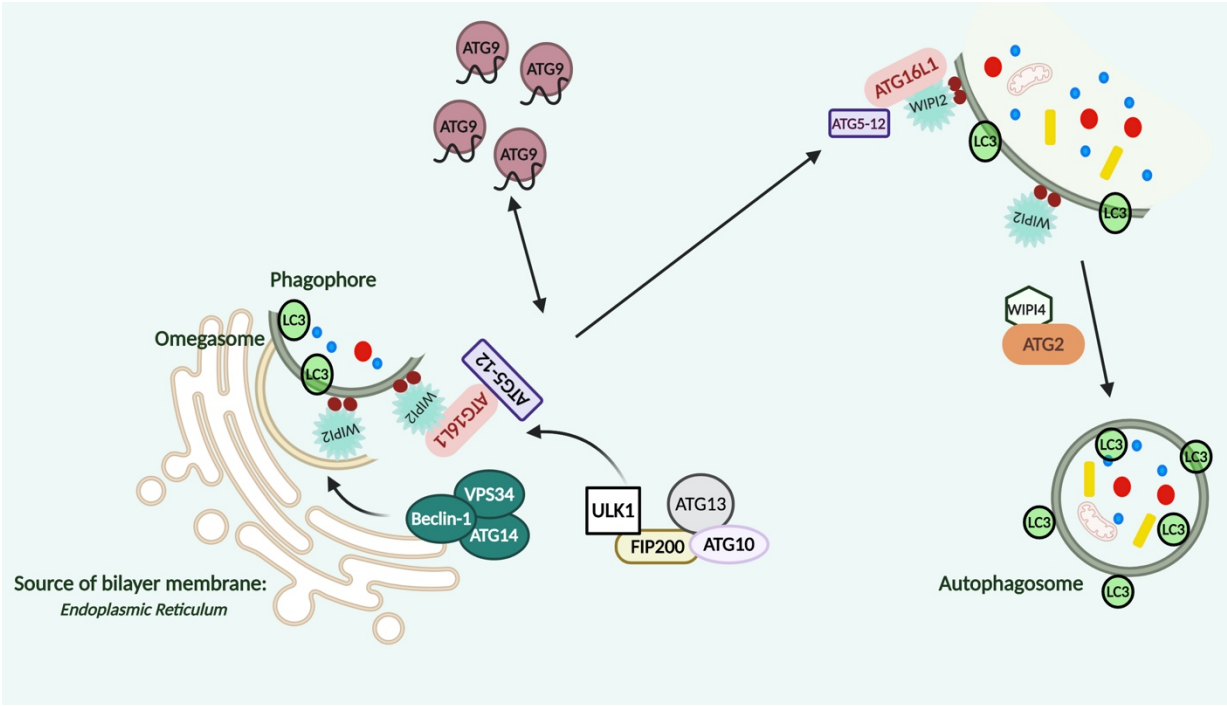


Figure 11: Mechanism of autophagosome formation: Adapted from (Mercer et al. 2018)

iii) Autophagosome Maturation and Fusion

Dephosphorylation and clearance of PI3P by the PI3P phosphatase Ymr1 on the completed autophagosome are essential for fusion of autophagosome and vacuole (Cebollero et al., 2012). Clearance of PI3P triggers the dissociation of the ATG machinery from the outer autophagosomal membrane, which makes the autophagosome competent for fusion.

On completion, autophagosomes and endosomes must be trafficked to the lysosomes, where they fuse by a Cczc and Mon1 dependent mechanism (Reggiori and Ungermann 2017). These proteins form a complex and participate in the homotypic vacuole fusion in the Cvt pathway (C.-W. Wang et al. 2003). These two proteins are part of a core machinery of proteins including SNAREs (Vam3, Vti1, Vam7, Sec18, Sec17, Rab GTPase, Class CVPS/HOPS) (Figure 12). As the external membrane of the autophagosome fuses with the lysosome, the inner membrane is released into the lysosomal lumen. Subsequently, it is degraded by the proteases in the lysosome, Pep4 and Prb1, which are active in acidic environment (Parzych and Klionsky 2018). The acidic environment has been a target for several pharmacological treatments, wherein, change in the pH of the lysosomes hampers the fusion of autophagosomes and lysosomes. In that sense, traditionally used autophagy blockers include inhibitors of lysosomal acidification such as Chloroquine (Mauthe et al. 2018) and Bafilomycin A (Mauvezin and Neufeld 2015).

Although autophagosomes are formed at the various phagophore assembly site across the cytoplasm, lysosomes are predominantly localized at the perinuclear region (Korolchuk and Rubinsztein 2011). Most commonly, dyneins help in the retrograde transport of autophagosomes. It has been demonstrated via knockdown of LC3, that trafficking of autophagosomes is impaired (Tammineni et al. 2017). This suggests a possible interaction of LC3 with the microtubules, presumably via MAPA1 and 2 (Halpain and Dehmelt 2006). Kinesins, like KIF5B, are essential for appropriate localization of autophagosomes. Late autophagosomes are transported to the cell periphery via the microtubule with the help of FYVE and coiled-coil domain-containing 1 (FYCO1). FYCO1 interreacts with small GTPase Rab7, LC3 and PI3P (F.

Guerra and Bucci 2016). It drives kinesin-dependent movement of autophagosomes by interacting with KLC2 light chain of kinesin1 (Pankiv et al., 2010; Raiborg et al., 2015).

UVRAG and RUBICON, members of the PI3K complex, also regulate the maturation and fusion of autophagosomes. While UVRAG promotes lysosomal fusion (Liang et al. 2006; Matsunaga et al. 2009), RUBICON has an inhibitory effect on this step (Q. Sun et al. 2011). UVRAG, localized at the ER, binds to the HOPS subunit, VPS16, and activates GTPhagophore assembly sitee Rab7. Under basal conditions, UVRAG is phosphorylated by mTORC1 which increases its interaction with RUBICON and prohibits VPS34 activity and binding to the HOPS complex (Figure 11) (Nakamura, et al, 2017).

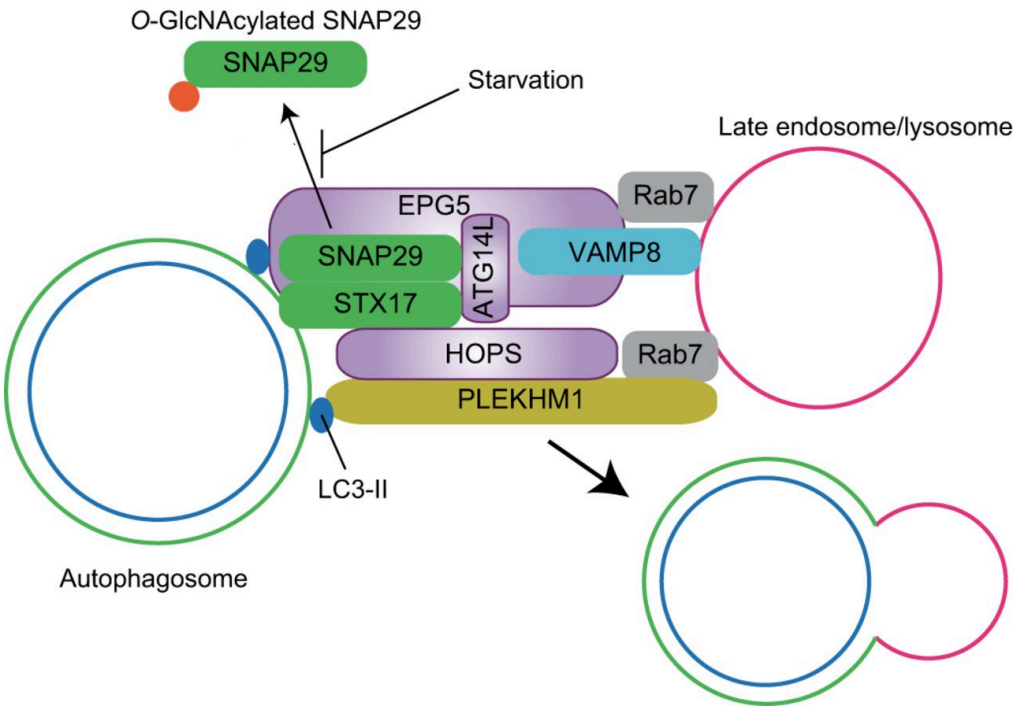


Figure 12: Protein complexes involved in the fusion of autophagosomes and late endosomes/lysosomes Adapted from Nakamura et al. 2017.

The fusion step employs other protein complexes like membrane-tethering complexes and SNARE proteins (soluble N-ethylmaleimide- sensitive factor attachment protein receptors). Rab GTPases recruit the tethering proteins to link the

two compartments together, while SNARE proteins drive the actual fusion of the two (Pylypenko et al. 2017; Lürick et al. 2018).

Rab Proteins

Ras-related protein in brain (Rab) GTPases are conserved proteins which regulate membrane trafficking. As mentioned above, they recruit cargo adaptor proteins to form the vesicles, motor proteins to traffic them and tethering proteins to help fuse them. As different Rab proteins act at different stages, they are believed to be markers for different stages of trafficking process (Vázquez-Martínez and Malagón 2011). Rab proteins are activated by specific guanine nucleotide exchange factors (GEFs) that bring about conformational changes. Rabs are negatively regulated by GTPase-activating proteins (GAPs) (Nakamura et al. 2017). Rab7 recruits its effectors Pleckstrin Homology And RUN Domain Containing M1 (PLEKHM1) and Rab Interacting Lysosomal Protein (RILP) that bind to the HOPS components VPS39 and VPS41, respectively (Wijdeven et al., 2016). Rab33, localized at the Golgi complex, negatively regulates formation of autophagosome by binding to ATG16, in a guanosine triphosphate-dependent manner (Itoh et al. 2008). Rab7 recruits another effector, the cholesterol sensor ORP1L, that binds to Rab7 in the presence of RILP and negatively regulates fusion (Wijdeven et al. 2016). At low levels of cholesterol, Oxysterol-Binding Protein-Related Protein 1 (ORP1L) interacts with the ER protein VAP-A to form the ER-autophagosome contact site. This prevents the recruitment of PLEKHM1 to Rab7, and HOPS complex, which results in a defective fusion. This complex with VAP-A, prevents the recruitment of dynein and/or dynactin by RILP and, thus, retrograde transport of late autophagosomes (Wijdeven et al. 2016). PLEKHM1 is also recruited by GABARAP to promote autophagosome–lysosome fusion (Nguyen et al. 2016)..

SNAREs

About 60 different SNARE proteins have been identified that are involved in the fusion process. Q-SNARE Syntaxin 17 (STX17), is recruited to the closed autophagosomes, and mediates fusion with lysosomes along with partner proteins, SNAP29 and VAMP8 (Figure 12) (Itakura et al., 2012). The O-linked N-acetylglucosamine (O-GlcNAc) modification of SNAP29 inhibits SNARE-dependent

fusion in a nutrient-dependent manner (B. Guo et al. 2014). Under nutrient rich conditions, STX17 is localized at the ER, but during starvation it is localized at ER-mitochondrial site (Hamasaki et al. 2013). This complex also binds to the STX17 and SNAP29 and aids in the binding with VAMP8 (Diao et al. 2015) (Figure 12).

Tethering factors

Membrane tethers facilitate docking and fusion by bridging opposing membranes and by stimulating SNARE complex formation. The HOPS complex functions as a tethering factor for autophagosome/lysosome fusion (P. Jiang et al. 2014). STX17 interacts with the HOPS complex, including VPS33A, VPS16, VPS11, VPS18, VPS39 and VPS41. Consistent with this, these HOPS subunits are recruited to STX17-positive autophagosomes upon starvation.

Ectopic P granules protein 5 (EPG5) is another Rab7 effector and tethering factor that determines the fusion specificity of autophagosomes with endosomes/lysosomes (Wang et al., 2016). EPG5 is recruited to lysosomes through Rab7 and R-SNARE VAMP7/VAMP8. EPG5 also binds to LC3/ through its LC3-interacting region (LIR) motif and to assembled STX17–SNAP29 complexes on autophagosomes. EPG5 stabilizes and facilitates the assembly of STX17–SNAP29–VAMP8 trans-SNARE complexes and, thus, is most likely to promote the fusion between autophagosomes and lysosomes (Kriegenburg et al. 2019)

Tectonin beta-propeller repeat- containing 1 (TECPR1) also functions as a tethering factor. TECPR1-depleted cells have impaired autophagic flux and demonstrate accumulation of p62/SQSTM1 and LC3-II (Shuang Chen et al. 2014). TECPR1 and ATG16 form mutually exclusive complexes with the ATG12– ATG5 conjugate, and TECPR1 binds PI3P upon association with the Atg12–Atg5 conjugate (Shuang Chen et al. 2014). Furthermore, TECPR1 localizes to lysosomes/autolysosomes and recruits the ATG12–ATG5 conjugate, which enables its binding to PI3P, thereby possibly facilitating autophagosome maturation and autophagosome–lysosome fusion.

A Recently discovered regulator of lysosomal fusion is phosphoinositide phosphatase inositol polyphosphate-5 phosphatase E (INPP5E) (Hasegawa et al. 2016; S. Nakamura, Hasegawa, and Yoshimori 2016). Inhibition of INPP5E causes impairment

of autophagosome–lysosome fusion INPP5E protein localizes to lysosome membranes, where it mediates the conversion of [PI(3,5)P2] to PI3P, crucial for fusion with autophagosomes (de Lartigue et al. 2009).

B.3 ROLE OF AUTOPHAGY IN VASCULAR DYSFUNCTION AND ATHEROSCLEROSIS

Autophagy in response to different stress stimuli in different cell types, like macrophages, vascular smooth muscle cells, and endothelial cells, has been implicated in the development and progression of atherosclerosis. An important factor predisposing to cardiovascular events is the propensity for an atherosclerotic plaque to rupture vs. remain stable. Studies have shown that basal autophagy in macrophages is important in maintaining the integrity of the fibrous cap and restricting lipid accumulation (Grootaert et al. 2018). Insufficient or inhibition of autophagy accelerates plaque burden or renders it unstable (Grootaert et al. 2018; X. Liao et al. 2012)

The past decade has seen increasing evidences of autophagy as a key player in the pathology of cardiometabolic diseases. Analyses of atherosclerotic plaques from symptomatic patients have exhibited low levels of LC3-II expression, compared to control patients, indicating dysfunctional autophagy in disease phenotype (Swaminathan et al. 2014). Other studies, analyzing macrophages isolated from human and murine plaques, have shown accumulation of another marker, p62 and presence of ubiquitinated proteins. In human samples, it was observed that p62, instead of colocalizing with LC3-II; mainly colocalized with the ubiquitinated proteins. The lack of colocalization with LC3-II was indicative of defective autophagy (Sergin, et al. 2015).

It is widely assumed that dysfunctional autophagy in the context of atherosclerosis, is due to the defect in the rate-limiting step of, lysosomal fusion. Defective autophagy is attributed to the accumulation of insoluble complexes formed by proteins and ox-LDL particles in the lysosomal structures.

Furthermore, oxidative stress can hinder the efficient activity of lysosomes, by causing the rupture of the lysosomal membrane and release of the lytic enzymes. This also results in defective removal of damaged mitochondria. The accumulated damaged mitochondria induce apoptotic pathways via the release of cytochrome C. Finally, cholesterol crystals accumulating in advanced atherosclerotic plaques can damage the lysosomal membrane (Sergin, et al.2015; Grootaert et al. 2018).

These findings suggest that, even though autophagy may be initiated by stimuli like reactive oxygen species, oxidized lipids, and shear stress, the defect in the pathway lies in the fusion and degradation steps, thereby impairing the autophagic flux (Grootaert et al. 2018).

B.3.1 Autophagy in Macrophages

Ox-LDL and oxysterols, via ER-stress, are an inducer of autophagy in macrophages. One of the key roles of autophagy in macrophages is cholesterol efflux from foam cells. Free cholesterol molecules are transported to the autolysosomes for degradation, by macroautophagy. Another pathway triggered is mitophagy, by different inflammasomes (Razani et al. 2012). ATG5-deficient macrophages are involved in increased generation of ROS, apoptosis and defective efferocytosis, cholesterol efflux, and increase inflammation due to excessive IL-1b secretion. These findings have been substantiated in macrophages from murine *ATG5*$^{-/-}$ *LDLr*$^{-/-}$ model, which exhibit a proatherogenic phenotype by promoting inflammation, necrosis and apoptosis (X. Liao et al. 2012).

B.3.2 Autophagy in Vascular Smooth Cells

4-hydroxynonenal (4-HNE), a product of lipid peroxidation, is known to activate autophagy in VSMCs, by inducing ER stress. Autophagy can also be modulated in VSMCs by exposure to different cytokines like TNF-α (Hill et al. 2008).
ATG7-deficient VSMCs promoted senescence, via increase in senescence associated β-galactosidase, high collagen secretion, migration, expression of inflammasomes like

TGF-β, IL1b and NLRP3. ATG7-deficient mice have shown an accumulation of p62 in VSMCs, which promotes the p16/RB-dependent senescence. Also, under oxidative stress, ATG7-deficient VSMCs exhibited an increase in the expression of NRF2 and ARE, which allowed them to be resistant to cell death induced by oxidative stress and promoted senescent phenotype (Vindis 2015)

PTEN-induced kinase 1 (PINK1) is a mitochondrial protein and a marker for mitophagy. Knockdown of *PINK1* by siRNA in VSMCs exhibited a cytotoxic response when exposed to oxLDL whereas PINK1 overexpression had an atheroprotective effect (Swiader et al. 2016). Formation of AGE, as a result of hyperglycemia and oxidative stress, plays an important role in atherogenesis. AGEs trigger autophagy by promoting VSMC proliferation through ERK, JNK and p38 signaling pathways, explaining the onset of atherogenesis in diabetes patients (Vindis 2015).

B.3.3 Autophagy in Endothelial Cells

i) Regulators of endothelial autophagy

Growth Factors

Autophagy is induced by inhibition of mTOR upon amino acid depletion or activation of AMPK upon glucose starvation. These two nutrient-sensing pathways converge on ULK1, a key initiator of autophagy, and regulate ULK1 activity by phosphorylating serine residues that either inhibit or activate the enzyme (Alers et al. 2012). Growth factors such as VEGF are known to trigger the Akt/mTOR pathway (Simons et al. 2016), which mediates the inhibitory phosphorylation of ULK1, suggesting that they slow down autophagy. In line with this, growth factor removal has been reported to activate autophagy (Lum et al. 2005). However, VEGF is also a potent stimulus for AMPK activation , which in turn may lead to ULK1 activation (Spengler et al. 2020).

VEGF stimulates autophagy via an AMPKα1-dependent mechanism. Autophagy activation was verified by VEGF-induced phosphorylation of ULK1 and its substrate ATG14 at sites known to be involved in the initiation of autophagy, S556 and S29,

respectively, by phosphorylation of ATG16L1, a component of the LC3B lipidation complex and by conjugation of LC3B, a marker of autophagosome formation (Yu et al. 2018). Furthermore, functional autophagy was confirmed by an increase in autophagic flux soon after VEGF stimulation and a decrease in p62 expression, which indicates its enhanced autophagic degradation in response to VEGF (Spengler et al. 2020).

Oxidized Lipids

Several studies have reported that endothelial cells exposed to ox-LDL showed an accumulation of autophagosomes, indicating a defect in the autophagy flux (C. Zhang et al. 2020; Yan-lin Zhang et al. 2010). Uptake of lipid particles by endothelial cells, can lead to splicing of the X-box binding protein 1 (*XBP-1*) mRNA, which in turn activates the transcription of Beclin-1 (Margariti et al. 2013).

Shear Stress

Shear forces are an important modulator of endothelial autophagy and leads to different phenotypes in endothelial cells (Jang et al. 2018). Autophagy is required for maintaining endothelial functions such as synthesis and release of nitric oxide, endothelial barrier function, secretion of anti-thrombotic factors, and angiogenesis (Spengler et al. 2020).

Studies have reported the regulation of shear stress in regulating several signalling pathways. For instance, laminar shear stress upregulates AMPK-dependent pathways by increasing the phosphorylation of AMPK, and inhibits mTOR-dependent pahways, thereby promoting autophagy in endothelial cells (F.-X. Guo et al. 2017).

Some major potential signaling pathways contributing to the regulation of endothelial autophagy by shear stress are described below.

a) The Primary Cilium

The primary cilium is an essential microtubule-based organelle, which, upon mechanosensing due to blood flow, triggers a signaling cascade in epithelial cells

(Orhon et al. 2015). The phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2- α (PI3KC2α) lipid kinase has been shown to participate in the regulation of primary cilium biogenesis and primary cilium associated signaling via specific PI3P synthesis. It has been demonstrated that the expression of lipid enzyme PI3KC2α, is increased during shear stress, and is responsible for the synthesis of a specific pool of PI3P at the vicinity of primary cilium, in response to fluid flow in kidney epithelial cells. Although, PI3KC2α is not implicated in the autophagic response to starvation, it is specifically required for the autophagic program associated with mechanical stimulation relayed by the primary cilium, but not essential for nutritional stress response (Boukhalfa et al. 2020).

PI3KC2α is a key regulator of primary cilia functions via the synthesis of a pool of PI3P. This in turn initiates the membrane mobilization and activation of Rab11a (Campa et al. 2018). Rab11a is stabilized by shear stress in ciliated cells, and that its mobilization is affected upon PI3KC2α knockdown, presumably as a consequence of the PI3KC2α dependent PI3P pool depletion. The cellular adaptation to shear stress mobilizes PI3KC2α- dependent PI3P synthesis without depending on VPS34 (Boukhalfa et al. 2020).

Although these findings hold true in the case of kidney epithelial cells, Vion, Kheloufi, et al, stated that primary cilium does not regulate endothelial autophagy. They showed that in human umbilical vein endothelial cells (HUVEC) lacking PECAM or KIF3a, the primary cilium does not serve a mechanosensing role in shear stress modulated endothelial autophagy (Vion et al. 2017).

b) TFEB pathway

Transcription factor EB (TFEB) is a basic helix-loop-helix transcription factor that regulates lysosomal biogenesis and autophagy function in many cell types (Lu et al. 2017). High SS induces TFEB nuclear translocation, which induces TFEB transcription in an autoregulatory loop (Settembre et al. 2013). Furthermore, when TFEB is phosphorylated by mTOR complex 1 (mTORC1), it is retained in the cytoplasm (Martina et al. 2012). Overexpression of TFEB in endothelial cells potently inhibited inflammation, reduces intracellular ROS by increasing the abundance of antioxidant

genes such as heme oxygenase 1 (HO1) and superoxide dismutase 2 (SOD2) (Lu et al. 2017). TFEB abundance in endothelial cells is increased by high SS and thus could contribute to endothelial atheroprotective effects in these conditions (Lu et al. 2017).

Mechanistically, TFEB suppresses IKK activity to upregulate IκBα level, leading to restrained nuclear translocation of p65. Also, KLF2 is a prerequisite upstream regulator of TFEB expression in endothelial cells. Therefore, under laminar flow, KLF-2/TFEB signaling axis imparts anti-inflammatory phenotype to endothelial cells. However, TFEB activity is repressed in case of diabetes mellitus due to increased production of IL-1b. Lack of TFEB activity also suppresses AMPK activation, and by extension endothelial autophagy. This could be a possible mechanistic explanation as to why diabetic conditions favour a pro-atherogenic phenotype in endothelial cells (Song et al. 2019).

c) The Rab4 Pathway

High shear stress increases autophagy flux, by upregulating Rab4 expression. Yao, et al, showed that Rab4 siRNA decreased autophagy and increased the adhesion of monocytes to HUVECs in high shear stress. These results indicate that Rab4 is involved in endothelial autophagy, which contributed to endothelial inflammatory response under shear stress (Yao et al. 2016).

d) The Nitric Oxide Pathway

In recent years, it was found that autophagy-dependent mediator such as NO, restrains the formation of autophagosome though various pathways. NO reduces phosphorylation of AMPK, leading to mTORC1 activation by TSC2 though IKK-b and inhibiting the activity of JNK1 and then ultimately impairs autophagy. NO also promotes the interaction Beclin 1/Bcl-2 and reduces the phosphorylation of Bcl-2 though inhibition of JNK1, and then damaging the formation of VPS34/Beclin 1 complex, eventually affect autophagy (Bharath et al. 2014)

e) The KLF pathway

KLF4 has been implicated in regulating endothelial autophagy. Hseih, et al, demonstrated that in treatment with Rapamycin or starvation induced autophagy in HUVECs coincided with an increase in KLF4 expression. This link was supported by the findings that showed increased LC3-I lipidation in case of increased KLF4 expression, while the opposite was observed in case of siRNA induced silencing of *KLF4*. They also stated that when cells were exposed to laminar shear stress in the absence of *ATG7*, upregulation of KLF4 was not able to increase expression of eNOS. Hence proving that endothelial function is in fact, partially regulated by KLF4 via the autophagy pathway (Hsieh et al. 2017).

A similar study by Vion, Kheloufi, et al, showed that when HUVECs were transduced with a lentivirus carrying shRNA-targeting *ATG5* and exposed to laminar flow, the overall KLF2 expression was reduced. They were able to infer that even though HUVECs were exposed to laminar flow, and that KLF2 expression should have been upregulated in these conditions, the defective autophagy pathway hindered the expression of KL2 (Vion et al. 2017).

ii) Functional Consequences Of Endothelial Autophagy

Regulation Of The Endothelial NO Pathway

Bharath, et.al demonstrated that shear stress induces NO generation in endothelial cells by promoting autophagy. Inhibition of autophagy via Atg3 knockdown, prevented shear-induced phosphorylation of eNOS Ser1117, a positive regulatory mechanism. This led to decrease in NO generation, amplified production of reactive oxygen species, and promoted a pro-inflammatory endothelial phenotype (Bharath et al. 2017).

Emerging evidence also suggests that endothelial autophagy might modulate the uncoupling of eNOS. For example, Caveolin-1, a critical determinant of eNOS coupling and NO bioactivity, is also a key regulator of endothelial autophagy (Shiroto et al. 2014; Nussenzweig et al. 2015).

Regulation Of Endothelial Senescence And Inflammation

High levels of laminar shear stress (> 15 dynes/cm^2) stimulate autophagy in endothelial cells, whereas disturbed/ turbulent flow impairs autophagic flux and promotes endothelial dysfunction (R. Li et al. 2015; Vion et al. 2017).

Shear stress also influences atherogenesis by regulating autophagy mediated anti-inflammatory and anti-senescent responses. Vion, Kheloufi et al. have demonstrated that in endothelial cells exposed to laminar flow, autophagy is initiated via the inhibition of mTOR and activation of AMPK pathways. Autophagy stimulation leads to the reduced expression of adhesion proteins like ICAM-1, VCAM-1 and cytokines MCP-1, IL-6, IL-12, RANTES, IL-10. Lack of ATG5 in endothelial cells in mice, showed an increase in the inflammatory (Vion et al. 2017)

One of the main characteristics that has been investigated in the context of autophagy regulated vascular ageing, is impaired endothelial-dependent relaxation of large arteries (Nussenzweig, Verma, and Finkel 2015). It is attributed to the increase in reactive oxygen species produced by the vasculature with increasing age, and is linked more specifically to mitophagy (Mei et al. 2015) Excessive load of oxidative stress has been linked to the exacerbation of several cardiovascular diseases, including atherosclerosis (Nussenzweig et al. 2015). Older endothelial cells express lower levels of Beclin-1 and higher levels of p62. The accumulation of p62 indcates that the autophagic clearance is impaired in these cells. However in studies using pharmacological interventions like Trehalose (LaRocca et al. 2012) or Spermadine (LaRocca et al. 2013), are able to reverse this effect by promoting autophagic flux. It has also been suggested that because nutrient availability largely regulates autophagy, caloric restriction could help ameliorate ageing and thereby, its effects on the vasculature. (Nussenzweig et al. 2015).

Other than vascular tone, another aspect affected by endothelial senescence is vascular permeability. In homeostatic conditions, the endothelial barrier regulates the entry of small molecules, inflammatory cells and nutrients from the plasma. The gap junctions regulate the vascular permeability in response to oxidative stress and inflammation. A study mentioned the degradation of the endothelial barrier,

especially in the blood-brain barrier, and reported increased levels of TNF-α, by senescent endothelial cells (Yang et al. 2018).

In summary, it can be inferred that shear stress exerted on endothelial cells, regulated autophagy in vascular endothelial cells, and thereby, the different cellular processes like inflammation, ageing, and apoptosis, all of which are essential to maintain homeostasis of the endothelial barrier.

B.4 AUTOPHAGY AND MICROTUBULE

Microtubules (MT) are one of the three main cytoskeletal structures in eukaryotes. They are composed of heterodimers of α and β tubulin. They are long, hollow, rod-like structures, ranging from 1μm-100μm in length, and 25nm in diameter. Each heterodimeric sub-unit is referred to as tubulin dimers, or tubulin.

One of the many functions of the microtubule network is to facilitate intracellular transport of cellular cargo like organelles and vacuoles. This is crucial for the efficient functioning and maintaining homeostasis in all cell types (Barlan and Gelfand 2016). MTs serve as tracks for different motor proteins as they transfer cargo across the cytosol, like kinesins at the plus-end and dyneins at the minus-end. MTs also contribute to the protein content of the cell by engaging in their sequestration, transport, release, and assembly; and thereby indirectly affecting the signal transduction pathways in the cell.

B.4.1 Role of Microtubule in Autophagosome Formation

Although there is little evidence suggesting the role of microtubules in autophagosome formation under basal conditions, others have reported the role of MTs in formation of autophagosomes under starvation (Köchl et al., 2006 ; Geeraert et al., 2010). Both isoforms, LC3 I and LC3 II, interacts with Microtubule-associated proteins like MAP1A, MAP1B (Yang et al. 2018) and C19ORF5 (Xie et al. 2011). Other proteins involved in autophagy, ATG5, ATG12, ULK1 and Beclin 1, were also found in these sub-cellular fractions. Geeraert also stated that in starved cells, pre-

autophagosomal structures, carrying WIPI-1 move along the MT filaments. MTs have also been implicated in the maturation of phagophores, as they aid in the fusion of ATG16-carrying vacuolar membranes. MTs are also responsible for the regulation of autophagosome formation via mTOR and PI3K complex. mTOR activity is regulated by the localization of lysosomes, which in turn is controlled by MTs ad two motor proteins: KIF2A and KIF1B (Korolchuk and Rubinsztein 2011). In conditions of starvation, these motor proteins colocalize with lysosomes, thereby leaving mTORC1 in active state. Active mTORC1 blocks autophagosome formation. Beclin-1, a component of the PI3K complex, is sequestered along the MT filaments by two proteins: Dynein light chain 1 and AMBRA1 or Bcl-2 like protein. Phosphorylated AMBRA1 releases Beclin-1 from the complex (Di Bartolomeo et al. 2010) while simultaneously, phosphorylation of Bcl-2 like protein, Bim, by JNK1 also leads to the release of Beclin-1 (Luo et al., 2012). Free Beclin-1 can now be recruited to omegasome membranes to complete the formation of the autophagosome.

B.4.2 Role of Microtubule in Autolysosome Formation

Autophagosomes are formed in the periphery of the cell, and they are transported along the microtubules, towards the lysosomes, positioned near the nucleus. Autophagosomes interact with dynein motor proteins and are transported in a retrograde manner.

Increase in acetylation of α-tubulin leads to increased interaction with motor proteins like kinesins and dyneins. Therefore, they facilitate the fusion step of autophagy, by favoring the retrograde trafficking of autophagosomes by dyneins, and anterograde trafficking by kinesins. Histone deacetylase 6 (HDAC6) is a known deacetylase of α-tubulin.

Vinblastine increases the acetylation of α-tubulin; however, it does not support polymerization of microtubules. This treatment led to blockage of two key steps in the autophagy pathway: conversion of LC3-I to LC3-II, and fusion of autophagosomes and lysosomes (Köchl et al. 2006). Paclitaxel has a similar effect on acetylation of α-tubulin, without interfering with the stabilization of the microtubules (Fass et al. 2006). Nocadazole reduces acetylation of α-tubulin, however, does not cause

depolymerization of microtubules. All three drugs, via different mechanisms block the conversion of LC3-I to LC3-II. This suggests that other forms of stable microtubules are involved in the conversion in autophagosome formation, however, acetylated microtubules are essential for the trafficking of autophagosomes or lysosomes (Xie et al. 2010).

C. HISTONE DEACETYLASES

C.1 Definition

Histone deacetylases or HDACs are a family of enzymes that catalyze the removal of acetyl groups from the N-terminal lysine residues of several histone and non-histone proteins (Seto and Yoshida 2014). Some of their non-histone targets are transcription factors, cell-surface receptors, signaling proteins, chaperone proteins, and cytoskeletal proteins ((Singh et al. 2010; Ocker 2010).

C.2 CLASSIFICATION

Human histone deacetylase enzymes are categorized into four classes, based on their homology to yeast HDACs (S.-Y. Park and Kim 2020). Histone deacetylase enzymes are broadly classified into two families based on the cofactors: Histone Deacetylases and Sirtuins (Seto and Yoshida 2014). Enzymes belonging to the HDAC family required bivalent metal ion, $Zn^{+2,}$ whereas members of Sirtuin family depend on nicotinamide adenine dinucleotide for their catalytic activity (S.-Y. Park and Kim 2020).

	CLASS	ENZYME	YEAST HOMOLOGY	LOCATION
Histone Deacetylas	I	HDAC 1, 2, 3,	Rdp3	Nucleus (Gregoretti, Lee, and Goodson 2004)
	II	HDAC 4, 5, 6, 7, 9 and 10	HDA1	Nucleus, Cytoplasm (Bjerling et al. 2002
	IV	HDAC 11	-	Nucleus, Cytoplasm (Gao et al. 2002; Yanginlar and Logie 2018)
Sirtuins	III	Sirtuin 1-7	Sirtuin2	Cytoplasm (Yoon and Eom)

Table 1: Classification of Histone Deacetylase Enzymes

Although the name suggests that they interact with histone proteins in the nucleus, HDACs have different non-histone targets, and can shuttle between the nucleus and the cytosol. For example, HDAC8 is present in the cytosol of smooth muscle cells, wherein it binds to α-actin component of the cytoskeleton. Class II of HDACs can be transported from the nucleus to the cytosol, depending on their interaction with shuttle proteins.

C.3 STRUCTURE OF HISTONE DEACETYLASE 6

HDAC6 is coded by a 1215 amino acid sequence, which in the 3-dimensional conformation, form two independent catalytic domains (Figure 13). The presence of two separate domains, is what sets this enzyme distinctly apart from the rest of the histone deacetylase family. These domains contain the zinc-finger ubiquitin-binding domain (UBZ domain) at the C-terminus. This compact structure consists of 5 anti-parallel β-strands, 2 α-helices, and 3 zinc ions with an aromatic pocket. Arg 1155 and Tyr 1156 residues act as gatekeepers, moving the ubiquitin binding site from an open

to a closed conformation (Hook et al. 2002). The zinc finger is able to recognize the free C-terminal diglycine motif of ubiquitin tag in protein aggregates and helps interact with HDAC6 (Ouyang et al. 2012). Peptide sequence containing Ser-Glu repeat at SE14 facilitates HDAC6 retention in the cytosol and interactions with tau protein(Bertos et al. 2004; Ding, Dolan, and Johnson 2008). HDAC6 shuttles between the nucleus and the cytosol with the help of two leucine-rich repeat sequences called nuclear export sequences (NES1 and NES2) (Simões-Pires et al. 2013).

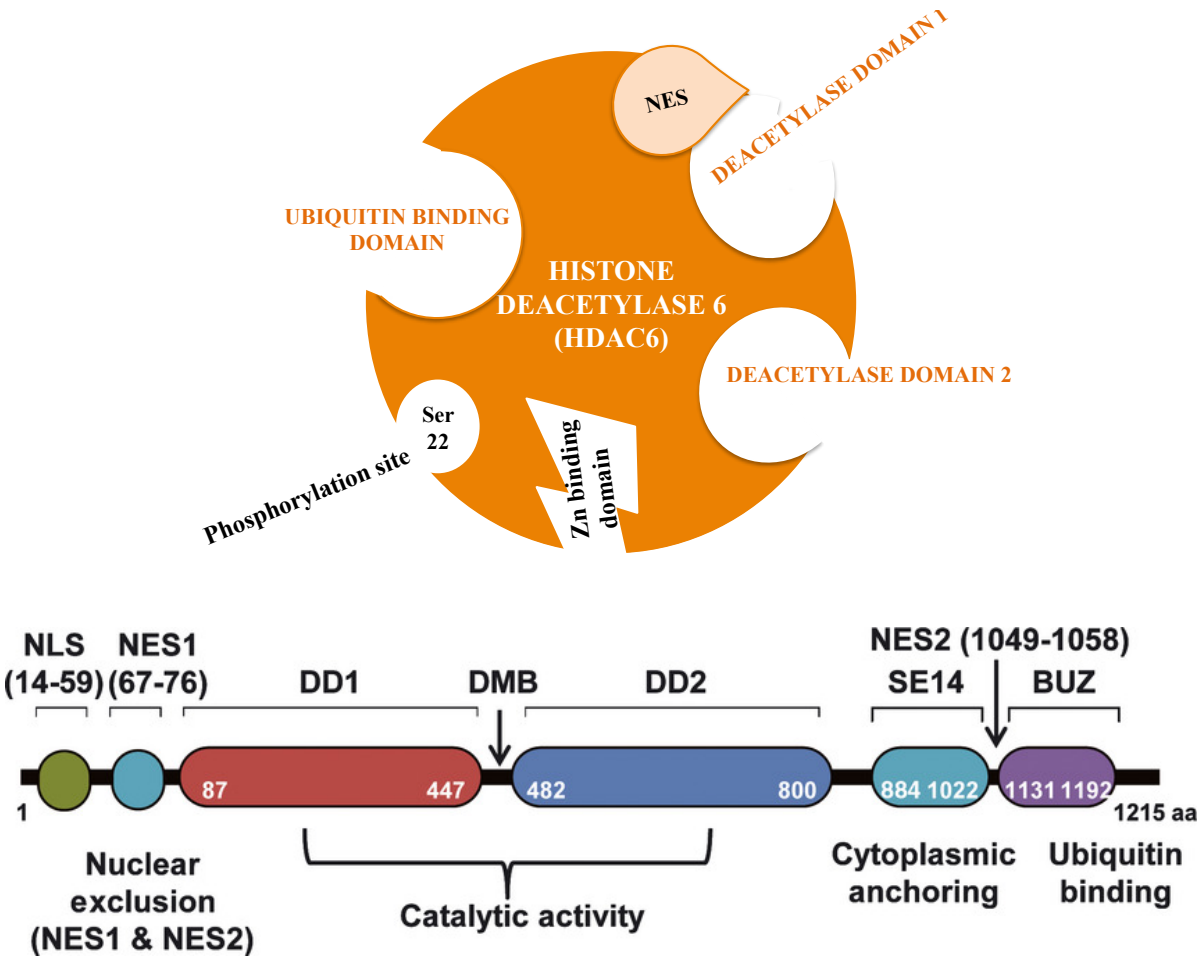


Figure 13: Structural organization of the different domains of Histone Deacetylase 6 (Li et al. 2013)

C.4 MECHANISM OF ACTION

C.4.1 Mechanism: Biochemical Overview

Class I, II, and IV HDACs are zinc-dependent enzymes. The zinc ion is positioned at the active site, coordinated by one histidine, two aspartates, and two water molecules (R. Wu et al. 2011). As a substrate, the acetamide-carbonyl group of the acetylated lysine replaces one water molecule and interacts with the tyrosine residue by forming hydrogen bonding. The tyrosine residue may undergo a conformational change to accommodate substrate binding. Following this, the other water molecule is deprotonated by a histidine nucleophile through the His–Asp couple. There are two histidine residues located near the active site. The first histidine has been suggested to act as a catalyst to activate the water molecule, while the second histidine functions to accept a proton as a base. After the oxygen of the deprotonated water attacks the lysine carbonyl group, oxyanions and tetrahedral intermediates are generated. Finally, when the second histidine serves as the proton-to-amine moiety, acetate and lysine are generated (Lombardi et al. 2011).

C.4.2 Mechanism: Molecular Pathways

HDAC6 is known for deacetylating cytoplasmic proteins like α-tubulin, cortactin, and HSP90. HDAC6 interacts with microtubules through its HDAC domain and is capable of deacetylating α-tubulin at lysine 40 (Figure 14). Deacetylation of microtubules and cortactin by HDAC6 influences microtubule-dependent and actin-dependent cell motility, respectively (Zilberman et al. 2009; Xiaohong Zhang et al. 2007).

Phosphorylation sites within HDAC6 as well as kinases that are responsible for phosphorylating HDAC6 have started to emerge. For instance, glycogen synthase kinase3β (GSK-3β) phosphorylates the Ser22 site located in the N terminus of HDAC6. It has been suggested that GSK-3β enhances HDAC6 deacetylase activity toward α-tubulin (Du et al. 2015).

HDAC6 can also be phosphorylated by Aurora A kinase, a centrosomal kinase involved in regulating mitotic entry. Phosphorylation of HDAC6 by Aurora A enhances the ability of HDAC6 to deacetylate α-tubulin to promote ciliary disassembly (Pugacheva et al. 2007). G protein-coupled receptor kinase 2 has also been shown to phosphorylate HDAC6 and stimulate its α-tubulin deacetylase activity (Lafarga et al. 2012; Penela et al. 2019).

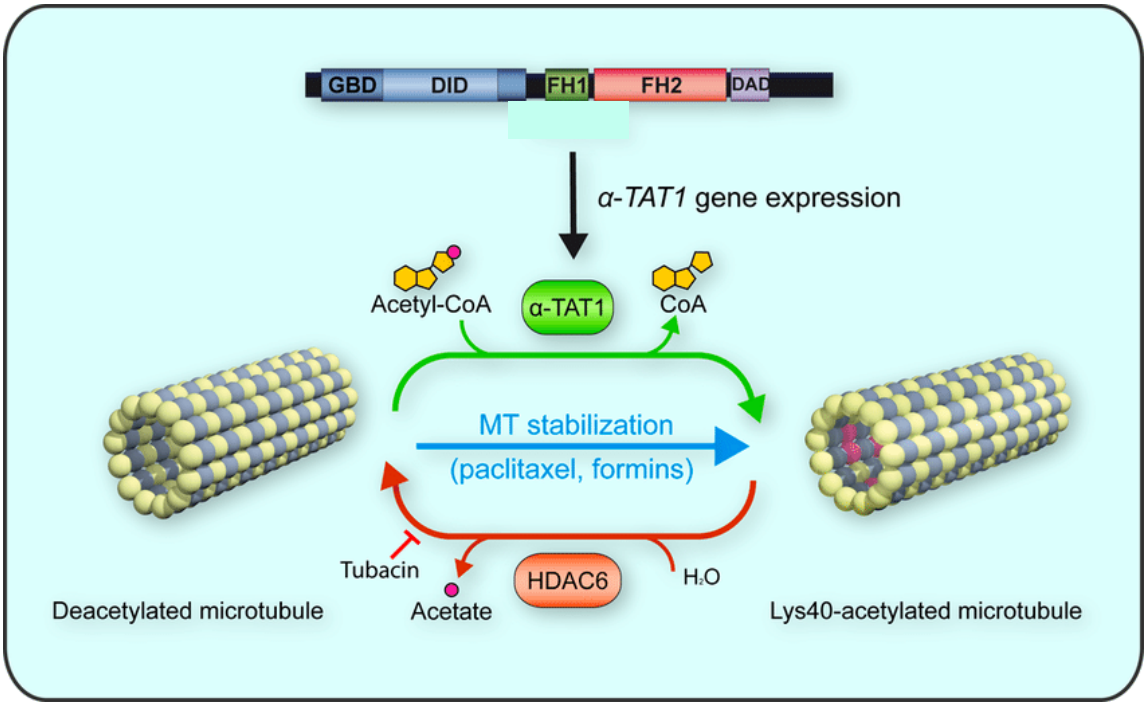


Figure 14: Regulation of Acetylation of α-Tubulin (Fernández-Barrera et al. 2018)

In addition to α-tubulin, phosphorylation of HDAC6 also alters its deacetylase activity toward other substrates, such as β-catenin. Protein kinase C (PKC) enhances HDAC6 deacetylase activity toward β-catenin and regulates its nuclear translocation and promoter binding (Penela et al. 2019; Du et al. 2015).

Tyrosine kinases such as epidermal growth factor receptor (EGFR) kinase have been shown to phosphorylate Tyr570 within the HDAC6 C-terminal deacetylase domain. This results in a decrease in the deacetylase activity of the enzyme, an increase in α-tubulin acetylation, and an altered EGFR trafficking (Lissanu Deribe et al. 2009; K. A. Williams et al. 2013). Ser1035 is a major ERK phosphorylation site in HDAC6. The EGFR-Ras-Raf-MEK-ERK signaling pathway stimulates cell migration via HDAC6

phosphorylation at Ser-1035. Apart from the Ser1035 site, Thr1031 is also a phosphorylation site in HDAC6 (Williams et al. 2013)

C.5 HDAC6 AND ITS IMPLICATIONS IN CARDIOVASCULAR DISEASES

Studies have linked HDAC6 to progression of atherosclerosis via its role in ROS production and lipid peroxidation. HDAC6 regulates the expression of NADPH oxidases (NOX), which are a major source of ROS as shown by Manea, et al, in macrophages (Manea et al. 2020). NADPH oxidases are similarly present in the cells of the vessel wall and the inflammatory cells. NOX4 is ubiquitously present in vasculature, whereas, NOX1 is mainly expressed in VSMCs and NOX2 is predominantly expressed in endothelial cells, adventitia and cells originating from the bone marrow (Konior et al. 2014; Vendrov et al. 2018; Clempus et al. 2007). NOX1 and 4 have been implicated in the regulation of ROS production and contributing to the early stages of atherosclerosis (A. V. Poznyak et al. 2020; Lassègue, San Martín, and Griendling 2012). NADPH-dependent production of ROS leads to the secretion of various cytokines by macrophages, like IL-6, TNF-α and IL-1β, by activating various transcription factors like MAPK-NF-κB/AP-1 (Youn et al. 2016).

Other than atherosclerosis, HDAC6 is known to play a role in the upstream events of plaque formation, which is myocardial infarction. Proteotoxicity in the heart, caused by accumulation of ubiquitinated protein aggregates, is a contributor in heart failure. Nagata, et al, showed that in rats, which underwent myocardial infarction, the HDAC6 content was higher in the initial weeks post-op, compared to the sham group. This indicated that the deacetylase activity of HDAC6 was higher post-myocardial infarction. HDAC6 is known to interact with heat shock response proteins like Hsp90 and HSF. In the failing heart, it is assumed that HSP90 is deacetylated due to markedly elevated HDAC6 activity and HSF1 is not activated, resulting in abolished HSP induction and impairment of protein homeostasis. These findings suggest that alterations in the heat-shock response mediated by an aberrant increase in deacetylase activity of HDAC6 is involved in the development of heart failure. (Nagata et al. 2019).

C.6 INHIBITORS OF HISTONE DEACETYLASE 6

Most HDAC inhibitors (HDACi) target the chelate interactions involving the Zn^{+2}. The first few pan-HDAC inhibitors to have been studied extensively and have clinical applications are histone deacetylase inhibitor/ HDACi, suberoylanilide hydroxamic acid (SAHA)/vorinostat and romidepsin (Lei Zhang et al. 2018).

The first significant small inhibitor of HDAC6 came from the diversity oriented synthetic (DOS) approach from the team of Schreiber and co-workers (Sternson et al. 2001) . A library of 7392-members was screened and revealed tubacin, a selective HDAC6i adhering to the canonical HDAC inhibitor scaffold and capable of elevating levels of acetylated α-tubulin (Haggarty, Koeller, Wong, Butcher, et al. 2003). Interestingly, tubacin is unable to alter gene expression or cell cycle progression, further confirming that the major substrates for HDAC6 are non-histone proteins as opposed to gene transcription modifiers. As tubacin was the first significant HDAC6 selective inhibitor identified, it has been used extensively as a chemical tool (Haggarty, Koeller, Wong, Grozinger, et al. 2003).

Butler et al., resulting from rational design using a homology model of HDAC6, reported a significant advancement in the development of drug like small molecules that were both potent and selective HDAC6i. Tubastatin A, a small molecule HDAC6 inhibitor with an IC50 of 15nM. Tubastatin A displayed the characteristic in vitro acetylation preference for increasing acetylated α-tubulin but not acetylated histone levels (Butler et al. 2010).

An interesting role of Tubastatin-A has been highlighted in the context of acute lung injury and endothelial barrier dysfunction. As the endothelial cells in the lungs are exposed to anoxia during acute lung injury, the HIF-1α pathway is activated (Bruhn et al. 2018). It is known to contribute to hypoxia-induced endothelial barrier damage. HIF-1α-induced cytoskeletal contraction along with decreased junction integrity , causing formation of paracellular gaps and consequential endothelial hyperpermeability (P. Wang et al. 2013).

The endothelial glycocalyx plays an important role as a regulatable barrier to transport of proteins, fluids and inflammatory cells, as well as in coagulation and as a mechanotransducer of SS on the vessel wall (Fu and Tarbell 2013; Zeng et al. 2018). During endothelial activation, as in the setting of hemorrhagic shock during lung injury, the glycocalyx layer is shed, the endothelium is prone to hypercoagulation, upregulation of adhesion molecules, and increased vascular permeability (Peng et al. 2013; Torres et al. 2013). Increased expression of ICAM-1, which is an inflammatory marker on endothelial cells, signifies endothelial dysfunction. Treatment of HUVECs with Tubastatin-A, the *in vitro* model for this study showed increased stability of the cytoskeleton, reduced vascular hypermermeability, ICAM-1 and HIF-1α expression, and thus prevented the effects of hemorrhagic shock on endothelial function like the shedding of the glycocalyx layer (Bruhn et al. 2018). This study illustrates an anti-inflamamtory role of Tubastatin-A and potential role in rescuing the endothelial barrier from dsfunction during inflammation.

AIMS AND OBJECTIVES

As previously described, endothelial cells exposed to low shear stress conditions present a defective autophagic activity (Figure 15). This defect makes them predisposed to pro-inflammatory and pro-senescent phenotypes, which in turn contributes to the development and progression of atherosclerotic lesions (Vion et al. 2017). In this study we aim to restore the autophagic flux in endothelial cells, otherwise lacking efficient autophagic due to exposure to low shear stress .

Our hypothesis is that in cells exposed to low shear stress, lack of autophagic flux is due to the defect in the rate-limiting step, the fusion betwen autophagosomes and lysosomes. Our stratgey is to induce conditions which would promote formation of autolysomes. A better trafficking system would aid the autophagsosomes to travel to the site of lysosomes and fuse with them. Autophagic vacuoles carry cargo throughout the cytosol across the microtubule network. Motor proteins help bind the vacuoles to the microtubules and facilitate their transport. Weak interactions with the microtubules leads to lack of cellular transport, and therefore accumulation of cellular by-products that should be degraded.

At the tissue level, promoting autophagy translates into an anti-inflammatory, anti-senescent and anti-apoptotic phenotype that prevents atherosclerotic plaque formation (Figure 15).

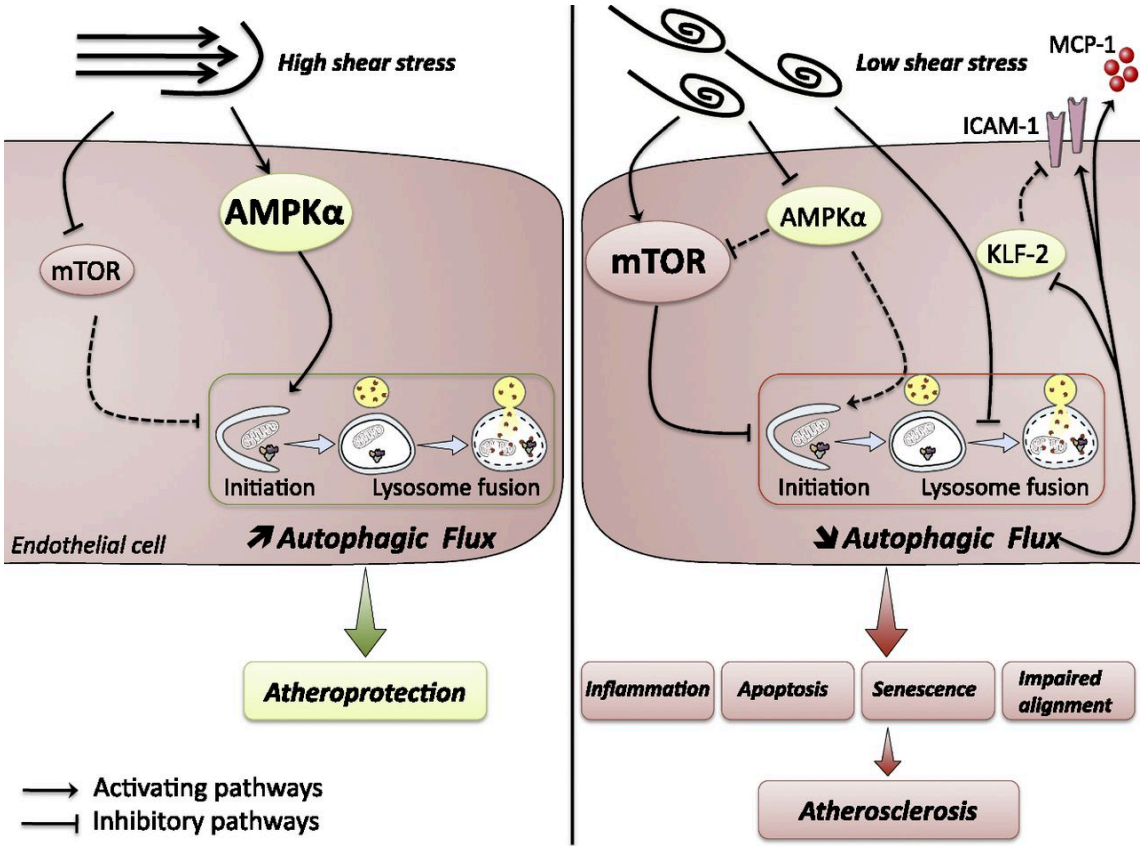


Figure 15: Schematic representation of the different links between shear stress, autophagy, and atherosclerotic plaque formation

Under high shear stress, endothelial autophagy is activated and plays an anti-apoptotic, anti-senescent, and anti-inflammatory role. Under low shear stress, a defect in endothelial autophagy occurs causing endothelial apoptosis, senescence, and inflammation, eventually increasing atherosclerosis development (Vion et al. 2017)

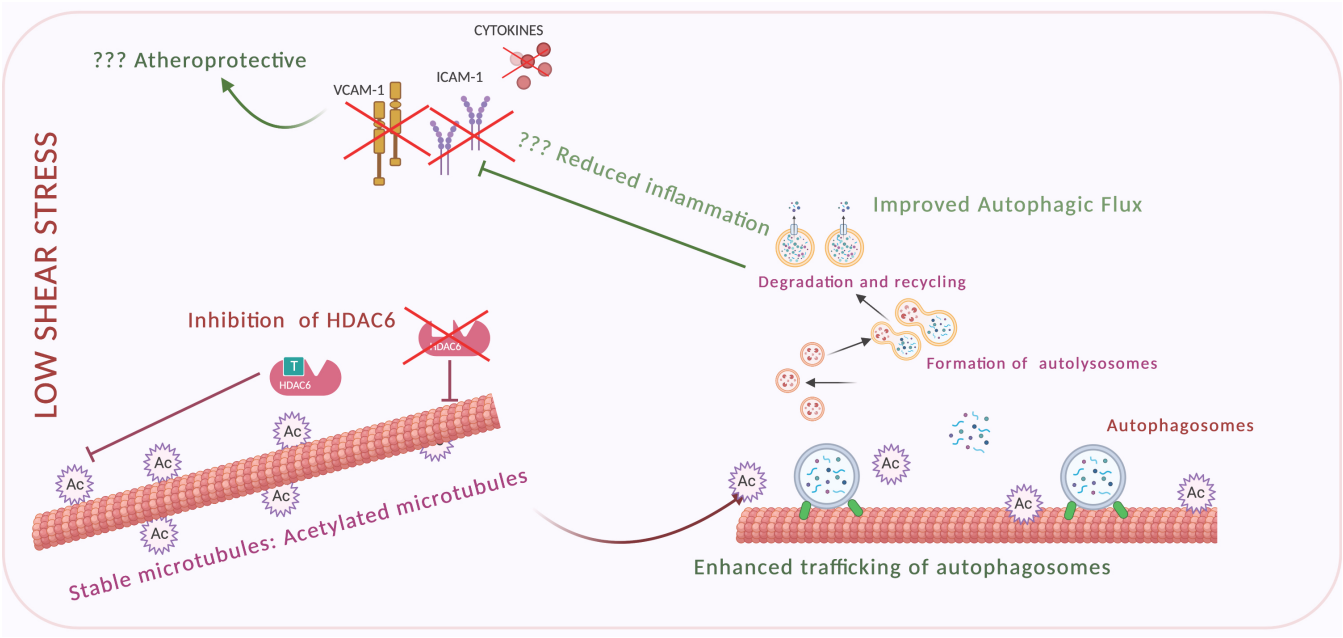


Figure 16: Schematic representation to highlight the strategy applied in our project, the expected outcomes and questions to be answered.

To restore autophagic flux and prevent atherosclerotic lesion formation, this study is divided into three main objectives:

- To promote autophagic flux by enhancing the stabilization of microtubules by increasing acetylation of α-tubulin via inhibition of the enzymatic activity of HDAC6 using pharmacological treatments (Tubastatin-A, SirReal2) and shRNA targeting *HDAC6*.

- To study the impact of inducing autophagy on endothelial inflammation.

- To evaluate the effects of *HDAC6* knockout *in vivo* on atherosclerosis development and progression in a double knockout *HDAC6*$^{-/-}$*ApoE*$^{-/-}$murine model.

MATERIALS AND METHODS

Culture Of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECSs from PromoCell (Heidelberg, Germany) were cultured on glass slides (SUPERFROST PLUS ™, Thermo Scientific) coated with 0.2% gelatin (Sigma-Aldrich-G1393) in Endothelial Cell Basal Medium (ECBM, PromoCell-C22210), supplemented with 2% Fetal Calf Serum, growth factors (0.4% ECGS, 0.1 ng/ml EGF, 1ng/ml ß-FGF), 90 µg/ml heparin, 1 µg/ml hydrocortisone, 10 µg/L Amphotericin B (Gibco™ - 15290026), 100 IU/mL Streptomycin (Gibco™ - 15140148) and 100 IU/mL Penicillin (Gibco™ - 15140148). HUVEC were used from passage 2-4 For this study, primary cells from 20 different donors were used.

HUVECs were plated at a density of 10,000 cells/cm^2 and media was changed every 48 hours until confluency was reached. Cells were cultured in a humid atmosphere, at 37°C, 5% CO_2 until the cells were 90-95% confluent.
In order to detach cells, they were treated with 0.025% Trypsin-EDTA (Gibco™ - 25300-054) in PBS (Gibco™ - 10010023) for 5min at 37°C, followed by washing with complete medium and centrifuging at 600g for 10min.

Lentiviral Transduction

Lentiviruses expressing inducible shRNA (Sigma-Aldrich, MISSION™ shRNA inducible vectors) were used to silence *HDAC6* and *ATG5* (Figure 16). The cells were infected with lentiviruses carrying sh*HDAC6*/sh*ATG5* or shControl at MOI 5 for 24 hours in the presence of Hexamethidine Bromide (8 µg/ml; Sigma-Aldrich - H9268). Transduced cells were selected using puromycin (Sigma Aldrich #P9620) at 1 µg/ml. HUVECs were treated for about 10 days with Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich - I6758) at 1mmol/mL.

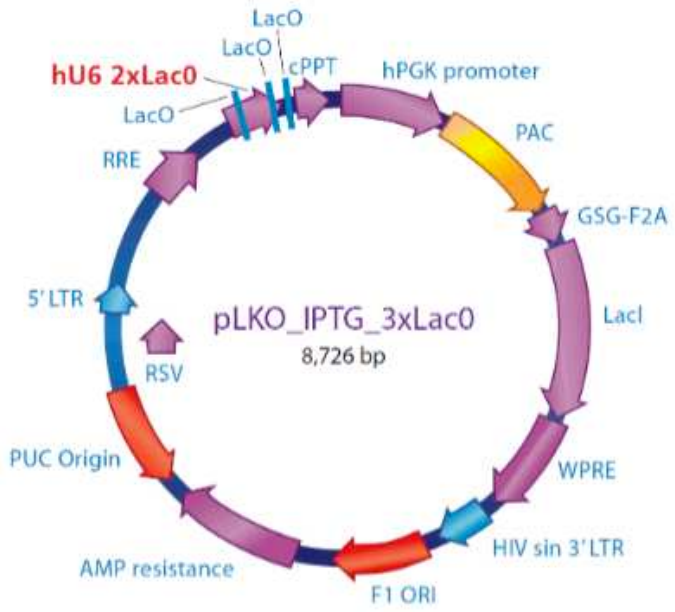


Figure 17: Schematic diagram of the shRNA Inducible Vector

In Vitro Shear Stress System

Confluent HUVECs, cultured on glass slides, were placed in a parallel plate chamber perfused with circulating medium for 24 hours, under laminar flow. The upper part of the chamber, a plexiglass block, comprised of two openings for entry and exit of flowing medium. Each block was connected to a reservoir containing 20 mL of culture medium, and together they served as a closed-circuit flow unit. Shear stress exerted on the cells was generated by regulating the flowrate of the medium using a peristaltic pump (Gilson) (Figure 17).

Shear stress was calculated by Poiseuille's law adapted for a rectangular channel, and was 20 dyne/cm^2 and 2 dyne/cm^2 for high shear stress and low shear stress respectively.

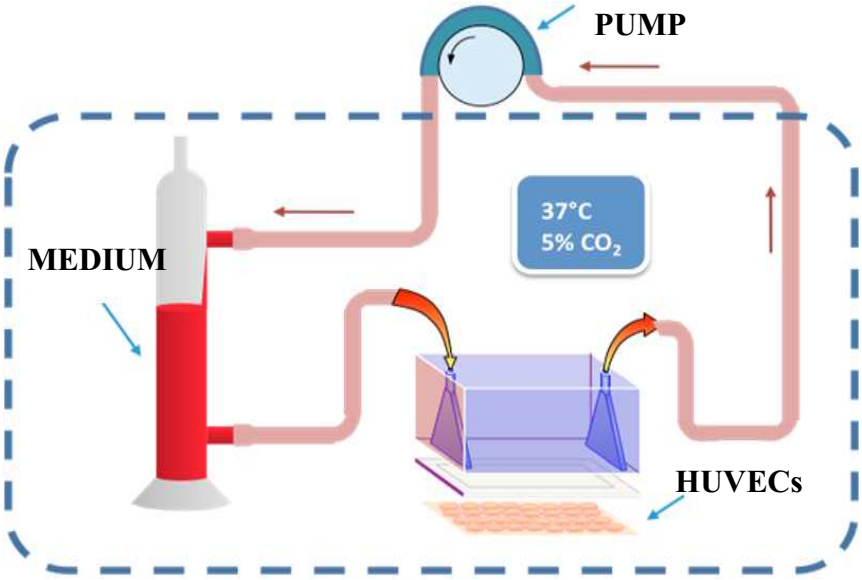


Figure 18: Schematic representation of the in vitro fluid shear stress system

$$\tau_w = (6\mu Q)/(Wh^2)$$

τ_w = Shear stress, applied at 0, 2 or 20 dyne/cm^2 for static, low and high shear stress, respectively, for our protocol.

μ = Viscosity of the medium, considered equal to water at 0.001 poise.

Q = Flowrate of the medium. It is variable in our shear stress system and adjusted using a peristaltic pump, to obtain the required shear stress.

W = Width of the chamber, equal to 2.5 cm in our system.

h = height; space between the cells and the surface of the block, equal to $2.5*10^{-6}$ cm in our system.

In most experiments, cells were treated with Tubastatin (3µM, Sigma-Aldrich), Rapamycin (0.5µM; Sigma-Aldrich) or Chloroquine (300µM; Sigma-Aldrich C6628) and exposed for 24 hours under shear stress conditions. For control conditions, HUVECs were treated with vehicle (Dimethyl Sulphoxide; 0.1 µl/ml, Sigma-Aldrich. For inflammation studies, the cells were treated with TNF-α (1ng/ml, Peprotech) for the last 12 hours of the experiment

Western Blot Analysis

Protein Extraction

Post shear stress, HUVECs were immediately washed twice with PBS and lysed in Radioimmunoprecipitation assay (RIPA) buffer consisting of 150 mmol/L NaCl, 50 mmol/L Tris HCl (pH=7.4), 2 mmol/L EDTA, 2 mmol/L activated orthovanadate, 0.5% deoxycholate, 0.2% Sodium Dodecyl Sulphate and supplemented with Complete™ Mini protease and phosphatase inhibitors cocktails (Roche, France). Then, the lysates were centrifuged for 15 minutes at 12500g, 4°C to remove cell debris. The supernatant was collected and stored at -80°C until further analysis.
Protein concentration was estimated by Lowry's protein assay method (DC™ Protein Assay, BioRad)

SDS-PAGE

Protein samples were denatured using buffer containing Tris-carboxyethyl phosphine hydrochloride (20x XT-Reducing Agent, BioRad-1610792) and separated based on molecular weight by electrophoresis in 4-12% gradient gels (Criterion™, BioRad-3450123). Proteins were then transferred on either 0.45 µm nitrocellulose (BioRad) or Polyvinylidene Fluoride (PVDF) membranes; following which the membranes were incubated with Ponceau Red to verify the efficiency of the transfer process. Membranes were blocked with 5% (w/v) milk or Bovine Serum Albumin (BSA) in TBS supplemented with 0.1% Tween-20.

Blotting

To detect the protein of interest, the membranes were then incubated with primary antibodies overnight at 4°C, with constant agitation. After three 10-minute washes, the membranes were incubated with secondary antibody coupled with Horseradish Peroxidase (anti-rabbit, anti-rat, anti-mouse, Amersham, GE Healthcare, 1/3000) for 1 hour at room temperature. Immunodetection was performed using Clarity™

Western ECL Substrate, and the chemiluminescent signal was revealed using the Las-4000 imaging system and quantified with MultiGauge software (Fujifilm, Japan).

ANTIBODY	HOST SPECIES	REF	DILUTION	DILUTION BUFFER
LC3-B	Rabbit	CST-2775S	1/1000	5% milk +TBST
GAPDH	Mouse	mab-374	1/20000	5% milk +TBST
ATG5	Rabbit	CST-12994S	1/1000	5% milk +TBST
ATG7	Rabbit	CST-2631S	1/1000	5% milk +TBST
p62	Mouse	ab56162	1/3000	5% milk +TBST
Beclin-1	Rabbit	CST-3495S	1/1000	5% milk +TBST
HDAC6	Rabbit	CST-7558S (D2E5)	1/1000	5% milk +TBST
phosphor-HDAC6	Mouse	ab61058	1/3000	5%BSA+TBST
Acetylated α-Tubulin	Mouse	Sigma T7451	1/3000	5% milk +TBST
Total α-Tubulin	Rat	ab6160	1/5000	5% milk +TBST
ICAM-1	Goat	R&D: AF796	1/1000	5% milk +TBST
VCAM	Rabbit	ab134047	1/3000	5% milk +TBST

Table 2: List of antibodies used in Western blot experiments

Immunofluorescence And Analysis

HUVECs, exposed to shear stress or control conditions, were fixed with either 100% ice-cold methanol for 20 minutes at -20°C or 4% (v/v) paraformaldehyde (PFA) in PBS, for 5 minutes at room temperature. Cells fixed with PFA were permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Permeabilized cells were blocked with 5% (w/v) BSA in PBS for 30 minutes and then incubated with primary antibody overnight at 4°C. The cells were washed with PBS and incubated with secondary antibody conjugated with fluorescent dye, Alexa Fluor®. Lastly, the nuclei were labeled with DAPI. The labeled cells were mounted on coverslips using Fluoromount-G®.

To perform *en face* immunostainong, aortas were harvested from mice and fixed with ice-cold 4% (v/v) paraformaldehyde (PFA) in PBS for 20 minutes. Residual fat attached to the aorta was removed and dissected along the entire inner side and outer curvature of the arch.

The aortas were then blocked with 5% (w/v) BSA in PBS, for 60 minutes at room temperature, and incubated with primary antibody for overnight, 4°C.
Following incubation with primary antibody, the aortas were washed multiple times and incubated with secondary antibody conjugated with Alexa Fluor® for 2 hours at room temperature. Subsequently, the aortas were washed with PBS and the nuclei were labeled with DAPI. Labeled aortas were transferred onto glass slides and mounted "*en face*".

ANTIBODY	HOST SPECIES	REFERENCE	FIXATION	DILUTION
LC3-B	Rabbit	CST-2775S	100% Methanol	1/400
VE-Cadherin	Goat	SCBT- (F-8) 9989	-4% PFA	1/400
HDAC6	Rabbit	CST-7558S (D2E5)	100% Methanol	1/100
phospho-HDAC6 (Ser22)	Mouse	ab61058	100% Methanol	1/200
Acetylated α-Tubulin	Mouse	Sigma T7451	4% PFA	1/500
Total α-Tubulin	Rat	ab6160	4% PFA	1/1000

Table 3: List of antibodies in immunofluorescence experiments

The slides prepared were examined using a Leica SP8 confocal microscope and analyzed with the ImageJ software.

HDAC6 Deacetylase Activity Assay

To determine the deacetylase activity of HDAC6, a fluorescence-based assay was performed. Deacetylation of a substrate by HDAC6 sensitizes it to the developer solution, which generates a fluorescent signal. The signal is analysed in a fluorimetric plate reader at 460nm. HUVECs were lysed in PBS buffer supplemented with - 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail (Thermo Fisher). Lysed cells were sonicated and centrifuged at 12500g for 15minutes, 4°C. The pellet was discarded, and the supernatant was transferred to fresh tubes. Protein concentration was estimated by Micro BCA assay (Thermo Scientific™ - 23235). For the assay, 15µg of lysates were diluted in 100µl of PBS and added to a 96-well plate. Where required, 1µL of the 100x HDAC6 inhibitor of the stock solution were added.

Vehicle (DMSO) was added to the controls at the same concentration. The plates were incubated at 37°C for 60 minutes. 5µL of substrate, specific to HDAC Class II, were added to the wells at a concentration of 1mM and incubated for 2 hours at 37°C. The substrate was bought from Bachem (I-1875; Boc-Lys (Ac)-AMC). 50µL per well, developer solution was added and incubated at 37°C for 20 minutes. The developer solution consisted of 1.5% Triton X-100, 3 µM TSA (Sigma-Aldrich, T8552), and 0.75 mg/mL trypsin (Gibco™ 15400054), diluted in 1x PBS. AMC fluorescence was measured using a plate reader, with excitation and emission filters of 360 nm and 460 nm, respectively. Background signals corresponding to the blank solutions was subtracted.

Monitoring Autophagic Flux Using mRFP-GFP-LC3

In order to observe the effect of shear stress on endothelial autophagic flux, we used a LC3 protein, fluorescently tagged with a RFP- GFP tandem, (generous gift from Dr F. Oury), according to Vion Kheloufi et al, PNAS 2017. The tandem allows us to observe different stages autophagy. At the autophagosomes stage, the LC3 protein is visualized as yellow signal, due to the overlapping of green and red fluorescence signal from GFP and RFP. However, when autolysosomes are formed, the acidic pH from lysosomes causes the quenching of GFP. Therefore, the red fluorescent signal corresponds to LC3 present only in the autolysosomes stage.

The cells were infected with lentiviruses carrying the plasmid, at MOI = 6, in the presence of Hexamethidine Bromide (8µg/ml; Sigma-H9268) for 24 hours. HUVECs were then exposed to shear stress for 24 hours with or without Tubastatin A (3µM; Sigma-SML0044). For experiments where autophagic flux was blocked, HUVECs were treated with Chloroquine (300µM) for the last 6 hours of the shear stress.
After exposure to shear stress, the cells were washed with PBS and fixed with 4% PFA for 5 minutes at room temperature, followed by labeling the nuclei with DAPI. The slides were mounted using Fluoromount-G® (SouthernBiotech). Images of the prepared slides were taken with the Leica SP8 confocal microscope and analyzed for LC3 positive signal using the ImageJ software.

ELISA

Levels of Monocyte Chemoattractant Protein-1 (MCP-1), released by HUVECs, in the media, were assessed by ELISA (R&D, Human MCP-1 Duo-Set DY279). The assay was performed according to manufacturer's protocol.

Animal Models

All the mice used in the study were of C57BL/6 genetic background. The double knockout model was generated by crossing HDAC6$^{-/-}$ mice, provided by T. McKinsey, and ApoE$^{-/-}$ (Charles River Laboratories). All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (no. 07430) and were approved by the institutional ethical committee (no. 02526.02).

Genotyping

Mice tails were snipped 2-3mm from the end and placed in 1.5ml tubes. Samples were stored in -20°C until processed. To each tail, 250 µL of lysis buffer (0.05M Tris HCl, pH8, 0.1M NaCl, 0.5%Tween 20, 50ml H2O) and 2µL of Proteinase K (ThermoScientific) were added. The tails were incubated overnight at 56°C in a water bath, followed by 30 minutes at 95°C. For the PCR mix, 10µL of DreamTaq polymerase, 2µL water, 1µL of each primer (Int9, Exo10, Zeo3 and Zeo1) were added to each well. 23µL of PCR mix and 2µL lysed tissue were added to each well.

Primer sequences for *HDAC6* are as follows:

- **Int9**: 5' CTG-GTT-CGT-CTG-AAG-ACA3'
- **Exo10**: 5' GTG-GAC-CAG-TTA-GAA-GCC 3'
- **Zeo1**: 5' CCA-TGA-CCG-AGA-TCG-GCG-AGC-A 3'
- **Zeo3**: 5' CGT-GAA-TTC-CGA-TCA-TAT-TCA-AT 3'

Primer sequences for *ApoE* are as follows:

- **IMR0180** : 5'-GCCTAGCCGAGGGAGAGCCG-3'
- **IMR0181** : 5'-TGTGACTTGGGAGCTCTGCAGC-3'
- **IMR0182** : 5'-GCCGCCCCGACTGCATCT-3'

Bone Marrow Transplant

8 weeks old *HDAC6$^{-/-}$/ApoE$^{-/-}$* and *HDAC6$^{+/+}$/ApoE$^{-/-}$* mice underwent Bone Marrow Transplant (BMT) procedure (Figure 18), in order to replenish the bone marrow in the mutated models with *HDAC6$^{+/+}$/ApoE$^{-/-}$* donor bone marrow. This allows us to focus on the role of endothelial cells and rule out the potential function of hematopoietic cells in progression of atherosclerosis.

4-6 weeks old donor mice (*HDAC6$^{+/+}$/ApoE$^{-/-}$*) were euthanized by cervical dislocation and the bone marrow was extracted from tibia and femur. The bone marrow was flushed and filtered. The recipient mice received radiation, 9.5 Gray, and were transplanted with donor bone marrow by retro-orbital injections the day after. The mice were allowed to recover for 4 weeks, whilst they received antibiotic treatment for the first two. After checking the efficiency of the myeloablation, the mice were put on High Fat Diet (HFD) until they were sacrificed.

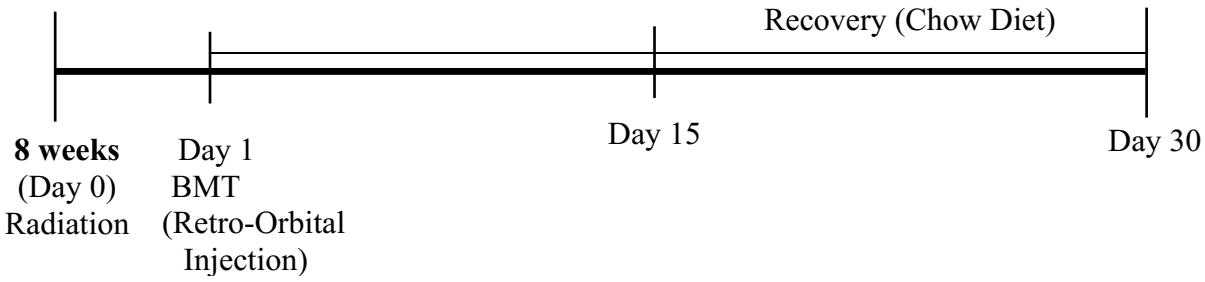


Figure 19: Timeline of the protocol for Bone Marrow Transplant in mice.

Estimation of Blood Cholesterol Levels

Before collecting blood, the mice were anesthetized with 2% Isoflurane. Blood (75µl) was collected by making a sub-mandibular puncture.

To prepare platelet-free plasma, the blood collected was centrifuged twice at 2500g for 15 minutes at room temperature. The cholesterol content was determined using Cholesterol FS 10' kit (Diasys).

Isolation of Aorta

The mouse was placed in the anesthesia induction chamber. The isoflurane flow was set to 2.5% (2.5% for induction and 2% for maintenance) and 2L/min of O_2. Post

induction, the mouse was taken out of the chamber and placed on its back on the surgical pad. The anesthesia was maintained by inhalation via a nose cone supplying isoflurane and oxygen.

The mouse was checked for suitable level of anesthesia: lowered breathing rate and lack of reaction to toe pinch.

The chest and abdomen were sprayed with 70% ethanol, and using dissection scissors (Phymep, ToughCut™ 14085-09), the abdomen was cut open along the linea alba. The abdominal organs were moved aside to expose the inferior *vena cava*. 500µL blood was drawn from the inferior *vena cava* using a 1mL syringe and 26G needle primed with sodium citrate.

Following blood collection, the diaphragm was cut, and the thoracic cavity exposed. The right atrium was nicked to release the blood. The heart was flushed with PBS via the left ventricle, using a 10 mL syringe and 25G needle. Followed by PBS, the heart was flushed with 1-2mL ice-cold 4% PFA and PBS to wash out the excess PFA.

To allow for better access to the aorta, the liver, lungs, trachea, oesophagus, thymus gland and pulmonary artery, were removed.

Using micro-dissection scissors (Phymep; Moria 9600, Moria 9601, Vannas 15000-10, Vannas 15000-00) and forceps (Moria MC31) the excess adipose tissue and adventita, surrounding the thoracic aorta and the carotids, were removed. The clean aorta was excised from the heart and placed in 4% PFA for 20 minutes on ice, followed by storage in ice-cold PBS.

Determination Of Atherosclerotic Plaque Size On *en face* Aorta

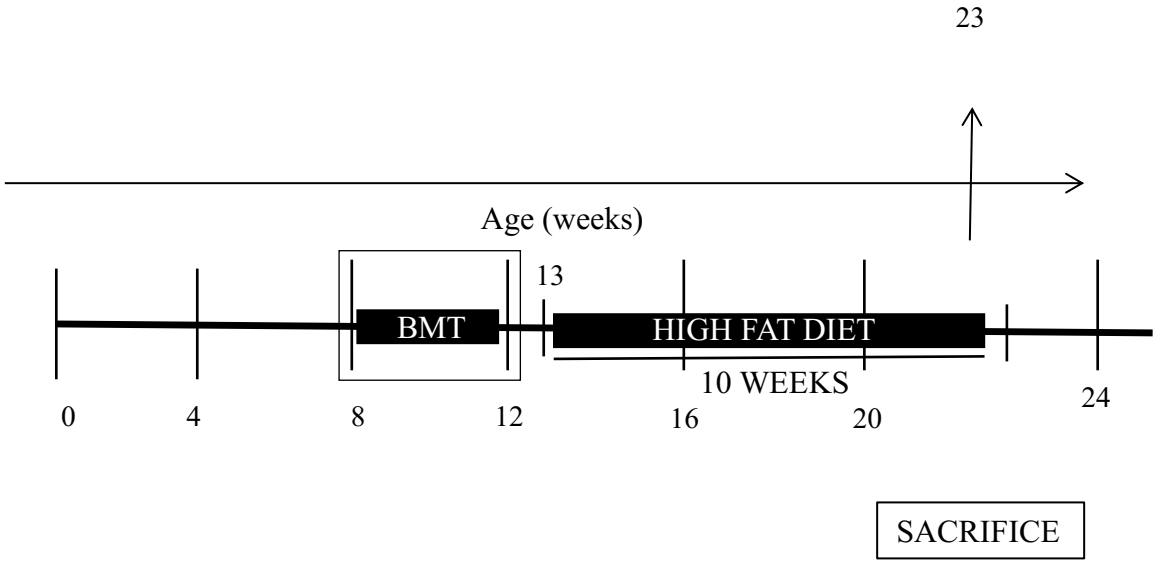


Figure 20: Timeline of the protocol to assess the progression of atherosclerosis in mice.

Mice, which underwent bone marrow transplant, at age 13 weeks, were put under high fat diet (D12079B, Research Diets; 20% proteins, 50% glucose, 21% lipids). After 10 weeks of high fat diet, the mice were sacrificed (Figure 19).

The aortas were then stained with freshly prepared Oil Red O dye (40% distilled water, 60% Oil Red O solution) for 20 minutes with constant agitation. The solution was prepared by dissolving the dye in isopropanol at a concentration of 5g/L. The aortas were washed with 75% ethanol for 5minutes to remove the excess stain and mounted "e*n face*" on glass slides and observed under microscope. Surface area occupied by the plaque was assessed using software, ImageJ.

Preparation of Bone Marrow

The femur and tibia were isolated from the hind legs of the mouse. Sterilized micronic tubes, with a pore at the bottom, were placed in 1.5 mL Eppendorf tubes. One bone was placed per tube and centrifuged at 10000g for 15 seconds at room temperature. This allows the bone marrow to pass through the bone and was collected in the 1.5 mL tubes. The cells collected were resuspended in 800µL PBS. This resuspension was passed through 40micron syringe filter. The filter was prepped by adding 5mL of PBS

and allowing it to pass through the filter. Once all the bone marrow cells from a mouse had been passed through the filter, the final volume was made to 20 mL with PBS. The samples were then centrifuged at 400g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in PBS to have a concentration of $10*10^6$ cells/ml. From this resuspension of bone marrow cells, 92µL was added to each well of 96-well conical bottomed plate. To the cells, 8.42 µL of antibody mix was added and incubated for 30 minutes at 4°C. The plate was centrifuged at 500g for 5 minutes at 4°C.

The supernatant was discarded, and the pellet was resuspended in 100µL of 1% SVF-PBS. The resuspension was transferred to micronic tubes and analyzed by flow cytometry.

Preparation of Spleen

Spleens were then weighed and rinsed twice with ice-cold 0.1 µm filtered 3% PBS-FBS. Placing them on 3% PBS-FBS pre-wetted 40 µm nylon cell strainers (Cat# 352340, Thermo Fisher Scientific, USA) suspended over a 50 ml polypropylene tube, the spleens were then mashed using the plunger end of a 1 ml syringe. The cell strainers were then rinsed four times with 1ml of 3% PBS-FBS. Cell suspension was then centrifuged at 500 *xg* for 10 minutes at 4°C. The supernatant was then carefully aspirated and discarded, and the pellet was then resuspended in 1 ml of 0.1 µm filtered PBS. 1 ml of red blood cell lysis buffer (Cat# R7757, Merck, USA) was added to the resuspension and incubated at 5 minutes at room temperature.

After the incubation, 18 ml of 3% PBS-FBS was added and suspension was centrifuged at 500 *xg* for 10 minutes at 4°C. Supernatant was then removed carefully, and pellet was resuspended in 3 ml of complete RPMI media that consisted of RPMI 1640 Medium with GlutaMAX and HEPES (Cat# 72400021, Thermo Fisher Scientific, USA) supplemented with 10% FBS, 1% P/S and 0.1% β-mercaptoethanol (Cat# 1610710, Bio-Rad, USA). Cells were then counted, and a final concentration of 10 x 10^6 cells/ml was obtained.

Flow Cytometry

Post-incubation, stained cells were transferred to a 96-well plate and placed on ice post-incubation. The following antibodies were used to detect the presence of splenic subpopulations:

ANTIBODY	FLUOROCHROME	REF	COMPANY
Anti-NK1.1	BV650	564143	BD Biosciences
Anti-CD11b	FITC	53-0112-82	eBioscience
Anti-Ly6C	BV785	128041	Biolegend
Anti-Ly6G	Pe-Dazzle594	127648	Biolegend
Anti-CD11c	PerCP-Cy5.5	560584	BD Biosciences
Anti-CD3e	BUV737	564618	BD Biosciences
Anti-CD4	BV510	563106	BD Biosciences
Anti-CD8a	BV605	563152	BD Biosciences
Anti-CD19	Pe-Cy5	15-0193-83	eBioscience
Anti-IgM	Pe-Cy7	406514	Biolegend
Anti-CD45R/B220	BV711	563892	BD Biosciences
Anti-CD23	PE	12-0232-81	eBioscience
Fixable Viability Dye	EF780	65-0865-14	eBioscience

Table 4: List of antibodies used in flow cytometry experiments

Here, immune cell populations are defined as follows: NK cells as $NK1.1^+$, neutrophils as $CD11b^+/Ly6C^+/Ly6G^+$, monocytes as $CD11b^+/Ly6C^+/Ly6G^-$, dendritic cells as $CD11b^+/Ly6C^+/Ly6G^-/CD11c^+$, macrophages as $CD11b^{lo/-}/Ly6c^+/Ly6g^-/F480^{hi}$, activated mature B lymphocytes as $CD19^+/IgM^+/B220^+$, follicular B lymphocytes as $CD19^+/IgM^+/B220^+/CD21^+$, marginal zone B lymphocytes as $CD19^+/IgM^+/B220^+/CD23^+$, cytotoxic T lymphocytes as $CD3^+/CD8^+$ and helper T lymphocytes as $CD3^+/CD4^+$.

Antibody incubation was performed on ice for 30 minutes and 100 µl of PBS-FBS 1% was added post-incubation. The plate was then centrifuged at 500 *xg* for 5 minutes at

4°C and supernatant was removed by inverting the plate. A final resuspension of splenocyte pellet with 100 µl of PBS-FBS 1% was performed before transfer to Micronic tubes (Cat# 2517810, Dutscher, France) for flow cytometry analysis.

Statistical Analysis

Data are expressed as mean ± SEM. In *in vitro* experiments, each 'n' represents one experiment performed on HUVECs from one individual donor. Statistical analysis was performed by using Wilcoxon test. Statistical analyses and figures were performed by using GraphPad Prism 7 software. All tests were two-sided and used a p-value < 0.05.

RESULTS

Regulation of Acetylation Of α-Tubulin By Shear Stress

Shear stress exerted on endothelium, is a physical stimulus, which modulates the expression and activity of different proteins. As previously described by McCue et al., in endothelial cells, shear stress regulates elongation and polarity of the cells via reorganization of the cytoskeleton. Therefore, the effect of different shear stress levels on microtubules in vascular endothelial cells was verified, by measuring the levels of expression of acetylated α-tubulin. Western blot data showed that, in HUVECs exposed to high shear stress for 24 hours, levels of expression of acetylated α-tubulin was signficantly higher, when compared to cells exposed to low shear stress (Figure 20.A).

Having established that shear stress regulates acetylation of α-tubulin, we followed the differential expression of acetylated on α-tubulin over short time courses - 0, 2, 4, 6, 10 hours (Figure 20.B). We observed that α-tubulin underwent acetylation and the highest level of expression for acetylated α-tubulin was at 2h and 4h for high shear stress and low shear stress, respectively.

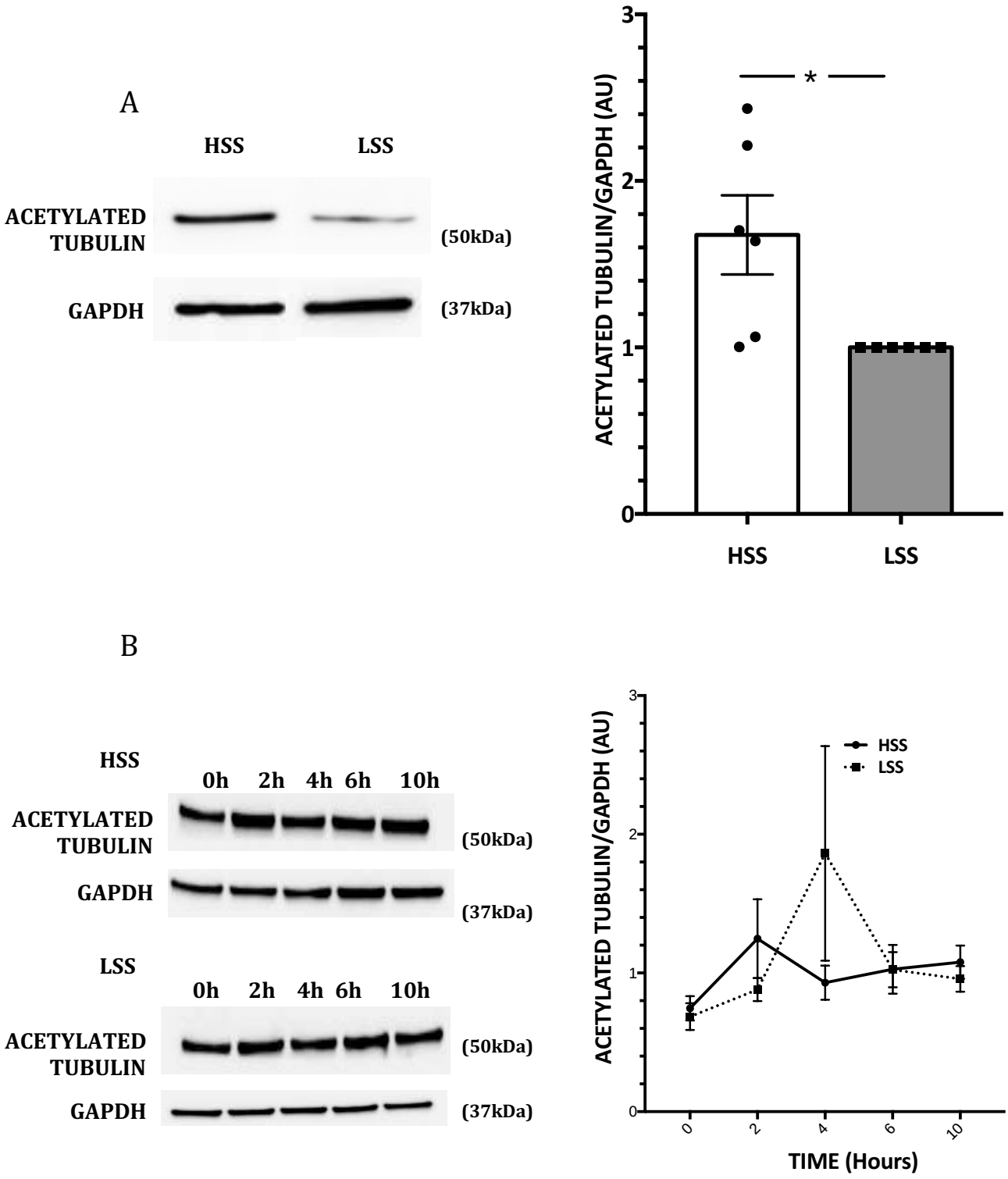


Figure 21: Regulation of acetylation of α-Tubulin by shear stress

(A) (Right panel) Analysis of expression of acetylated α-tubulin in lysates of HUVECs exposed to either high shear stress (HSS) (20 dyn/cm^2) or low shear stress (LSS) (2 dyn/cm^2) for 24 hours (n=6). **(B)** (Left panel) Representative western blots and (right panel) analysis of acetylated α-tubulin expression over 0,2,4,6,10 hours for HSS (i) and LSS (ii). Expression of acetylated α-tubulin over 2, 4, 6 and 10 hours under different shear conditions (n=6). Each 'n' represents experiments performed on HUVECs from different donors. *$p \leq 0.05$ (Wilcoxon t-test).*

Regulation of Deacetylase Activity Of HDAC6 By Shear Stress

We hypothesized that; in endothelial cells, shear stress may regulate deacetylation of α-tubulin via the deacetylase activity of HDAC6. Therefore, the effect of shear stress on HDAC6 activity was assessed. The deacetylase activity of HDAC6 was determined by a fluorimetric assay (Lemon et al. 2011) in HUVECs exposed to 20-dyne/cm^2 vs 2 dyne/cm^2. The enzymatic activity of HDAC6 in HUVECs exposed to high shear stress was 50% lower than that of HUVECs exposed to low shear stress (Figure 21). HDAC6 interacts with α-tubulin when it is phosphorylated at Ser22 by GSK-3β (Sigeng Chen et al. 2010). The expression and localization of both; the total and phosphorylated forms of HDAC6 in HUVECs was observed by immunofluorescence. Cells were exposed to either high shear stress or low shear stress. No visible difference in the expression levels of total-HDAC6 under high or low shear stress was observed. However, HUVECs exposed to low shear stress exhibited higher levels of phosphorylated-HDAC6, compared to high shear stress conditions Figure 22. A-B). Presence of higher levels of phosphorylated HDAC6 is indicative of higher α-tubulin deacetylase activity of the enzyme under low shear stress.

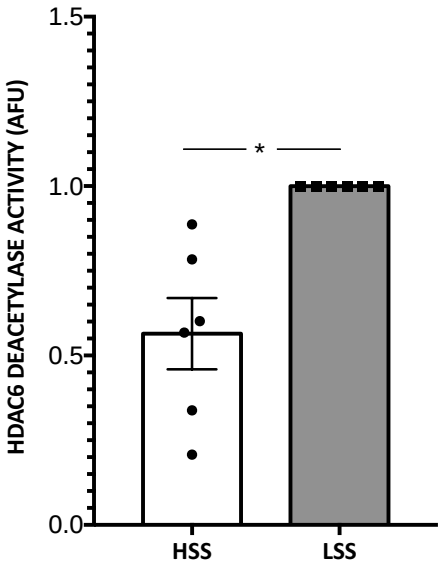


Figure 22: Regulation of deacetylase activity by shear stress

Analysis of HDAC6 enzymatic activity in HUVECs exposed to high shear stress (HSS) (20 dyn/cm^2) or low shear stress (LSS) (2 dyn/cm^2) for 24 hours (n=6) Each 'n' represents experiments performed on HUVECs from a different donor. ** $p \leq 0.05$ (Wilcoxon t-test).*

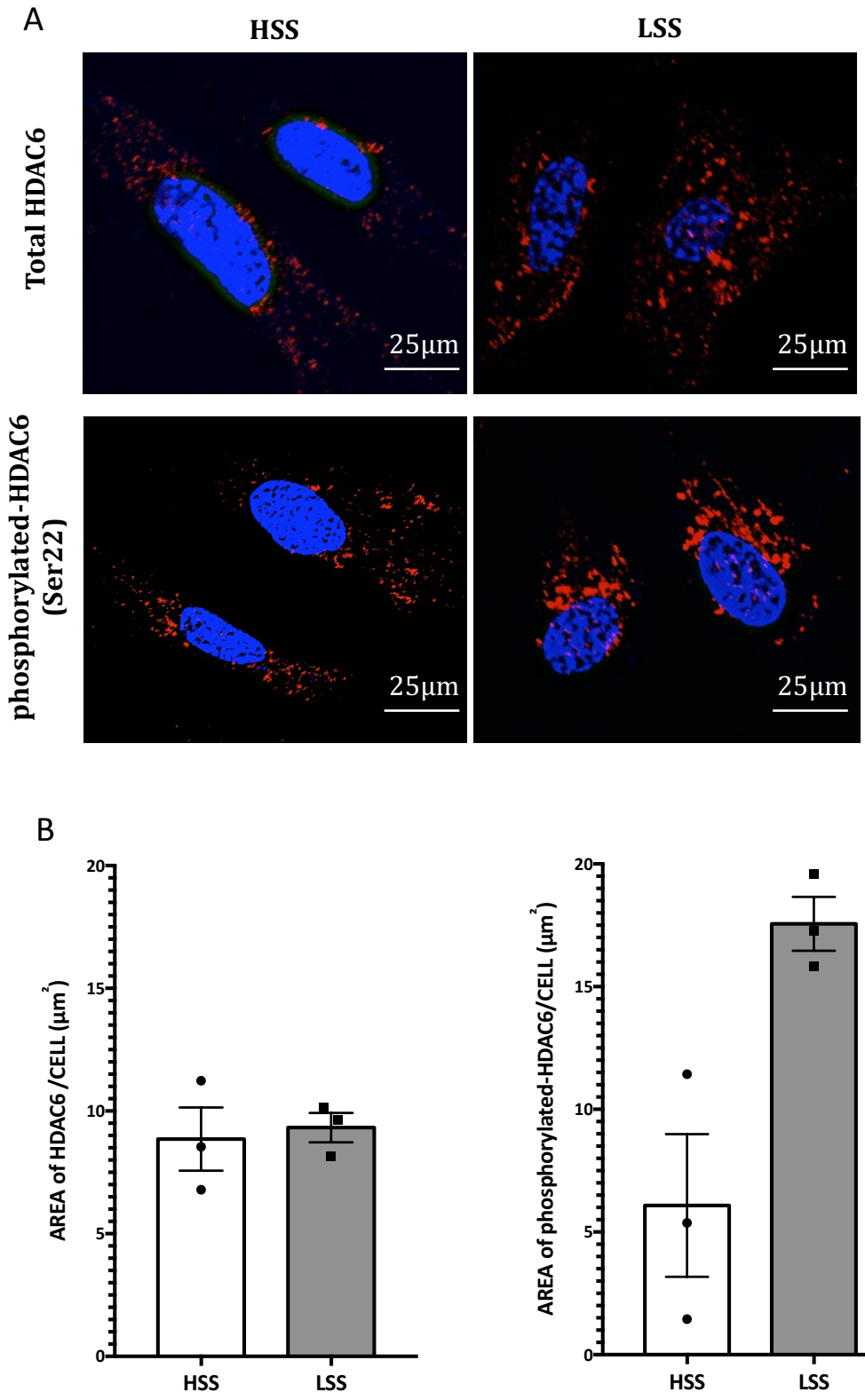


Figure 23: Expression of Total HDAC6 and phosphorylated (Ser22) regulated by shear stress **(A)** Representative images and **(B)** analysis of expression of HDAC6 and phosphor-HDAC6 by Immunofluorescence. HUVECs exposed to HSS (20 dyn/cm^2) or LSS (2 dyn/cm^2) for 24 hours (n=3), were labeled with HDAC6 or phospho-HDAC6 antibody, and the area/cell (µm^2) was analyzed. Top panel- Red= Total HDAC6; blue= DAPI. Bottom panel- Red= phosphorylated HDAC6; blue= DAPI.

Effect of Pharmacological Interventions on Acetylation of α-Tubulin

To evaluate the effect of inhibiting HDAC6 deacetylase activity on the acetylation of α-tubulin, HUVECs were treated with a known HDAC6 inhibitor, Tubastatin-A and with a Sirtuin2 inhibitor, SirReal2. HUVECs were exposed to low shear stress, under Tubastatin-A (3μM) treatment, SirReal2 (5μM) or vehicle (DMSO at 0.1μL/ml). Acetylated α-tubulin expression and autophagy levels (LC3-II/LC3I or LC3II/GAPDH), were measured by western blot (Figure 23.A) and expressed in terms of ratio to GAPDH. Under Tubastatin-A treatment, HUVECs exposed to low shear stress exhibited a significantly higher expression of acetylated α-tubulin (Figure 23.B), as well as LC3-II expression (Figure 23.C). However, exposure to SirReal2 showed no significant increase in acetylation of α-tubulin, nor affected autophagy levels in HUVECs (Figure 23.B-C).

Following this first set of experiment, we continued to perform experiments using Tubastatin-A only to enhance the levels of α-tubulin acetylation (Figure 24). The localization and amount of acetylated α-tubulin was analyzed in cells exposed to low shear stress. Similar to the Western blot data; levels of acetylated tubulin were significantly higher in HUVECs treated with Tubastatin-A.

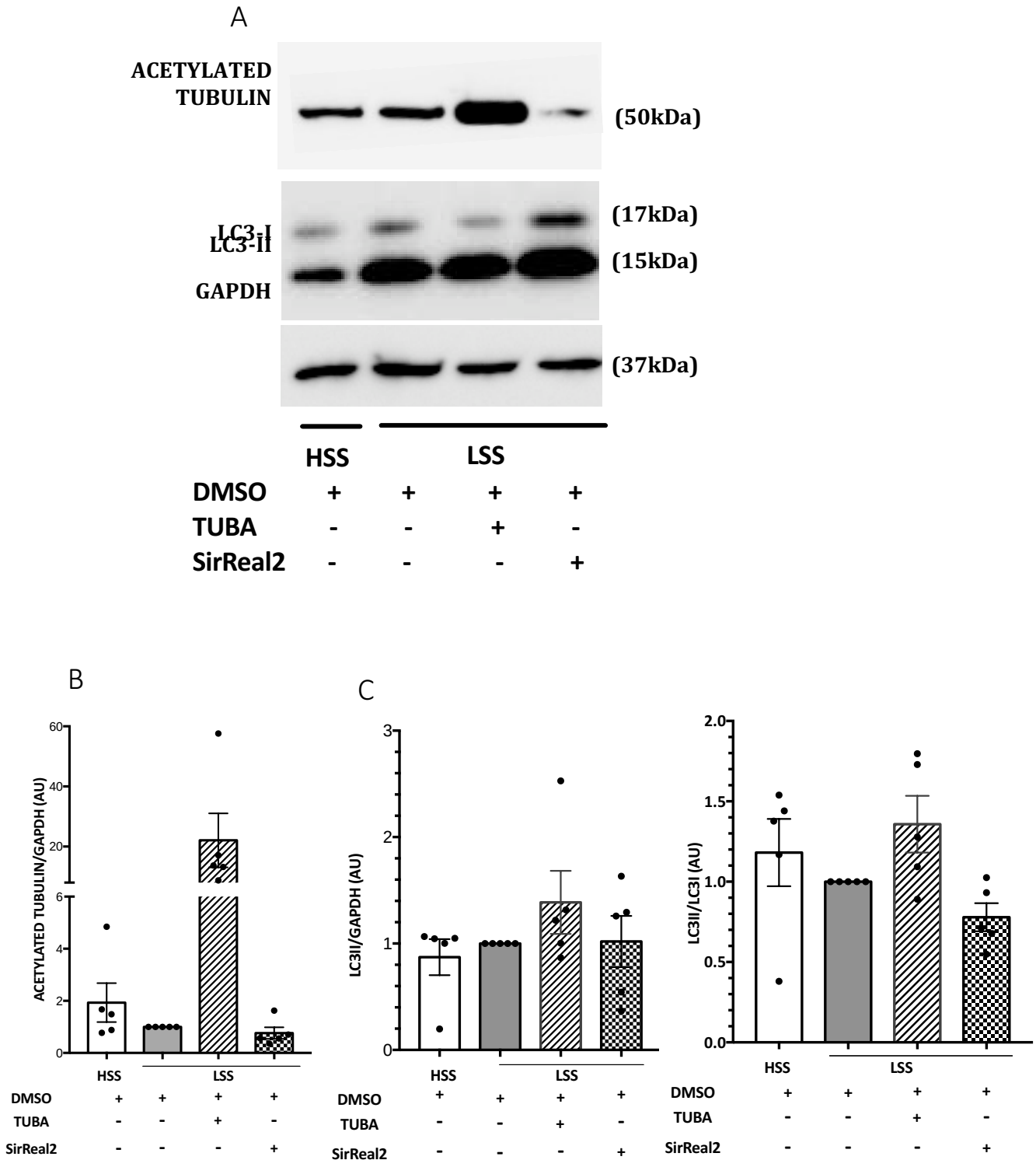


Figure 24: Effect of SirReal2 and Tubastatin-A on acetylation of α-Tubulin

(A) Representative western blot of acetylated α-tubulin, LC3-II, LC3-I and GAPDH in HUVECs exposed to high shear stress (HSS) (20 dyn/cm^2) or low shear stress (LSS) (2 dyn/cm^2) for 24 hours **(B)** Analysis of expression of acetylated α-tubulin, normalized to GAPDH. **(C)** Analysis of expression of LC3-II, normalized to GAPDH, and the ratio of LC3II/LC3I, to estimate the level of autophagy;(n=5).

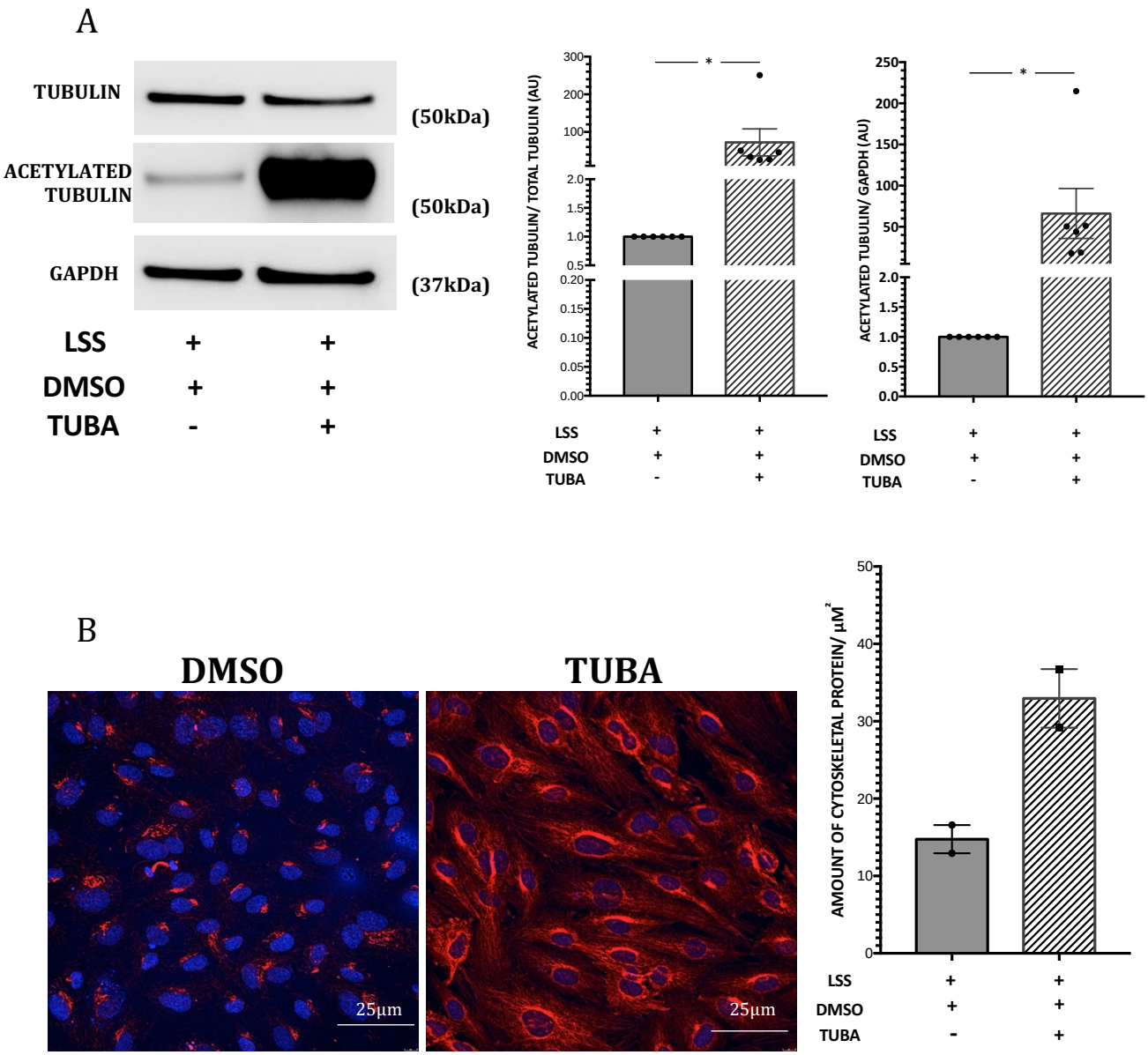


Figure 25: Effect of Tubastatin-A on acetylation of α-tubulin

(A) (Left Panel) Representative western blot and (Right panel) analysis of Acetylated α-tubulin in HUVECs exposed to low shear stress (LSS) (2 dyn/cm^2) and treated with either vehicle (DMSO: 0.1µl/ml) or Tubastatin-A (3µM) (n=6) ** $p \leq 0.05$ (Wilcoxon t-test).* **(B)** (Left panel: Red= Acetylated α-tubulin; blue= DAPI) Representative image and (Right panel) analysis of amount of acetylated α-tubulin, by immunofluorescence in HUVECs exposed to LSS (n=2).

HDAC6 Inhibition Increases Conversion of LC3-I To LC3-II in Low Shear Stress Conditions

As previously described by Mackeh et al., a stable microtubule network is necessary for autophagic flux, as it is required for the efficient formation of autophagosomes as well as their fusion with lysosomes. To observe the effect of inhibiting the deacetylase activity of HDAC6, and subsequent stabilization of α-tubulin, on endothelial autophagy, we first measured the levels of essential autophagy proteins: LC3, p62 and ATG5.

A lower LC3II/LC3I ratio and overall expression of LC3-II was observed in HUVECs exposed to low shear stress compared to high shear stress conditions. Treatment of cells with Tubastatin-A, in low shear stress conditions, increased by 2 fold the LC3II/ LC3I ratio when compared to control conditions (Figure 25.A). Similar results were obtained when localization and expression of LC3-II was observed by immunostaining. By quantifying the area of LC3-positive punctae (Figure 26), we found that the amount of autophagic structures was lower in cells exposed to low SS compared to high shear stress, in agreement with our previous results (Vion et al, 2017). Treating cells with Tubastatin-A, in low shear stress conditions, increased by 2-fold the area of LC3-positive structures per cell. For essential autophagy proteins such as ATG5, there was no difference observed between the different conditions of high SS or low SS or Tubastatin-A treated HUVECs exposed to low shear stress (Figure 25.B).

We then analyzed the expression of p62 as an indicator of autophagic flux (Figure 25.C). p62 is an autophagy adaptor protein that is responsible for the trafficking of ubiquitinated cargo and subsequently, their degradation (Bjørkøy et al. 2005; Pankiv et al. 2007). Along with the cargo, p62 is also degraded in the autolysosomes. Hence, a decrease in the expression levels of p62 may indicate a higher autophagic flux. On an average, HUVECs treated with Tubastatin-A and exposed to low shear stress showed no difference in expression of p62, compared to control conditions (Figure 25.C).

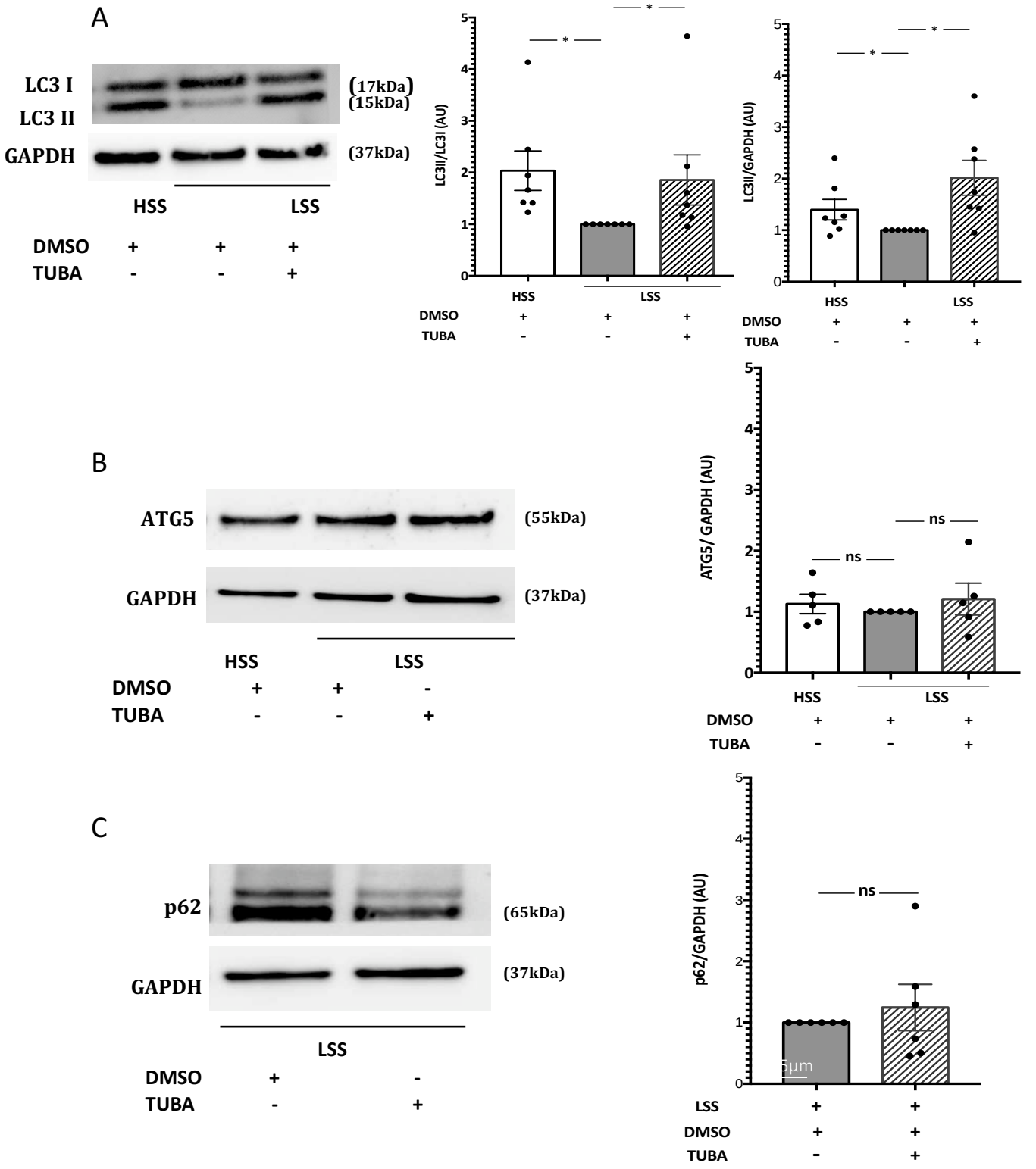


Figure 26: Effect of Tubastatin-A on Endothelial Autophagy

(Left panel) Representative Western blot and (right panel) analysis of **(A)** LC3II/LC3 ratio and LC3II/GAPDH : **(B)** ATG5 ; **(C)** p62 in lystaes from HUVECs treated with vehicle (DMSO at 0.1µl/ml) or Tubastatin A (3µM) and exposed to high shear stress (HSS) and/or low shear stress (LSS) for 24 hours (n=6) **p ≤ 0.05 (Wilcoxon t-test).*

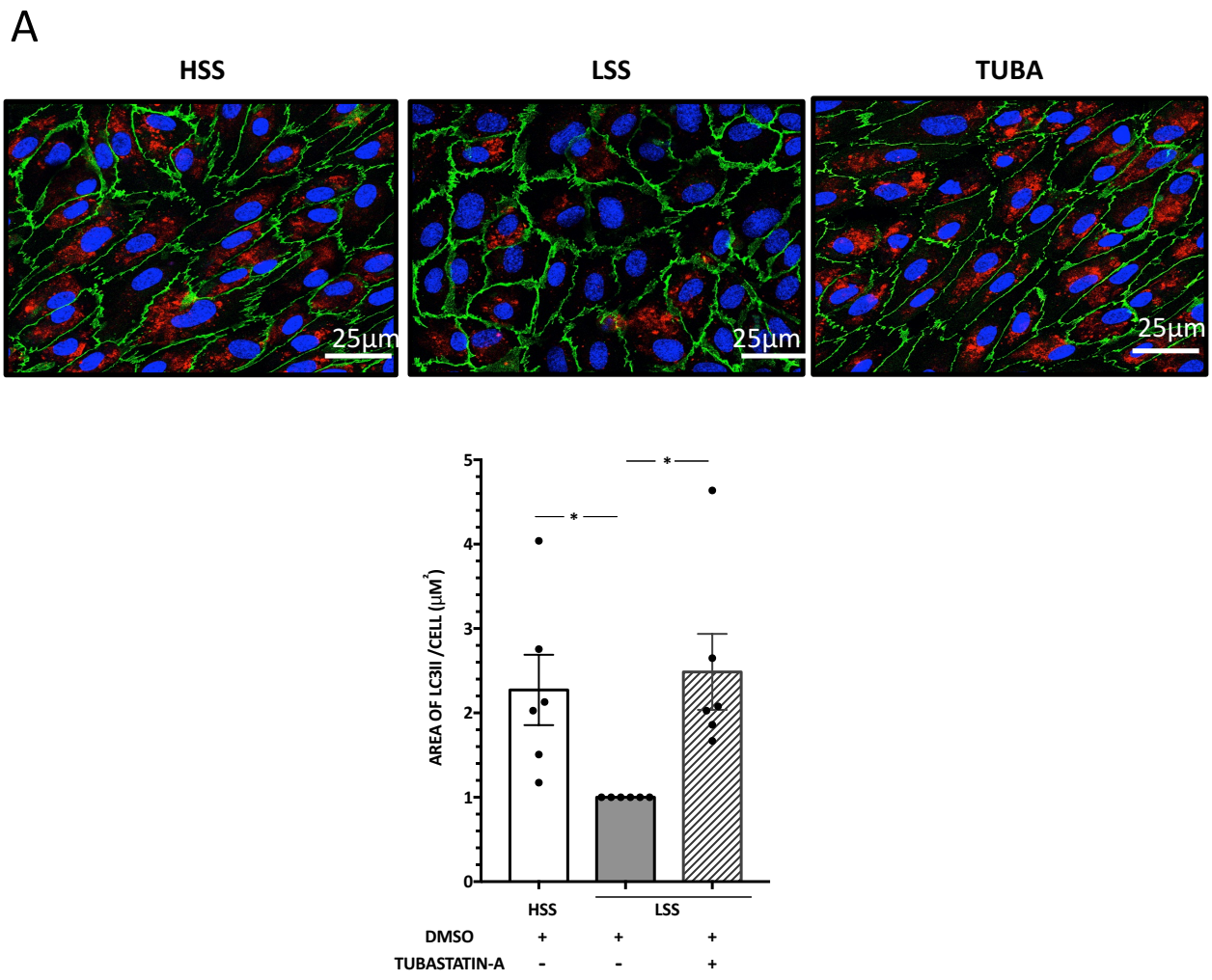


Figure 27: Tubastatin-A increases LC3-II punctae in HUVECs exposed to low shear stress

Immunofluorescence labeling of LC3-II in HUVECs exposed to shear stress (HSS) (20 dyn/cm^2) or low shear stress (LSS) (2 dyn/cm^2), and treated with vehicle (DMSO at 0.1µl/ml) or Tubastatin A (3µM) (n=6). Top panel- Red= LC3-II, green= Ve-Cadherin, blue= DAPI.* *$p \leq 0.05$ (Wilcoxon t-test).*

HDAC6 Inhibition Reverses Defect In Endothelial Autophagic Flux In Low Shear Stress Conditions

We next observed the consequences of microtubule stabilization on autophagic flux. To do so, we used a tandem LC3-B fluorescently tagged with mRFP and GFP. HUVECs were transduced with lentiviral particles carrying this plasmid, to allow us to assay the autophagic flux. As described before, the yellow fluorescent signal indicates the autophagosome stage, whereas the red fluorescent signal is indicative of autolysosomes due to quenching of the GFP signal in low pH environments (Figure 27.A). We evaluated autophagic flux by quantifying the ratio of autolysosomes to autophagosomes per cell (Figure 27.B) . HUVECs were treated with either Rapamycin (1µM), Tubastatin-A (3µM) or vehicle (0.1 µl/ml DMSO), and exposed to either high or low shear stress. Cells exposed to high shear stress conditions had approximately twice the number of autolysosomes than those exposed to low shear stress conditions (Figure 27.D). Treatment with Rapamycin, a known autophagy inducer, resulted in a slight increase in autophagic flux in low shear stress conditions. However, it did not prove to be as efficient as high shear stress conditions. In HUVECs treated with Tubastatin-A under low shear stress conditions, we observed a pro-autophagic phenotype, as the number of autolysosomes formed was twice as high as in untreated cells. In order to confirm that stabilization of microtubules does indeed facilitate the increase in autophagic flux, HUVECs exposed to low shear stress were treated with vehicle (0.1 µl/ml DMSO) or Tubastatin (3µM), and with Chloroquine (300µM) for the last 6 hours of the shear stress experiment (Figure 28.A). Chloroquine is a known to inhibit autophagy flux by blocking the fusion of autophagosomes and lysosomes (Mauthe et al. 2018). In low shear stress conditions with Chloroquine treatment alone, the ratio of autolysosomes/ autophagosomes was inversed (Figure 27.C-D), with no visible difference in the total number of autophagy vacuoles generated, when compared to low shear stress control conditions. This suggests that the flux was blocked by Chloroquine.

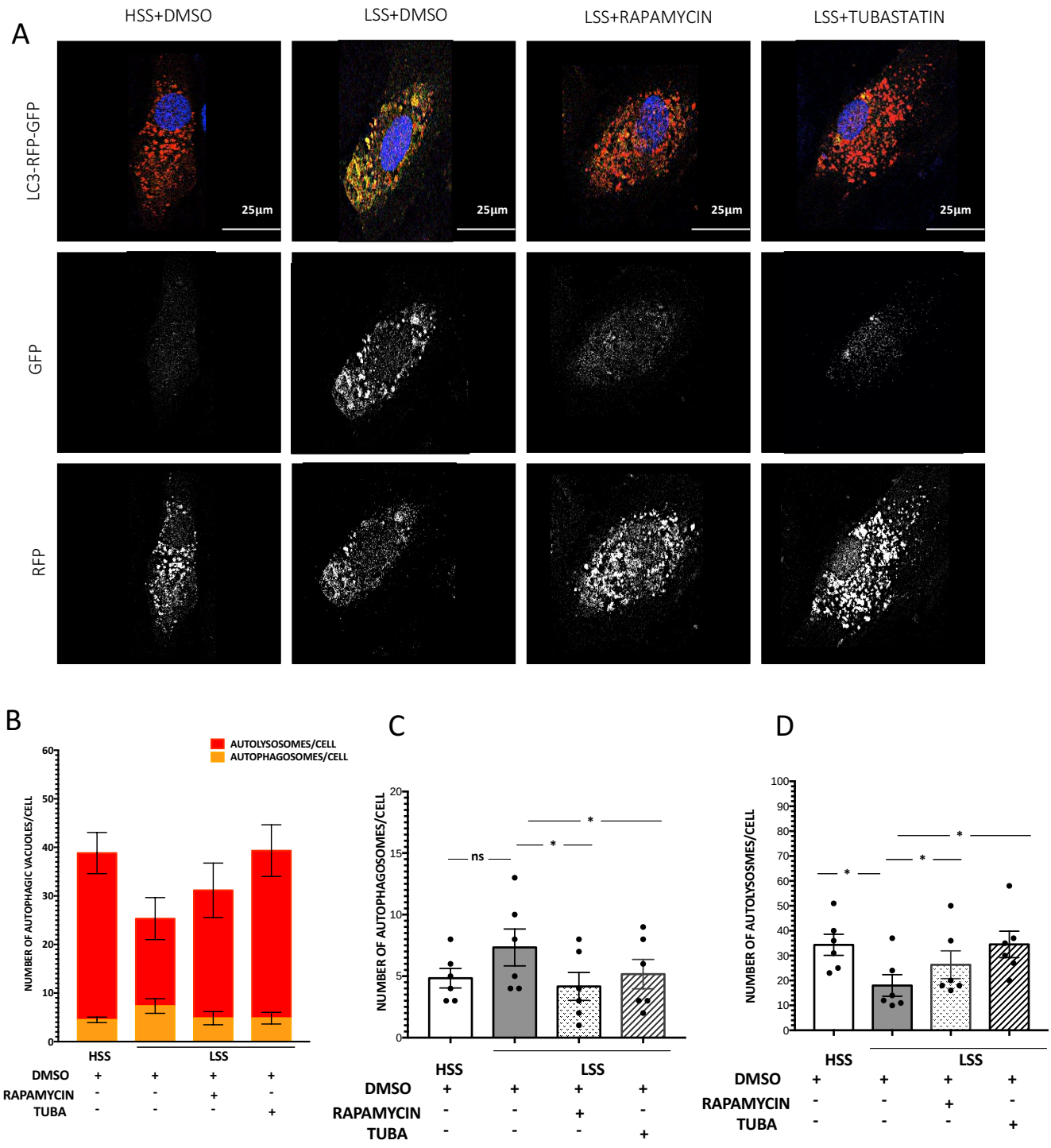


Figure 28: Autophagic flux is restored in HUVECs exposed to low shear stress and treated with Tubastatin-A.

(A) Representative confocal microscopy images of HUVECs infected with lentivirus carrying the tandem mRFP-GFP-LC3 and exposed to high shear stress (HSS) or low shear stress (LSS) for 24 hours after being treated with DMSO (0.1µl/ml) or Tubastatin (3µM). Autophagosomes and autolysosomes are denoted by yellow and red signals, respectively. Scale bar: 25µm.

(B) Quantification of effect of Tubastatin treatment on autophagic flux in HUVECs. Autophagic flux is represented as the ratio of autolysosomes/autophagosomes. The yellow bars correspond number of autophagosomes per cell and red bars correspond to number of autolysosomes per cell. On an average, about 100 cells were analyzed, per condition, for each experiment (n=6). **(C)** and **(D)** represent the number of autophagosomes/cell and autolysosomes/cell respectively.* *p ≤ 0.05 (Wilcoxon t-test).*

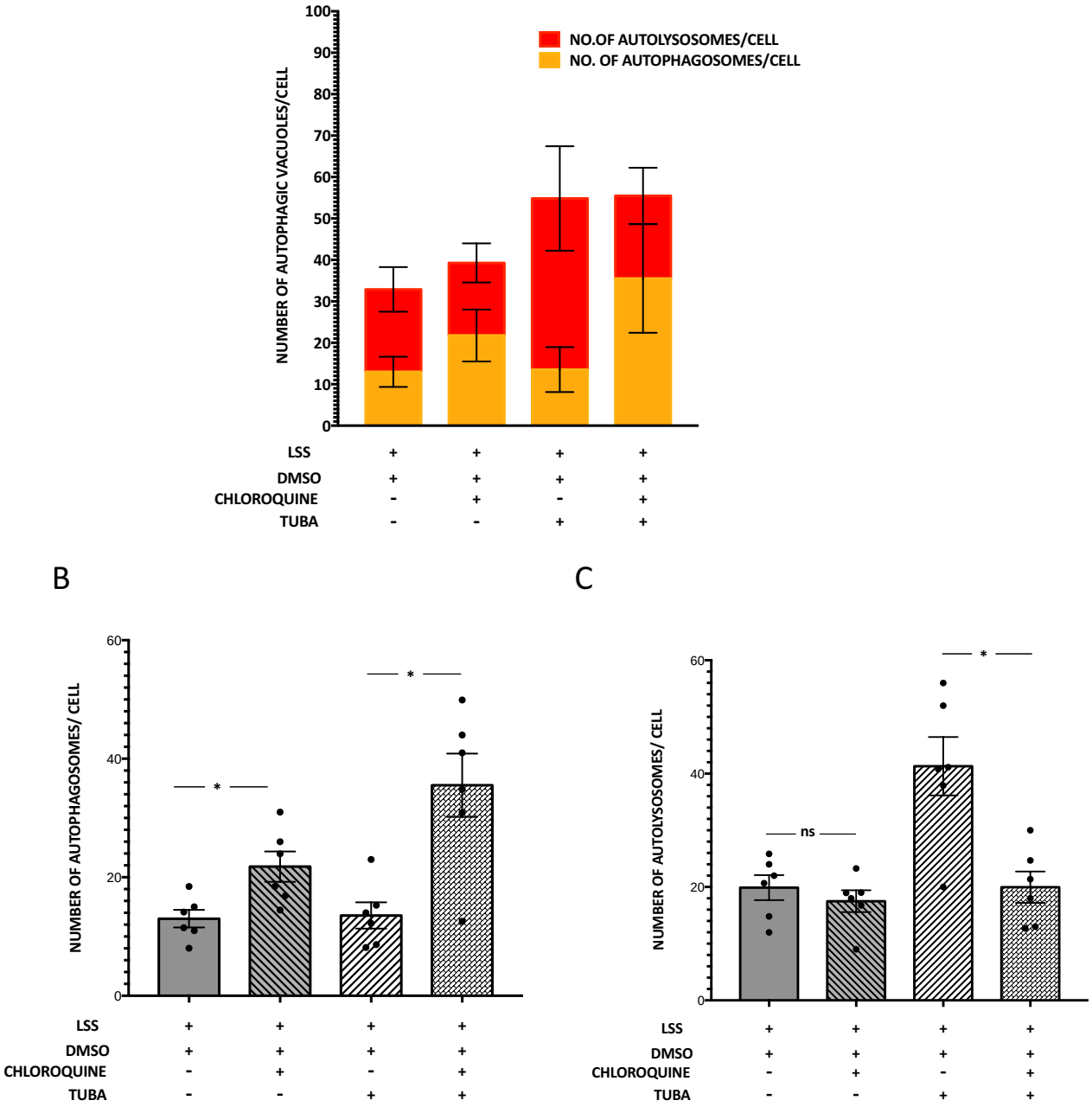


Figure 29: Autophagic flux blocked in HUVECs exposed to low shear stress by Tubastatin-A in the presence of Chloroquine.

(A) Autophagic flux is represented as the ratio of autolysosomes/autophagosomes. The yellow bars correspond number of autophagosomes per cell and red bars

correspond to number of autolysosomes per cell. On an average, about 100 cells were analyzed, per condition, for each experiment (n=6). **(B)** and **(C)** represent the number of autophagosomes/cell and autolysosomes/cell respectively.*$p \leq 0.05$ *(Wilcoxon t-test).*

Tubastatin-A Has Anti-Inflammatory Effect On Endothelial Cells Exposed To Low Shear Stress

Since Tubastatin-A reverses the defective autophagic flux in low shear stress conditions, we wanted to observe if this translated to an endothelial anti-inflammatory phenotype. To do so, we treated HUVECs with TNF-α at 1ng/ml for the last 12 hours of the shear stress experiment.

Using Western blot analysis, we assessed the expression of ICAM-1 and VCAM (Figure 29.A-B). Firstly, as expected, a pronounced inflammatory response is observed in conditions where the cells were treated with TNF-α, compared to their corresponding negative control conditions. HUVECs exposed to low shear stress, showed significantly higher levels of expression of inflammatory markers, ICAM-1 and VCAM-1, compared to high shear stress as well as cells exposed to low shear stress and treated with Tubastatin-A.

We then quantified the levels of MCP-1 released by HUVECs into the conditioned media (Figure 29.C). Similar to the expression profile of inflammatory proteins, we observed a 5-fold increase in the release of MCP-1 under the low SS, when compared to high shear stress conditions. However, when HUVECs are treated with Tubasatin-A, the levels of MCP-1 released in the presence of TNF-α is significantly reduced.

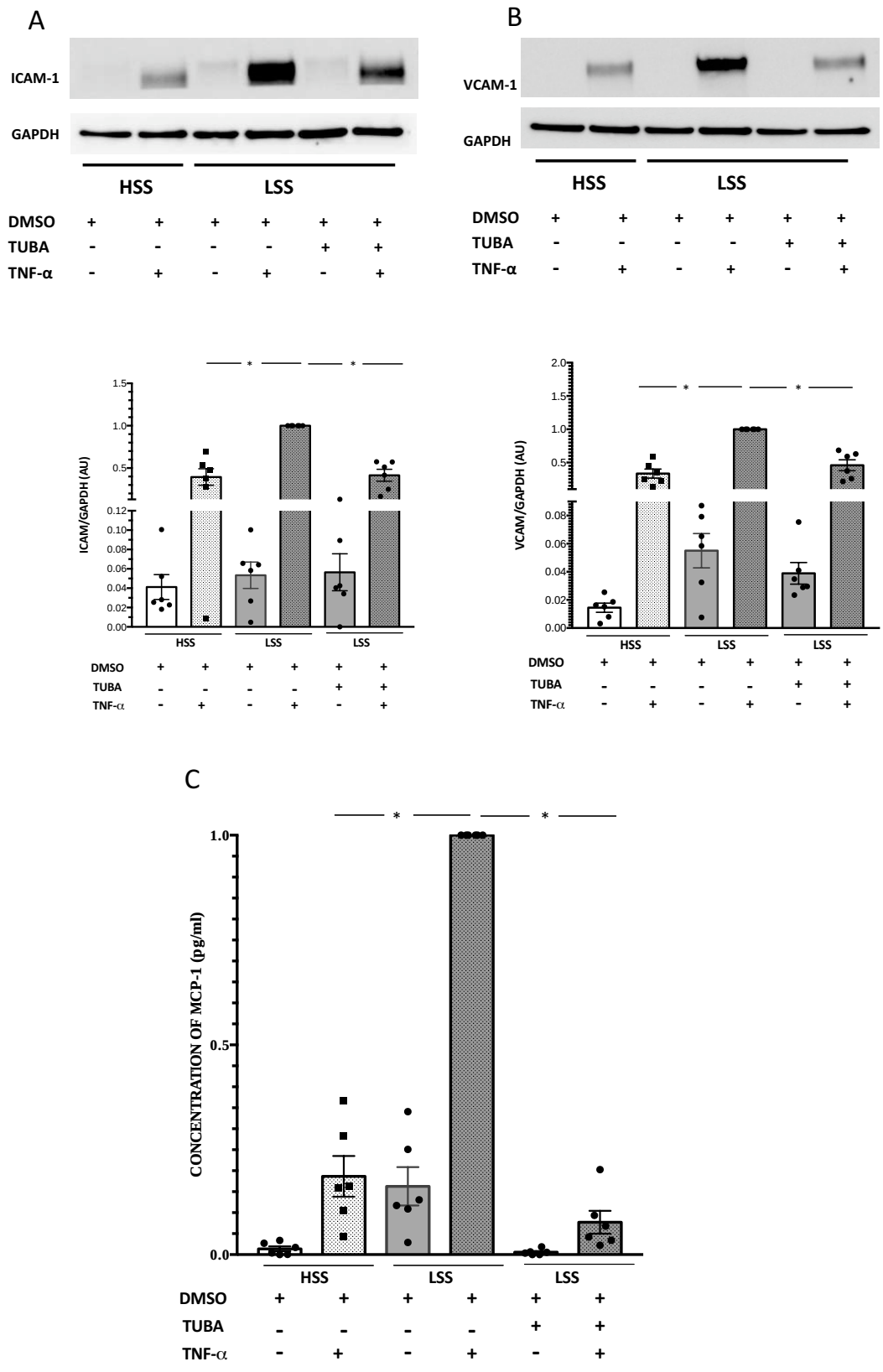


Figure 30: Tubastatin-A has anti-inflammatory effect on endothelial cells exposed to low shear stress

Representative Western blots and analysis of inflammatory markers **(A)** ICAM-1 and **(B)** VCAM-1, by Western blot in HUVECs exposed to high shear stress (HSS) (20 dyn/cm^2) or low shear stress (LSS) (2 dyn/cm^2), and treated with vehicle (DMSO at 0.1µl/ml) or Tubastatin A (3µM) (n=6). **(C)** Analysis of levels of MCP-1 released in the supernatant media by HUVECs, by ELISA (n=6).*$p \leq 0.05$ (Wilcoxon t-test).*

Tubastatin-A Does Not Rescue Inflammation in ATG5 Deficient HUVECs Exposed to Low Shear Stress

As shown above, Tubastatin-A is able to reduce inflammation in endothelial cells. However, to establish the link between the two effects of Tubastatin-A: restoration of autophagic flux and attenuation of inflammation, we used endothelial cells lacking ATG5, an important player in the autophay pathway. Endothelial cells were infected with lentiviruses carrying the shRNA against *ATG5*. We were able to observe an approximately 60% reduction of the expression of the protein, as observed by Western blot (Figure 30.A-B). When we assesed the levels of autophagy by estimating the levels of LC3II/LCE3, we observed a lack of autophagy, indictaed by the low LC3II/LC3I ratio in ATG5-deficient cells (Figure 30.C). We were also able to reproduce the effect of Tubastatin-A on autophagy; when we analyzed the LC3II/LC3I ratio in the control cells (Figure 30.C).

To elucidate the effect of Tubastatin-A in conditions of autophagy deficiency on inflammation, we analyzed the levels of expression of ICAM-1 by Western blot and concentration of MCP-1 released by the cells (Figure 30.D-E). Between the control cells treated with vehicle or Tubastatin-A, in the presence of TNF-α, we observed the same effect as reported earlier. That is, Tubastatin significantly reduces the expression of ICAM-1, as well as the release of MCP-1. Similar to what has been reported previously by Vion, et al, we observe that lack of autophagy leads to increase in inflammation by increasing the expression of adhesion molecules like ICAM-1, and cytokine release like MCP-1. However, in autophagy deficient cells, this effect of Tubastatin-A is no longer observed.

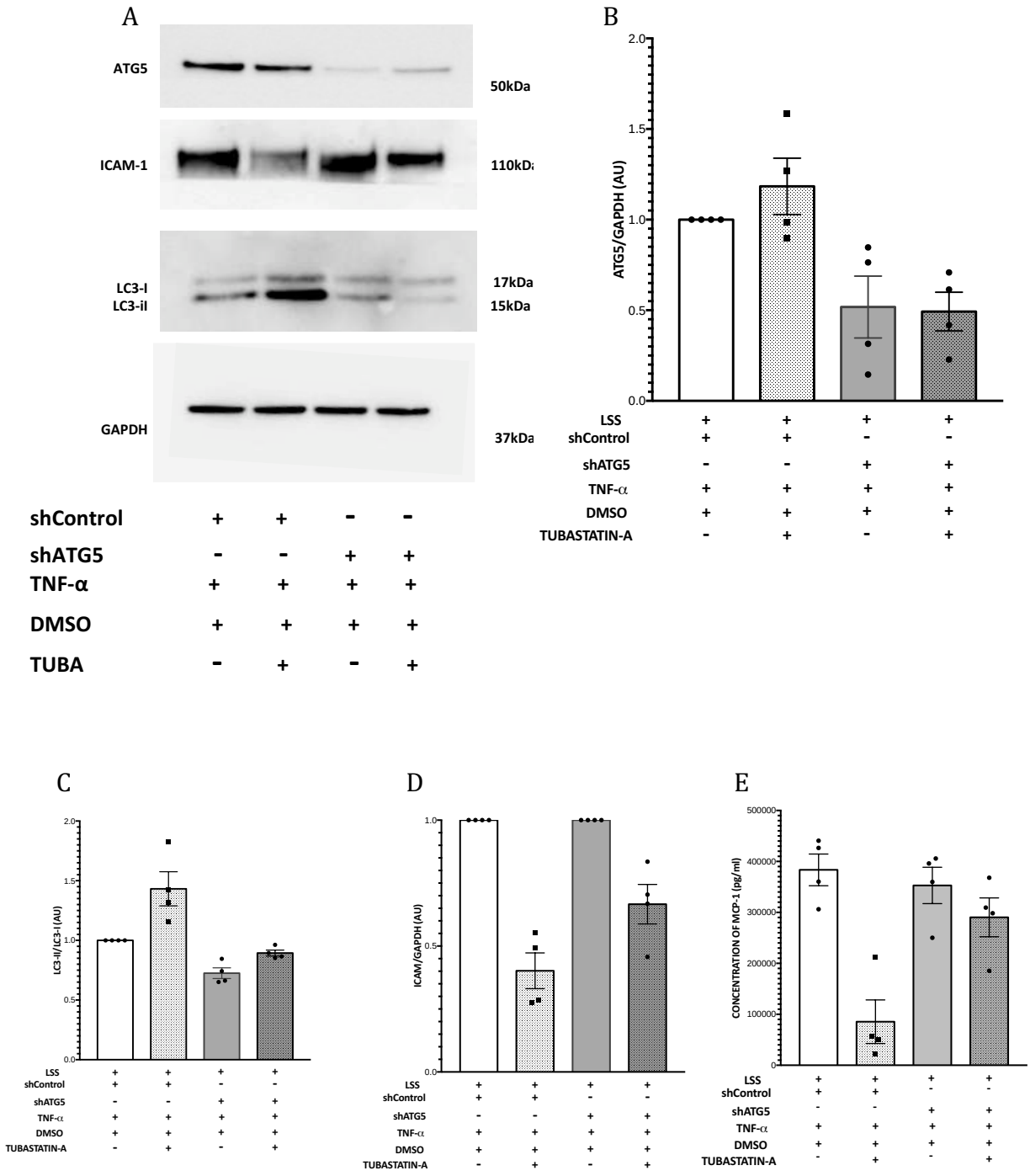


Figure 31: Tubastatin-A does not have anti-inflammatory effect on endothelial cells exposed to low shear stress in autophagic deficient conditions.

(A) Representative Western blots and analysis of inflammatory markers **(B)** ATG5, **C)** LC3II/LC3I, **(D)** ICAM-1 by Western blot in HUVECs exposed to low shear stress

(LSS) (2 dyn/cm^2), and treated with vehicle (DMSO at 0.1µl/ml) or Tubastatin A (3µM) (n=6) in the presence of TNF-α.
(E) Analysis of levels of MCP-1 released in the supernantant media by HUVECs, by ELISA (n=4).

Knockdown of HDAC6 in HUVECs Increases Autophagy Levels and Suppresses Inflammatory Phenotype

We then evaluated the impact of HDAC6 knockdown in endothelial cells and its downstream effects on autophagy and inflammation. This would also allow us to establish the link that it is essential to inhibit the deacetylase activity of HDAC6 in order to restore autophagy and reduce inflammation, and might prove to be helpful to validate our *in vivo* data, if we were to observe similar anti-inflammatory effects. We knocked down the HDAC6 protein in HUVECs using lentiviruses carrying the shRNA. We validated the efficiency of the knockdown by analyzing the expression of HDAC6 protein and its target, α-tubulin (Figure 31A-C).

We were able to observe an approximately 30% reduction in the expression of HDAC6 and about 50% increase in the expression of acetylated α-tubulin. Following this, we assessed the effects of HDAC6 knockdown on endothelial autophagy and inflammation. LC3II/LC3I ratio was also observed to have increased in conditions of HDAC6-deficient cells (Figure 31.D), while expression of ICAM-1 and concentration of MCP-1 was reduced (Figure 32.A-B).

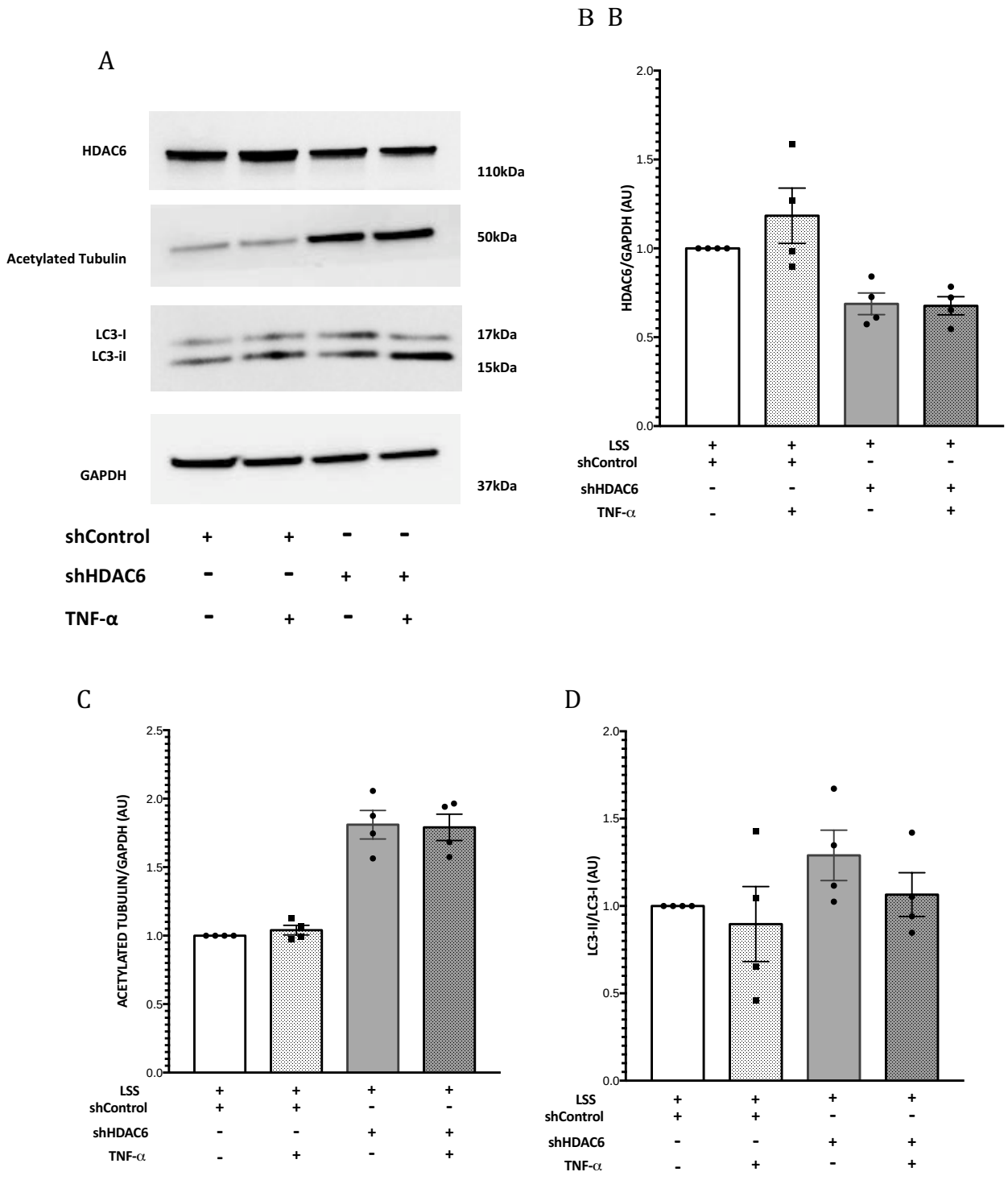


Figure 32: Knockdown of HDAC6 in endothelial cells increases autophagy

(A) Representative Western blots and analysis of **(B)** HDAC6, **(C)** Acetylated α-tubulin and **(D)** LC3II/LC3I, by Western blot in HUVECs exposed to LSS (2 dyn/cm²), in the presence or absence of TNF-α (n=4).

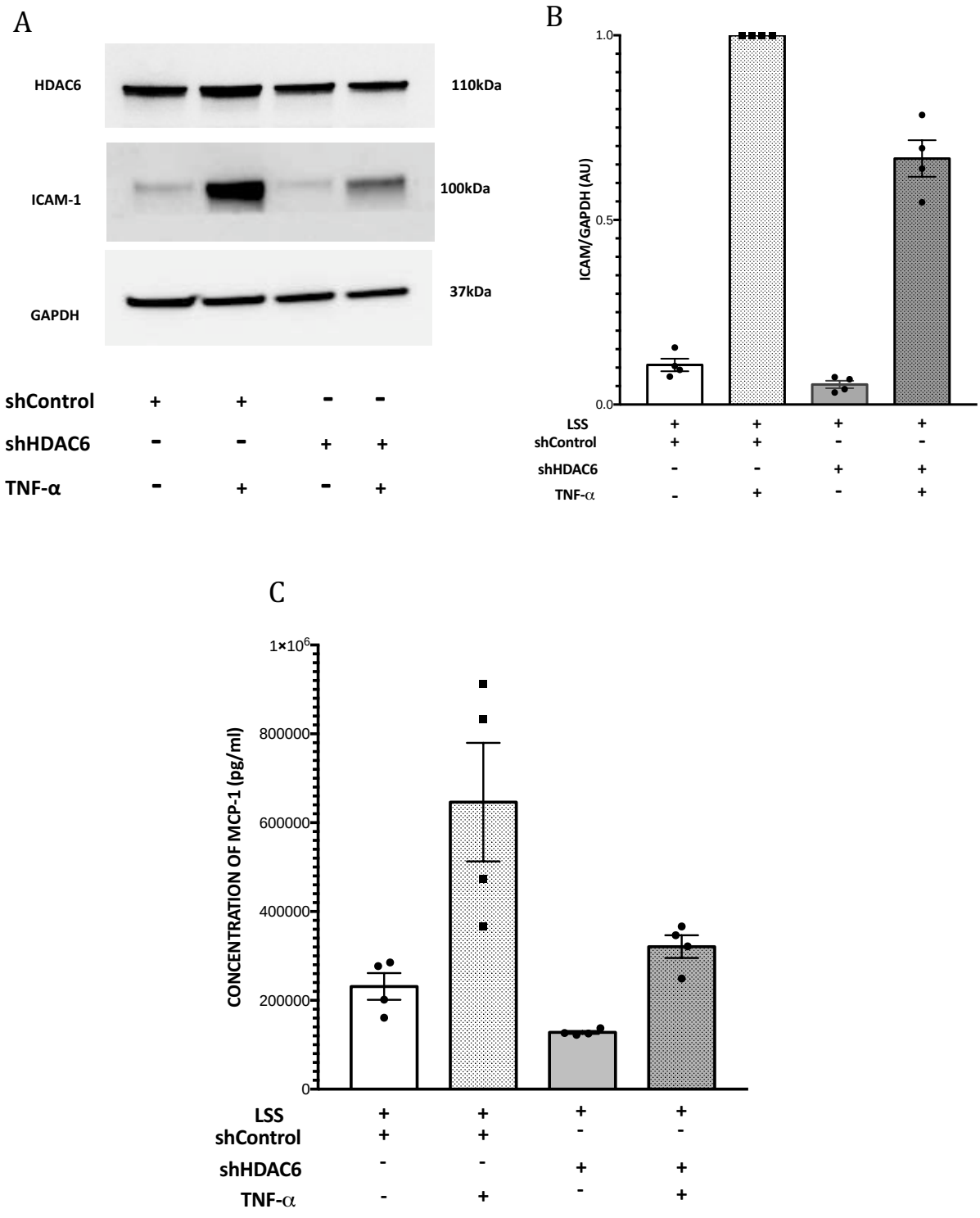


Figure 33: Knockdown of HDAC6 in endothelial cells reduces inflammation

(A) Representative Western blots and analysis of **(B)** ICAM-1 and **(C)** concentration of MCP-1, released by cells, shControl or shHDAC6, with or without TNF-α treatment.

Deletion Of HDAC6 In Vivo Leads To Reduction Of Atherosclerotic Plaque Size

To evaluate the effect of *HDAC6* deletion on atherosclerosis *in vivo*, we generated a knockout murine model on an *ApoE*$^{-/-}$ background, *HDAC6*$^{-/-}$*/ ApoE*$^{-/-}$. Mice with genotype, *HDAC6*$^{+/+}$*/ ApoE*$^{-/-}$, were used as control. All mice underwent irradiation followed by bone marrow transplant from *ApoE-/-* donor mice and were fed Western diet for 10 weeks.

We compared cholesterol levels between the two groups of mice, as well as before and after Western diet regime (Figure 33.A). Overall, there is a 2-fold increase in the cholesterol levels at the end of the regime, but there were no statistical differences in cholesterol levels between the two groups after 10 weeks regimen. There were no differences in blood cell count (Figure 33.B) determined by the Hemavet cell counter or cell populations in the spleen and bone marrow (Figure 34), determined by flow cytometry, between the two groups of animals.

Atherosclerotic plaques in the aorta were stained with Oil Red O. We assessed the -progression of atherosclerosis by quantifying the size of the plaques in different areas of the aorta. In the double knockot mice, ze observed approximately 40% decrease and about 20% decreaese in the plaque size; in the aortic arch and the descending aorta respectively, when compared to the wild-type.

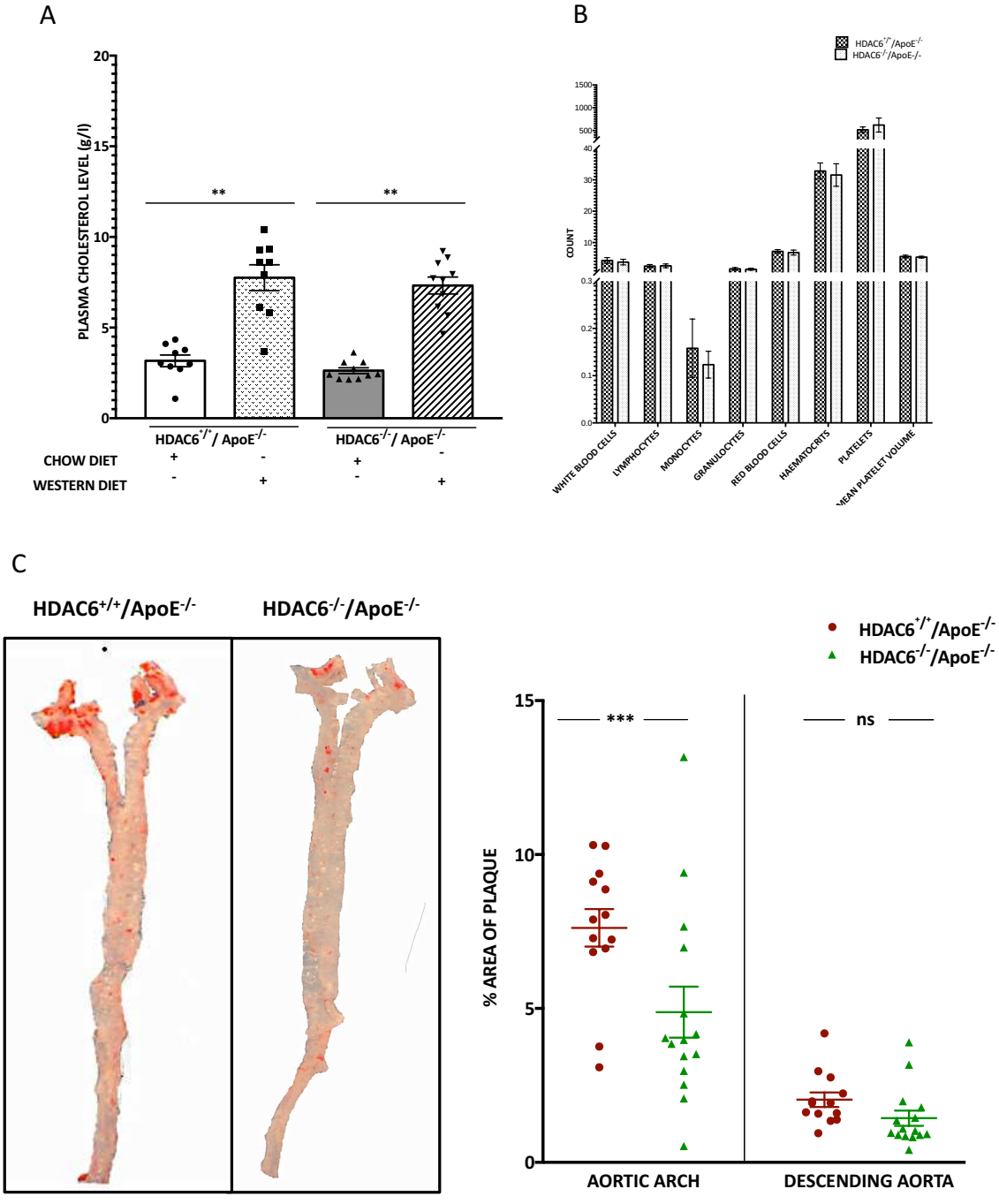


***Figure 34: Deletion of HDAC6 in vivo leads to reduction of atherosclerotic plaque size*(A)** Analysis of plasma cholesterol levels compared betweenthe two groups, *HDAC6$^{+/+}$ ApoE$^{-/-}$* and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, before and after the Western diet (*HDAC6$^{+/+}$ ApoE$^{-/-}$* :n=9; and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, n=10) **(B)** Number of cells for different populations in the blood, compared between *HDAC6$^{+/+}$ ApoE$^{-/-}$* and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, (*HDAC6$^{+/+}$ ApoE$^{-/-}$* :n=4; and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, n=5). **(C)** Representative images of aorta stained with Oil Red-O and the analysis between the two groups of mice; in the aortic arch and descending aorta (*HDAC6$^{+/+}$ ApoE$^{-/-}$*:n=9; and *HDAC6$^{-/}$ /ApoE$^{-/-}$*, n=10).

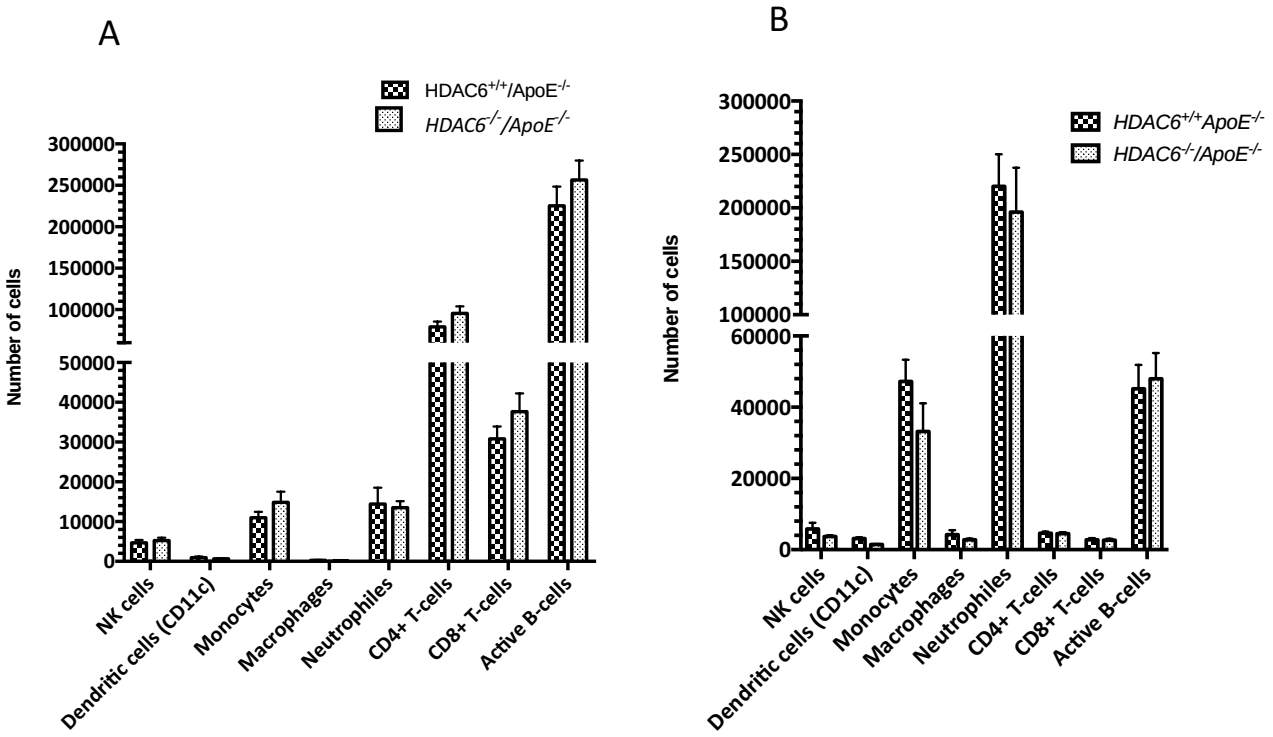


Figure 35: Immune cell profile of the mice determined by flow cytometry

Comparison of the number of cells detected for each population, in the **(A)** spleen and **(B)** bone marrow of *HDAC6$^{+/+}$ ApoE$^{-/-}$* and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, (*HDAC6$^{+/+}$ ApoE$^{-/-}$* :n=6; and *HDAC6$^{-/-}$ ApoE$^{-/-}$*, n=6)

DISCUSSION

The present study explores a strategy to restore defective autophagic flux in endothelial cells exposed to low shear stress caused by turbulent flow. In order to do so, we aimed to enhance the autophagic flux in endothelial cells by favoring the fusion of autophagosomes and lysosomes, by enhancing the stability of the cytoskeleton. Our experiments have shown that increased acetylation of α-tubulin, via inhibition of the deacetylase activity of HDAC6, maintained a stable cytoskeletal network, which led to increase in fusion of autophagosomes and lysosomes. This in turn led to increase in the autophagic flux while also promoting an anti-inflammatory phenotype in endothelial cells. These findings were then corroborated in an *in vivo* model, *HDAC6*$^{-/-}$*/ApoE*$^{-/-}$, where a decrease in the size of the atherosclerotic lesion was observed in the inner curvature of the aortic arch and the carotid bends.

A. Effect of Tubastatin-A on Endothelial Autophagic Flux

Shear stress regulates endothelial microtubule stability by modulating acetylation of α-tubulin. Shear stress also influences endothelial polarity and alignment by redistributing microtubule network and reorganizing the microtubule organizing centre (Bailey et al. 2017; McCue et al. 2006). Acetylated α-tubulin is one of the characteristic markers of stabilized microtubules. Acetylation of α-tubulin at Lys40 marks stabilized microtubule structures and helps regulate microtubule dynamics (Y.-H. Wang et al. 2010). McCue, et al, reported that shear stress regulates stabilization of microtubules via increase in acetylation when exposed to high shear stress. They exposed porcine aortic endothelial cells to 15 dyne/cm^2 and observed upregulation of acetylation of α-tubulin within the first hour and this increase in acetylation was further sustained at the end of the 24-hour exposure to high shear stress. In our experiments, wherein we exposed endothelial cells to shear stress of 2 or 20 dyne/cm^2, we also observe a time-dependent modulation in the levels of acetylation of α-tubulin. However, our data shows that levels of expression of acetylated α-tubulin in HUVECs were different in high and low shear stress. While under high shear stress, we observed a peak at the initial 2-hour time point, for HUVECs exposed to low shear stress, maximum levels of acetylated α-tubulin were

observed at a 4-hour time point. As we observed that under different shear stress conditions, high and low, the level of acetylation of α-tubulin varies over time. This suggests that upstream regulator of acetylation of α-tubulin may also be regulated by shear stress (D.-Y. Lee and Chiu 2019). One of the suggested upstream regulators of tubulin is GSK-3β (McCue et al. 2006).

McCue, et.al hypothesized that GSK-3β could regulate acetylation of α-tubulin under shear stress conditions. However, as the transient increase in acetylation of α-tubulin did not coincide with the expression levels of GSK-3β, it was inferred that there might be other modulators of α-tubulin acetylation involved. This highlighted another underlying mechanism that could regulate acetylation of α-tubulin: regulation of deacetylase enzymes. One such well known cytosolic deacetylase which interacts with α-tubulin, and targets α-tubulin at Lys40 is HDAC6 (Lemon et al. 2011; Skultetyova et al. 2017; Asthana et al. 2013). Little is known about the regulation of HDAC6 by shear stress, however, it has been reported that shear stress may modulate the activation of the deacetylase activity of HDAC6 via its phosphorylation. When phosphorylated by GSK-3β at Ser22, HDAC6 undergoes a conformational change that increases its interaction with α-tubulin. Therefore, we looked at the levels of the deacetylase activity of HDAC6 in HUVECs exposed to different shear stress conditions. The first set of data, based on the fluorometric assay, revealed that under low shear stress conditions, HDAC6 has higher deacetylase activity. In the next set of experiments, analysis of expression levels of phosphorylated-HDAC6 in high vs low shear stress, gave a similar result to the fluorometric assay. We observed higher expression of phosphorylated-HDAC6 in low shear stress conditions. Taking these two sets of data, we were able to infer that under low shear stress, HDAC6 has a higher deacetylase activity via increased phosphorylation by kinases like GSK-3β. This leads to increased removal of acetyl motif from amino group on Lys40. Together, the data indicates that HUVECs exposed to high shear stress, have lower levels of HDAC6 activity, higher levels of acetylated α-tubulin and a more stable cytoskeletal network, compared to HUVECs exposed to low shear stress, which is similar to what has also been reported by McCue, et al (McCue et al. 2006).

This study initially tested two different pharmacological interventions to increase acetylation of -tubulin. The first being a HDAC6 inhibitor, Tubastatin-A. It is a

compound with an IC50 of 4nM and a high specificity, about 100-1000 fold higher specificity for HDAC6, when compared to other members of the HDAC family (Butler et al. 2010). It inhibits the deacetylase activity of HDAC6 and therefore causes hyperacetylation of α-tubulin (Leyk et al. 2017; Butler et al. 2010). Another strategy that was used to increase acetylation of α-tubulin was inhibiting the activity of Sirtuin2, a NAD+-dependent deacetylase of α-tubulin (Szabó et al. 2017). One of the commonly used inhibitors against Sirtuin2 is Sirtuin2 rearranging ligand or SirReaL2, which has been shown to hyperacetylate α-tubulin (Rumpf et al. 2015; Swyter et al. 2018). The optimal concentration to be used for these pharmacological treatments was optimized by testing several doses (not shown). The optimal concentration to be used for these pharmacological treatments were optimized by testing several concentrations (not shown). Even though the recommended IC50 of Tubastatin-A is 15nM, endothelial cells under shear stress responded to the treatment only at 3μM.

When we analyzed by Western blot the levels of acetylation of α-tubulin under low shear stress conditions, Tubastatin-A was able to successfully increase the levels of acetylated α-tubulin. Surprisingly, SirReal2 had little to no effect on acetylation of α-tubulin, even though different concentrations were tested. Since Tubastatin-A had a potent effect on acetylation of α-tubulin by inhibiting the activity of HDAC6 than SirReaL had by inhibiting Sirtuin2, for further experiments we used Tubastatin-A as a treatment.

Several studies have reported that HDAC6 is essential for selective autophagy (Mazzetti et al. 2020; Seigneurin-Berny et al. 2001; Scherzinger et al. 1997). HDAC6 binds to ubiquitinated substrates via its ubiquitin-binding zinc finger and helps the formation of protein aggregates by trafficking them across the microtubule network (Kawaguchi et al. 2003), especially in the case of neurodegenerative disorders like Parkinson's', Huntington's' or Alzheimer's' disease (Kawaguchi et al. 2003). Recently, the role of HDAC6 was also mentioned in the formation of NLPR3 inflammasomes in immortalized bone marrow derived macrophages (iBMDMs). In this study, they illustrated the role of HDAC6, wherein it facilitates the clearance of large ubiquitinated proteins aggregates in a retrograde manner by dyenin adapters. These protein aggregates, that are too large to be degraded by the proteasomal pathway,

form aggresomes near the microtubule-organizing center (MTOC), to be further degraded by autophagy. HDAC6 supports NLRP3 inflammasome activation by its scaffolding role in a dynein-mediated transport without the need for its deacetylase catalytic activity (Magupalli et al. 2020). HDAC6 has also been implicated in regulating oxidative stress through chaperone-mediated autophagy in neurons (Su et al. 2015). Hyperacetylation of Hsp90 favors chaperon-mediated autophagy, and HDAC6 inhibition leads to increased release of ROS in response to hypoxia-ischemia in neurons suffering from spinal cord injury.

Although several studies have mentioned the essential role of HDAC6 In chaperone-mediated autophagy and aggresome formation, HDAC6 inhibition has proved to boost macroautophagy. Majora, et al, have shown that in the context of Cockayne syndrome, HDAC6 inhibition using SAHA/Vorinostat in fibroblasts, led to increased fusion of autophagosomes and lysosomes (Majora et al. 2018). Similarly, Tubastatin-A treatment in chronic kidney disease led to enhanced autophagy via upregulation of TFEB. TFEB, in conditions of HDAC6 inhibition is hyperacetylated and translocated to the nucleus (Brijmohan et al. 2018).

Overall, the inhibition of HDAC6 remains an elusive subject, as it depends on variables like cell type, targeting cellular pathways and proteins that interact with HDAC6 via its several domains. However, we aimed to elucidate its role in macroautophagy, specifically improved trafficking of autophagic vacuoles along a stable microtubule network, and their fusion with lysosomes for degradation of their contents.

Hence, we assessed the consequences of microtubule stabilization on autophagic flux. First, we observed an overall increase in autophagy in endothelial cells exposed to low shear stress and treated with Tubastatin-A, when compared to control conditions. However, the levels of expression of essential autophagy proteins like ATG5, ATG7, Beclin-1 were not affected by Tubastatin-A or shear stress in our experimental model. When we studied the effect of Tubastatin-A treatment on the fusion step of the autophagy pathway, we observed a higher number of autophagosomes generated per cell in high shear stress conditions as well as their fusion with lysosomes to form autophagolysosomes, when compared to HUVECs exposed to low shear stress conditions. This effect of shear stress on endothelial

autophagic flux that we observed in the present study was in accordance with what was reported by Vion, et.al 2017 (Lee et al. 2010). When HUVECs were treated with Tubastatin-A, we not only observed an increase in the number of autophagosomes per cell, but also a significant increase in the number of autolysosomes. All of the above data put together, suggests that increasing α-tubulin acetylation by HDAC6 inhibition, increases the turnover of the autophagic vacuoles by promoting the fusion of autophagosomes with lysosomes. Interestingly, the total number of vacuoles counted per cell was as high as that in cells exposed to high SS. This shows that under Tubastatin-A conditions, not only the fusion step is enhanced, but we hypothesize that interactions with stable microtubules help accentuate the number of autophagosomes generated at the initial steps of the pathway.

As previously mentioned, several experimental studies have reported the impaired formation of autophagosomes as well as autolysosomes in conditions where the microtubule was disintegrated using drugs like taxol or nocadazol (Geeraert et al. 2010; Köchl et al. 2006). Acetylation at Lys40 causes conformational changes in α-tubulin and stabilizes the microtubule network. This also leads to enhanced interactions with motor proteins like kinesin-1 and dyenin-1, which promote cellular trafficking (Reed et al. 2006; Badding and Dean 2013). Alterations in their interactions, either due to destabilization of microtubules or mutations in the motor proteins, can overall attenuate the trafficking process. Although this process is not well described in endothelial cells exposed to shear stress, Geerart, et. al reported the link between hyperacetylation of α-tubulin and starvation-induced autophagy (Geeraert et al. 2010). Enhanced MAPK/JNK phosphorylation activates KIF1/kinesin family member 1-dependent mechanisms and promotes autophagy. MAPK/JNK signaling increases the dissociation of Beclin-1 from the BCL2 inhibitor and promotes its association with factors such as microtubules required for initiating autophagosome formation (Bánréti, Sass, and Graba 2013). The main protein complexes like Beclin-1, class III PtdIns3K, ATG5-12 complex and LC3-II, move along microtubules as they promote the formation of phagophores and autophagosomes (Geeraert et al. 2010). Mature autophagosomes, via LC3-II -dyenin interaction, are trafficked centripetally to the MTOC to fuse with lysosomes (Bánréti et al 2013). Although not tested in our study, we could perform a similar experiment wherein

destabilization of microtubules, using drugs like vinblastine or nocodazole, would render the effect of Tubastatin-A ineffective on autophagy. This would allow us to demonstrate that indeed stable microtubule network is essential for trafficking of autophagosomes.

In a study aiming to inhibit HDAC6 in neurons and improve cognition in patients with Alzheimers' disease, Tubastatin-A increased acetylation of α-tubulin, and improved axonal transport of mitochondria via kinesins and dynein motor proteins to promote autophagy. Hyperphosphorylated tau protein, a consequence of tau and HDAC6 interaction, destabilizes the microtubule network. Tubastatin-A was also able to inhibit the upstream kinase of HDAC6, GSK-3B. This is due to the regulation of the Akt/GSK-3b/mTOR signaling axis (Ling Zhang et al. 2014).

Zheng, et.al demonstrated the role of Tubastatin-A in promoting autophagic flux via stabilization of microtubules in axonal neurons to recover from spinal cord injury. Their results stated that inhibition of HDAC6 by Tubastatin A caused increased acetylation, thereby stabilizing the microtubule network. The stable microtubule network helps increase the interactions of Syntaxin17 and VAMP8. These proteins are present on the membrane surface of mature autophagosomes and late lysosomes. They form the STX17- SNAP29-VAMP8 trans-SNARE complexes to promote fusion of autophagosomes and lysosomes (Bánréti et al 2013). They also observed that Tubastatin-A induced fusion and autophagic flux was partially blocked by pretreatment with autophagic flux inhibitor, Bafilomycin-A1. It was also stated that trafficking of lysosomes along detyrosinated microtubules is Kinesin-1 dependent (Liu et al. 2019). This interaction promotes intracellular trafficking of lysosomes to fuse with autophagosomes and form autolysosomes. Using Nocadazole treatment or overexpression of HDAC6, they observed lowered levels of fusion of autophagosomes and lysosomes, and accumulation of lysosomes around nucleus due to the attenuated trafficking (Zheng et al. 2020).

Another interesting interaction highlighting the role of HDAC6 in regulation of autophagic flux has been demonstrated by Jiang, et.al in prostate cancer cellsl. They observed that overexpression of p62 attenuates autophagic flux by increased interaction with HDAC6 and reduced acetylation and stability of microtubules,

which causes an impaired trafficking of autophagosomes to fuse with lysosome and a blockade of the flux (Jiang et al. 2018).

B. Inflammation

Atherosclerosis is a chronic inflammatory disease where endothelial dysfunction plays an important role in the initiation step. In order to observe the effect of Tubastatin-A treatment on endothelial inflammation, we analyzed the expression levels of inflammatory markers, like 1-1, VCAM-1 and MCP-1. Western blot analysis of ICAM-1 and VCAM-1, under TNF-a induction, showed that Tubastatin-A decreased the expression of adhesion molecules by about 50% and promoted an anti-inflammatory phenotype in HUVECs exposed to low shear stress. Similarly, by ELISA we were able to estimate the concentration of MCP-1 released by endothelial cells. The cytokine profile of HUVECs treated with Tubastatin-A under low shear stress, was similar to that of HUVECs exposed to high shear stress. Overall, this led to us to infer that Tubastatin-A treatment promotes an anti-inflammatory phenotype in endothelial cells.

To link the restoration of autophagic flux as the link due to which we observe an attenuated inflammatory effect on endothelial cells, we knocked down ATG5 protein in HUVECs by transducing a lentivirus carrying the shRNA. Lack of ATG5 should inhibit initiation of autophagy, and formation of autophagosomes, indicated by reduced LC3II/LC3I ratio. With very few autophagic vacuoles generated, a stable microtubule network shall not have sufficient autophagosomes to traffic in order to fuse with lysosomes and enhance autophagic flux in endothelial cells. HUVECs transduced with shControl, exhibited a similar inflammatory phenotype as reported above. The cells treated with Tubastatin-A under low shear stress showed a reduced inflammatory status compared to the control cells exposed to just low shear stress. However, no significant difference in the expression of adhesion molecules, like ICAM-1 was observed between control and knockdown cells exposed to low shear stress. Although Tubastatin-A was able to rescue the pro-inflammatory phenotype, as mentioned in previous experiments, it was not able to have the same effect on the knockdown cells. There was no difference in the expression of adhesion molecules

or concentration of MCP-1 released by cells, between the knockdown cells treated with vehicle or Tubastatin-A.

Hence, we inferred that even though Tubastatin-A hyperacetylates α-tubulin by inhibition of deacetylase activity of HDAC6, and stabilizes the microtubule network, the lack of endothelial autophagy does not allow Tubastatin-A to render enhanced fusion of the autophagic vacuoles to increase the flux and favor an anti-inflammatory phenotype.

These results were also corroborated with the experiment where we observed that decreased expression of HDAC6 led to an increased lipidation of LC3-I to form LC3-II, a marker of autophagy induction. Consequentially it led to a diminished inflammatory status of endothelial cells, that is reduced expression of ICAM-1 and VCAM-1. Overall, we were able to link the effect of hyperacetylation of α-tubulin with enhanced autophagic flux, that imparts reduced expression of ICAM-1 and cytokine release, which are, in fact the initial responses to endothelial damage in the vessel wall.

Several studies have shown that HDAC6 significantly impacts the production of inflammatory cytokines. Inhibition of HDAC6 has been shown to downregulate the production of IL-6, IL-1β, and TNF-α in murine models of inflammatory disease, such as a a Freund's complete adjuvant-induced mouse model of inflammation, and a collagen antibody-induced arthritis mouse model of synovial inflammation (Ran and Zhou 2019). HDAC6 inhibition has also been reported to prolong survival in murine models of systemic inflammation and injury (Jinyan Yu et al. 2016). In contrast, HDAC6 deficiency has been shown to increase expression of IL-17 in endothelial cells (Yan et al. 2017).
Although these findings have been reported in the context of different disease conditions or cell types, it still allows extrapolating and perceiving a general idea of the effect of HDAC6 inhibition on the production of cytokines.

Another suggested mechanism of action is via the activation of caspase3 (Jinyan Yu et al. 2016). Caspases, including caspase 3 contribute to TNF-α induced endothelial cell dysfunction such as barrier disruption and hyperpermeability (Sawant et al.

2013; Zehendner et al. 2011; Shi et al. 2016). Caspase activation mediates apoptosis and redistribution of junction proteins like ZO-1 and VE-Cadherin (Sawant et al. 2013; Zehendner et al. 2011; Shi et al. 2016). HDAC6 inhibition by HDAC6 inhibitors or siRNA knockdown can reduce caspase 3 activation by promoting protein acetylation (Shi et al. 2016. Microtubule disruption leads to increased apoptotic signaling and caspase-3 activation (Carew et al. 2012; Cheng et al. 2013). The decreased caspase 3 activation by HDAC6 inhibition could be due to its protection against microtubule disassembly by α-tubulin acetylation. Although we did not evaluate whether or not HDAC6 inhibition decreases endothelial apoptosis in low shear stress conditions, inhibition of endothelial caspase 3 would likely contribute to the decreased atheroprone phenotype we observe with Tubastatin-A.

C. Effect of HDAC6 Deletion *In Vivo*

In order to observe the consequences of lack of HDAC6 on atherosclerotic plaque formation, we used a double knockout murine model, *HDAC6*$^{-/-}$*/ApoE*$^{-/-}$. Similar to previous studies that have used the *HDAC6*$^{-/-}$ model we observed that these mice are viable, and have shown no signs of major defects (Yu Zhang et al. 2008; Lemon et al. 2011). These mice underwent bone marrow transplantation to eliminate the effects of *HDAC6* deletion in inflammatory cells that maybe recruited to the site of lesion. The mice were kept on high fat diet to induce atherosclerosis. Post diet, we first verified if the deletion of *HDAC6* caused any adverse effects on the cell count in different sub-populations in the blood, spleen and bone marrow. In order to do so we employed flow cytometry techniques and the Hemavet cell counter, we obtained similar results. Inferring from both these techniques, the global knockout of *HDAC6* did not cause any alterations in the number or activity of different types of inflammatory cells. Hence, moving on we were able to base our inferences on the role of vascular endothelial cells and their functions in response to lack of HDAC6 activity and lowered plaque formation. Next, we analyzed the lesion size by Oil Red-O staining and the total plasma cholesterol levels. Interestingly, we observed a 60% decrease on the lesion size at the aortic arch in the *HDAC6*$^{-/-}$ *ApoE*$^{-/-}$, compared to the control littermates, *HDAC6*$^{+/+}$ *ApoE*$^{-/-}$.

The data from measuring the plasma cholesterol levels suggest that HDAC6 deletion has no effect on cholesterol metabolism or cholesterol efflux. This shows that in both conditions, presence and lack of HDAC6, endothelial cells are exposed to the risk of hypercholesteremia, which could ameliorate the development of atherosclerosis. However, only in the knockout model do we observe an atheroprotective phenotype. This could partially be due to the restoration autophagic flux in endothelial cells exposed to low shear stress, and their ability to maintain lipid homeostasis (Torisu et al. 2016).

The role of HDAC6 has been explored in the context of hypertension (Chen et al. 2019), which is a risk factor for atherogenesis. Leucker, et.al, are one of the few groups to have reported that HDAC6$^{-/-}$ mice showed improved endothelial function, and that inhibition of deacetylase activity may help prevent or reverse the process of plaque formation (Zhao et al. 2020; Leucker et al. 2017). Their study highlights the role of HDAC6 inhibition, either via tubacin or siRNA, in restoring the endothelium-dependent vasorelaxation. HDAC6 inhibition blocks the inhibitory effects of OxLDL on cystathionine γ-lyase and eNOS activity indicating that it may be atheroprotective. Cystathionine γ-lyase is a major source of vasodilator, H_2S in blood vessels and its role has been implicated in hypertension, endothelial dysfunction, and accelerated atherosclerosis (Mani et al. 2013; G. Yang et al. 2008). A similar study was published by Chen, et. al, where they used Tubacin, HDAC6 inhibitor, to stimulate endothelial NO production and improve endothelial function. However, this study focuses on the role of Tubacin in maintaining the stability of the eNOS mRNA rather than inhibiting the expression or activity of HDAC6 (J. Chen et al. 2019). Although Tubacin is a potent small molecule with specificity for HDAC6, the more recent drug, Tubastatin-A, has been reported to show significantly higher specificity against the HDAC isoforms (Butler et al. 2010) Thus, we must take into account the fact that the findings reported using molecules like Tubacin or Trichostatin or SAHA, could also be attributed to the activity of members of the deacetylase family. Also, the structure of Tubacin with characteristics like high lipophilicity and complexity in synthesis makes it a tool better used for basic research than for clinical investigations. The lack of translational quality of this

compound makes it a less preferred tool for studies investigating the effects of HDAC6 inhibition.

To summarize, our findings suggest that in low shear stress conditions, endothelial cells lack a stable microtubule network, as inferred from the lack of acetylated α-tubulin expression in HUVECs exposed to low shear stress. This could be due to the regulation of the deacetylase activity of HDAC6 by shear stress. The lack of a stable microtubule network affects cellular trafficking manifold. A labile microtubule network does not facilitate the long-lasting interactions of cargo with the motor proteins, and subsequently their trafficking is disrupted. A key step in autophagy is the fusion of the autophagosomes to the lysosomes. Lack of a stable microtubule network in endothelial cells exposed to low shear stress, hampers its trafficking to lysosomes, and consequently, the recycling and clearance of cellular products.

Active HDAC6 has proved to be instrumental in identifying and transporting ubiquitinated proteins to be degraded by the proteosomal pathway by several studies. However, this study presents the benefits of inhibition of HDAC6 deacetylase activity specifically. Inhibition of deacetylase activity allows increased acetylation of α-tubulin, thereby facilitating better trafficking and increased autophagic flux. Also, the cytoskeleton is important in mechanotransmission of the signals received from physical stimuli. Different conformation of the cytoskeletal network can relay the signals in different manner, leading to difference in stimulation of signaling pathways and gene expression. These put together implies the essential role the microtubule network plays in signal transduction and transmission along with trafficking.

High shear stress favors an anti-inflammatory phenotype by promoting autophagy via a network of several signaling cascades. Low shear stress, on the contrary promotes a pro-inflammatory phenotype by down regulating autophagy. HDAC6 inhibition by Tubastatin-A is able to overcome the obstacles of defective autophagic flux and restore it in endothelial cells that would otherwise lack autophagy. Restoration of autophagy helps rescue the cells from a pro-inflammatory status and partially restores endothelial function (Figure 35). These findings from the *in vitro*

experiments were substantiated in the *in vivo* experiments, wherein we observed a significant reduction in plaque size in *HDAC6* knockout model compared to the control mice.

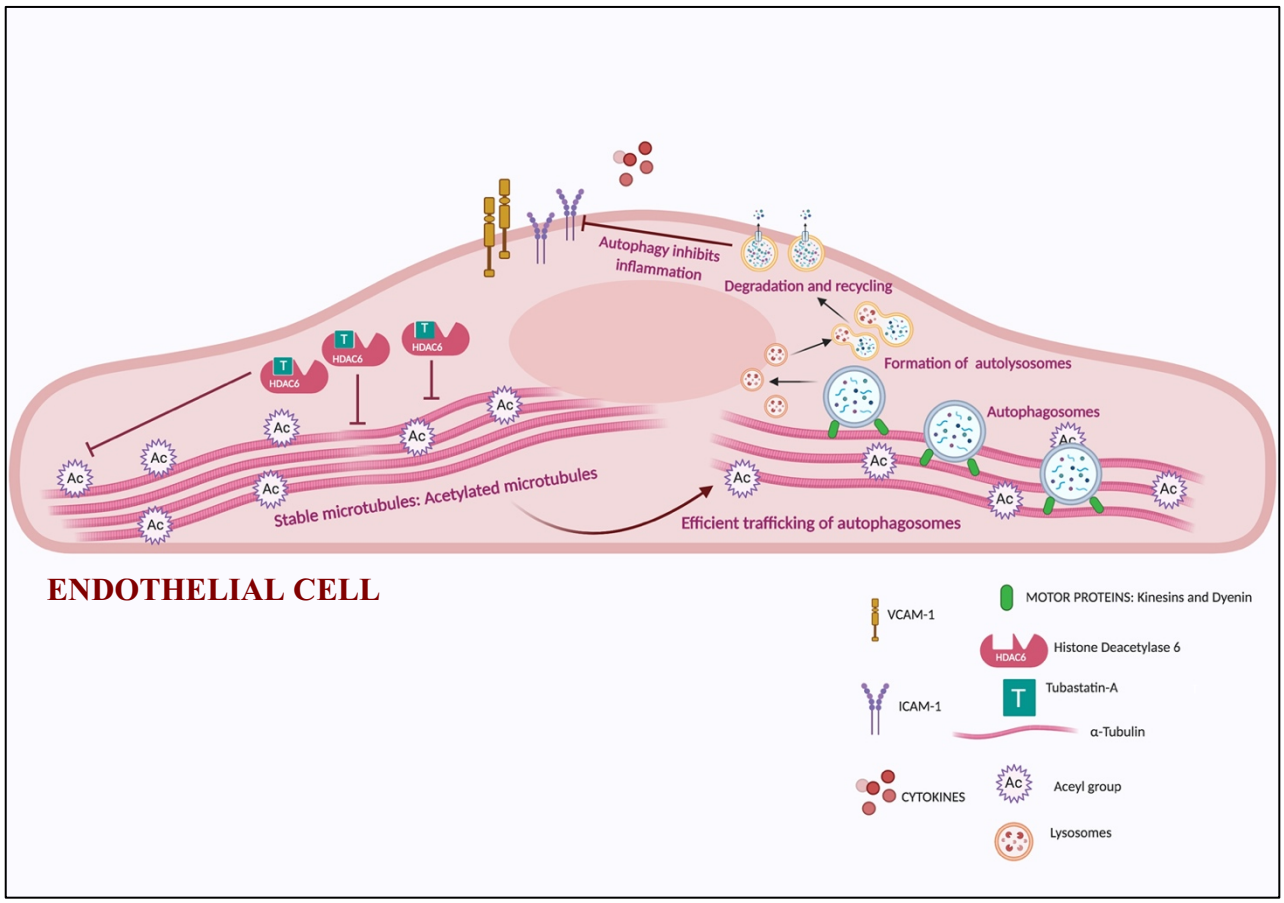


Figure 36: Schematic representation of the mechanism regulating microtubule stabilization, autophagic flux and inflammation.

This study demonstrates that inhibition of HDAC6 by Tubastatin-A increases acetylation of α-tubulin. This indicates stabilization of microtubule network, which promotes fusion of autophagosomes and autolysosomes by improved trafficking of autophagsosomes. Stabilized microtubules help maintain interactions with the motor proteins that carry the cargo across the cytosol. Formation of autolysosomes and further degradation of cellular waste helps maintain endothelial function. Through various signaling pathways and transcription factors, autophagy helps promote an anti-inflammatory response of endothelial cells, by attenuating the expression of adhesion molecules like ICAM-1, VCAM-1 and cytokine release.

LIMITATIONS AND PERSPECTIVES

We have yet to identify the possible candidates for upstream regulators which regulate HDAC6 deacetylase activity under different shear stress conditions, like GSK-3β or atypical protein kinase C, and establish their role in endothelial autophagy by HDAC6 modulation. We have not demonstrated the effect of Tubastatin-A on the interactions between endothelial cells and leukocytes, a key step in atherogenesis. This could be illustrated by analyzing monocyte adhesion on endothelial cells.

One of the consequences of an increase in autophagic flux is the anti-inflammatory phenotype of endothelial cells, which could help prevent atherosclerotic lesion formation in areas predisposed to lesion development. However, we have not yet elucidated the different molecular mechanisms that could play a role in linking shear stress, autophagy and inflammation mediated by altered HDAC6 activity.

The main limitation of our study is the murine model that we used. Being a global knockout, we are not able to entirely overlook the consequences of the deletion of HDAC6 in other cell types, especially in the vasculature like the smooth muscle cells. Though we have replaced the bone marrow cells of all mice with wild-type bone marrow, in order to rule out the role of immune cells in the development and progression of the plaque. For future experiments, we could develop and work with a model carrying an endothelial-specific knockout.
Some of the *in vivo* experiments that could shed a light on the plausible pathways are establishing the effect of HDAC6 deletion on endothelial autophagy by *en face* staining of the aorta for LC3-II punctae, as well as determination of plaque size at the aortic sinus. We would also be assessing the expression of the different inflammatory receptors and cytokines released by the endothelial cells that could substantiate the data regarding the plaque size by linking it to restoration of endothelial autophagic flux in the HDAC6 KO model.

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SUMMARY

RESTAURATION DU FLUX AUTOPHAGIQUE DANS LES CELLULES ENDOTHÉLIALES VASCULAIRES POUR PRÉVENIR LE DÉVELOPPEMENT DE PLAQUES ATHÉROSCLÉROSE

SUMMARY - FRENCH

Les lésions athéroscléreuses se développent préférentiellement dans les bifurcations et les courbures artérielles, où les cellules endothéliales sont exposées à un flux sanguin turbulent, et par conséquent à de faibles contraintes de cisaillement. Dans ces conditions, les cellules endothéliales expriment un phénotype pro-inflammatoire et libèrent des cytokines. La fonction des cellules endothéliales est principalement régulée par des facteurs de transcription induits par la contrainte de cisaillement, et un défaut dans leur activité pourrait faciliter la pathogenèse de l'athérosclérose. Ces facteurs modulés par des stimuli mécaniques sont définis comme des éléments sensibles à la contrainte de cisaillement (SSRE) et ils subissent différentes adaptations en réponse à la contrainte de cisaillement exercée. De plus, ces cellules présentent également un phénotype sénescent et apoptotique. Notre équipe a précédemment démontré qu'une activité autophagique défectueuse pouvait être le lien entre de faibles contraintes de cisaillement et une inflammation endothéliale accrue, car elles favorisent un phénotype athéroprone. L'autophagie est un mécanisme catabolique cellulaire responsable du recyclage des organites et des protéines endommagés. Il existe une large gamme de substrats qui peuvent être dégradés par autophagie, des petites protéines aux organites à double membrane, comme les mitochondries. Initialement, le concept d'autophagie a été introduit comme un processus `` d'auto-alimentation '' pour dégrader les protéines mal repliées et les organites endommagés, mais il a ensuite été déduit comme une réponse adaptative au stress cellulaire, comme l'épuisement des nutriments ou des facteurs de croissance, l'hypoxie ou l'infection. . En cas de stress, la cellule se désintègre et recycle des composants non essentiels pour fournir des nutriments et de l'énergie. Au fil des ans, la recherche a impliqué l'autophagie dans la physiopathologie de plusieurs maladies telles que les maladies neurodégénératives, les maladies auto-immunes, le cancer et les troubles métaboliques. Il a été montré qu'un manque de certaines

protéines clés de l'autophagie, comme l'ATG5, conduit au développement de la plaque en raison d'une inflammation et d'une sénescence accrues, dans des zones de l'aorte normalement protégées. La fusion des autophagosomes et des lysosomes est une étape critique de l'autophagie, qui est responsable du recyclage et de la dégradation efficaces des déchets cellulaires. Le manque de fusion conduit à l'accumulation d'autophagosomes transportant les sous-produits cellulaires en tant que cargaison. Diverses études ont démontré qu'un microtubule stable est essentiel pour une fusion efficace des autophagosomes et des lysosomes, pour former des autolysosomes. La stabilisation des microtubules, en particulier de l'α-tubuline, est régulée par plusieurs modifications post-traductionnelles. Une de ces modifications post-traductionnelles clés est l'acétylation de l' α-tubuline. L'acétylation de l'α-tubuline à Lys40 marque les structures de microtubules stabilisées et aide à réguler la dynamique des microtubules. Cette étude teste l'hypothèse selon laquelle l'inhibition de la désacétylase endothéliale HDAC6 stabilise l'α-tubuline et favorise ainsi la fusion efficace des autophagosomes et des lysosomes, pour augmenter le flux d'autophagie endothéliale dans des conditions de faible contrainte de cisaillement. En conséquence, cela pourrait conduire à un phénotype athéroprotecteur malgré des conditions de contrainte de cisaillement défavorables. Pour restaurer le flux autophagique et prévenir la formation de lésions athéroscléreuses, cette étude est divisée en trois objectifs principaux: 1) Promouvoir le flux autophagique en améliorant la stabilisation des microtubules en augmentant l'acétylation de l'α-tubuline via l'inhibition de l'activité enzymatique de l'HDAC6 à l'aide de traitements pharmacologiques (Tubastatine-A, SirReal2) et shRNA ciblant HDAC6. 2) Etudier l'impact de l'induction de l'autophagie sur l'inflammation endothéliale. 3) Évaluer les effets du knock-out HDAC6 in vivo sur le développement de l'athérosclérose et la progression dans un modèle murin à double knockout HDAC6 - / - ApoE - / -Notre étude teste l'hypothèse selon laquelle l'inhibition de la désacétylase endothéliale HDAC6 stabilise l'α-tubuline et favorise ainsi la fusion efficace des autophagosomes et des lysosomes, pour augmenter le flux autophagique endothéliale dans des conditions de faibles contraintes de cisaillement. En conséquence, cela pourrait conduire à un phénotype athéroprotecteur,s malgré des conditions de contraintes de cisaillement défavorables. Nous avons d'abord évalué si les contraintes de cisaillement régulaient l'acétylation de l'α-tubuline et observé que des contraintes de

cisaillement élevées augmentaient l'acétylation de l'α-tubuline, par rapport à des faibles contraintes de cisaillement. Nous avons ensuite confirmé cela en estimant l'activité de désacétylase de l'HDAC6, et observé que sous de fortes contraintes de cisaillement, l'HDAC6 a une activité de désacétylase inférieure par rapport aux conditions de contraintes de cisaillement faibles, en raison de moins d'activation par les kinases respectives. Nous avons d'abord inhibé l'activité de la désacétylase endothéliale dans les HUVEC exposées à des conditions de faibles contraintes de cisaillement en utilisant un inhibiteur de l'HDAC6, la Tubastatin-A, et un inhibiteur de la Sirtuin 2, SirReal2. Alors que le traitement à la Tubastatin-A a entraîné une augmentation des niveaux d'acétylation de l'α-tubuline, SirReal2 n'a pas modifié l'acétylation de l'α-tubuline. La Tubastatin-A a conduit à une augmentation des niveaux d' α-tubuline acétylée d'environ 50 fois et a considérablement amélioré l'expression de la protéine LC3-II, par rapport à LC3-I, et le nombre de structures positives à la LC3-II par immunofluorescence. En utilisant un une protéine de fusion LC3, couplé à une RFP et à une GFP, nous avons pu surveiller le flux d'autophagique endothélial. Nous avons observé que de fortes contraintes de cisaillement favorisaient le flux autophagique dans les cellules endothéliales, par rapport aux cellules exposées à de faibles contraintes de cisaillement. Cela a été indiqué par le nombre plus élevé d'autolysosomes formés dans des conditions de contraintes de cisaillement élevées. L'absence de fusion des autophagosomes et des autolysosomes dans des conditions de faibles contraintes de cisaillement a été inversée en utilisant une molécule connue stimulant l'autophagie, la rapamycine. Enfin, nous avons observé qu'en présence de Tubastatin-A, les cellules endothéliales présentaient un flux autophagique amélioré par rapport aux conditions de contrôle. Cependant, cet effet de la Tubastatin-A a été altéré lorsque nous avons utilisé la chloroquine, un inhibiteur de flux autophagique. L'absence de fusion en présence de chloroquine, suggère que la Tubastatin-A favorise en fait la fusion et la formation d'autolysosomes en stabilisant le réseau de microtubules. Suite à ces résultats, nous avons évalué l'effet de la Tubastatin-A sur l'inflammation endothéliale. Nous avons analysé l'expression de marqueurs inflammatoires endothéliaux comme ICAM-1 et VCAM-1. Ce sont des molécules d'adhésion qui aident au recrutement des leucocytes et à leur transmigration à travers la barrière endothéliale. Nous avons également évalué les niveaux de cytokine, MCP-1, libérés dans le surnageant. Le MCP-1 est une protéine

chimioattractive qui facilite le recrutement des monocytes sur le site de la lésion ou de la blessure. Nous avons observé que dans des conditions de contraintes de cisaillement élevées, l'expression d'ICAM-1 et de VCAM-1 par les cellules endothéliales, était significativement plus faible par rapport à de faibles contraintes de cisaillement, ce qui est conforme à ce qui a été rapporté précédemment par notre équipe. Cependant, dans les cellules endothéliales traitées à la Tubastatin-A et exposées à de faibles contraintes de cisaillement en présence de TNF-α, l'expression des marqueurs inflammatoires, ainsi que la quantité de MCP-1 libérée dans le milieu conditionné, a été significativement réduite. Par conséquent, nous avons pu déduire que la Tubastatin-A est capable de favoriser le flux autophagique et de réduire l'inflammation, imitant le phénotype des cellules endothéliales exposées à de fortes contraintes de cisaillement. Afin de relier nos résultats au flux autophagique et à l'inflammation endothéliale, nous avons inhibé le processus autophagique en bloquant l'expression de la protéine ATG5, par l'utilisation d'un shRNA. Le manque d'ATG5 inhiberait l'initiation de l'autophagie. La réduction du nombre d'autophagosomes annulerait ainsi l'effet de la Tubastatin-A sur l'étape de fusion. Nous avons observé qu'en infectant des HUVEC avec des lentivirus portant le shRNA ciblant l'ATG5, nous avons pu réduit l'expression de la protéine de 50%. Cela a également conduit à une autophagie réduite dans les cellules endothéliales. Alors que dans des conditions contrôle, le traitement à la Tubastatin-A a conduit à un phénotype anti-inflammatoire, il n'a pas pu réduire l'inflammation dans les conditions déficientes en ATG5. Cela confirme que la Tubastatin-A favorise un phénotype anti-inflammatoire en modulant l'autophagie dans les cellules endothéliales exposées à des conditions de faibles contraintes de cisaillement. Nous avons ensuite confirmé nos données en renversant l'expression de HDAC6 dans les HUVEC à l'aide d'un shRNA, ce qui a entraîné une réduction d'environ 30% de l'expression de la protéine. Le manque d'HDAC6 a conduit à une acétylation accrue de l'α-tubuline, à la conversion de LC3-I en LC3-II et à une expression réduite des marqueurs inflammatoires, ICAM-1 et MCP-1. Dans l'ensemble, nos résultats *in vitro* suggèrent que dans des conditions de faibles contraintes de cisaillement, une altération du flux autophagique peut être inversée en facilitant la fusion des autophagosomes et des lysosomes via un réseau de microtubules stabilisés. Cela entraîne également une réduction de l'inflammation des cellules endothéliales. Suite à nos expériences in

vitro, nous avons évalué les effets de la délétion de HDAC6 in vivo. Nous avons généré un modèle murin à double knockout pour notre étude, *HDAC6*$^{-/-}$*/ApoE*$^{-/-}$ et l'avons comparé au groupe témoin composé de compagnons de portée avec le génotype, *HDAC6*$^{+/+}$*/ApoE*$^{-/-}$. Nous voulions évaluer l'effet du manque d'HDAC6 sur le développement de la plaque d'athérosclérose. Nous avons également réalisé une greffe de moelle osseuse chez les souris afin que toutes les cellules immunitaires portent le gène HDAC6. Cela nous permettrait d'éliminer les effets de la délétion de HDAC6 dans les cellules immunitaires, sur le développement de la plaque d'athérosclérose. Dans un premier temps, nous avons évalué les taux de cholestérol plasmatique des souris dans les deux groupes suivant un régime riche en graisses pendant 10 semaines. Bien que les taux de cholestérol plasmatique aient augmenté de manière significative après le régime pour les souris des deux groupes, nous n'avons observé aucune différence lors de la comparaison des taux de cholestérol entre les deux groupes de souris. Nous n'avons pas non plus observé de différences dans les différentes populations de cellules dans le sang, la rate et la moelle osseuse. Nous n'avons pas observé de changements drastiques dans le développement de l'athérosclérose dans l'aorte descendante (ou la région de forte contrainte de cisaillement) des souris, entre les deux groupes. Cependant, nous avons observé une différence significative dans la formation de plaque d'athérosclérose dans l'arcade (région de faibles contraintes de cisaillement), qui était réduite d'environ 40 à 50% chez les souris chimériques *HDAC6*$^{-/-}$*/ApoE*$^{-/-}$. Pour résumer, nos résultats suggèrent que dans des conditions de faible contrainte de cisaillement, les cellules endothéliales n'ont pas de réseau de microtubules stable, comme cela est déduit du manque d'expression d'α-tubuline acétylée dans les HUVEC exposées à une faible contrainte de cisaillement. Cela pourrait être dû à la régulation de l'activité désacétylase de HDAC6 par contrainte de cisaillement. L'absence d'un réseau de microtubules stable affecte le collecteur de trafic cellulaire. Un réseau de microtubules labiles ne facilite pas les interactions durables de la cargaison avec les protéines motrices, et par la suite leur trafic est perturbé. Une étape clé de l'autophagie est la fusion des autophagosomes avec les lysosomes. L'absence d'un réseau de microtubules stable dans les cellules endothéliales exposées à une faible contrainte de cisaillement, entrave son trafic vers les lysosomes et, par conséquent, le recyclage et l'élimination des produits cellulaires. L'inhibition de l'HDAC6 par la tubastatine-A est capable de

surmonter les obstacles d'un flux autophagique défectueux et de le restaurer dans les cellules endothéliales qui autrement manqueraient d'autophagie. La restauration de l'autophagie aide à sauver les cellules d'un statut pro-inflammatoire et restaure partiellement la fonction endothéliale Pris dans leur ensemble, ces résultats indiquent que l'inhibition de l'HDAC6 peut être une stratégie intéressante pour restaurer le flux autophagique endothélial en stabilisant les microtubules et pour favoriser un phénotype endothélial athéroprotecteur malgré un cisaillement défavorable conditions de stress.

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EXPERIENCE

08/2017 – Present: PhD Project, Pathophysiology, speciality Cardiovascular.
INSERM : Paris Centre de Recherch Cardiovasculair

- Project Management for primary project: ***'Restoration of Autophagy Flux in Vascular Endothelial Cells to Prevent Atherosclerotic Plaque Formation'***
- Secondary project: ***'Role of Autophagy During Endothelial Extracellular Vesicles Biogenesis'***
- **Co-supervision of students** : Emilie Bacquard, Brevet de Technicien Supérieur
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  Loic Sibeuwou, Pharmacy Student
  Internship for Master 1: June-August 2019

  Dalia Ghoul, Biological Science
  Internship for Master 2: January-June 2020
- Apprentis Chercheur: Teach and supervise 3 high school students over a period of 6 months, as they work on a project employing basic laboratory techniques.

04/2016- 07/2016: Masters' Thesis, University of Leeds, Leeds, UK

- ***Selection and characterisation of Adhirons targeting the ErbB family and FGFR3 for cancer diagnostics*** **(April'16-July'16).**

12/2014- 05/2015: Bachelor Thesis

- ***Characterization of fibrinolytic protease from marine Streptomyces rubiginosus VITPSSM.***
- **Genbank entry** for characterising marine actinomycetes- *Streptomyces rubiginosus* VITPSSM, 16S ribosomal RNA gene, partial sequence, 735 bp, linear genomic, KR233809.

2013-2014: Internships

- Trainee at Jawaharlal Nehru Cancer Hospital and Research Centre: Experienced in animal handling, cytogenetics and clinical microbiology.

EDUCATION

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Pathophysiology, speciality Cardiovascular

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Coursework: Institute Pasteur, Paris
Animal Training (Rodents and Lagomorphs)- Regulatory Course for Project Designers

08/2015 – 10/2016 : **Master of Science**
Infection, Immunity and Human Diseases
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Awarded the **GREAT Leeds-India scholarship**

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07/2011 – 06/2015 : Bachelor of Technology
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SKILLS AND COMPETENCY

- **Wet Lab Techniques** : Cell culture (bacterial and mammalian), viral transduction, transfection, immunofluorescence and histology on cardiac tissue, Confocal microscopy, Flow Cytometry, PCR, protein extraction and purification, design and produce recombinant plasmids, isolation of DNA and RNA.

- **Animal Experimentation**: Animal model development, Animal handling, intra-peritoneal, retro-orbital and sub-mandibular injections, isolation and dissection of organs.

- Oral Presentation skills

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CONFERENCES

2017 : Club Francophone de l'Autophagie (CFATG), Paris

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Restoration Of Endothelial Autophagic Flux In Vascular Endothelial Cells Exposed To Low Shear Stress

Shruti Chatterjee, Marouane Kheloufi, Pierre-Michaël Coly, Chantal M. Boulanger
(Poster presentation ; First author)

- **Building the Cell, Paris (26-29 September)**

(Poster presentation; First author)

Restoration Of Endothelial Autophagic Flux In Vascular Endothelial Cells Exposed To Low Shear Stress

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Restoration Of Endothelial Autophagic Flux In Vascular Endothelial Cells Exposed To Low Shear Stress

Shruti Chatterjee, Marouane Kheloufi, Pierre-Michaël Coly, Chantal M. Boulanger
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PUBLICATIONS

- Comparative study of death rate kinetics by sterilization reaction on B. subtilis and E.coli (PUBLISHED : Journal of Chemical and Pharmaceutical Research, 2014, 6(5):1095-1100)
- Design of a Novel genetically Engineered Vaccine against *Mycobacterium leprae* (PUBLISHED : Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry, 2014).

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- Resourceful, reliable and responsible
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- Analytical
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