IDENTIFICATION OF POTENTIAL ATHEROGENIC miRNA MARKERS IN EXOSOMES FORMED IN *IN VITRO* VASCULAR SMOOTH MUSCLE CELL AND ENDOTHELIAL CELL CULTURE MODELS

by

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Koç University

Graduate School of Health Sciences

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ABSTRACT

Identification of Potential Atherogenic miRNA Markers in Exosomes Formed in an *in vitro* Vascular Smooth Muscle Cell and Endothelial Cell Culture Models

Fatıma Hacer Kurtoğlu Babayev

Master of Science in Cellular and Molecular Medicine

August 4, 2023

Approximately 20% of all deaths worldwide may be attributed to cardiovascular disease, and atherosclerosis is the leading cause of these deaths. Atherosclerosis is an inflammatory disease that leads to lipid accumulation in the blood vessel, and molecular changes in the endothelial and vascular smooth muscle cells, leading to fatty streak formation and vessel narrowing by the plaque growth. In atherosclerosis, the lesion develops slowly in the vessel wall and its rupture may cause a sudden heart attack or stroke. Early diagnosis is essential to prevent these complications. Intercellular communication is important in the early stages of cardiovascular diseases, and exosomes play a crucial role in this type of communication. Exosomes are vesicles produced by cells that carry proteins and genetic material; mRNA, small RNA (miRNA and lncRNA), protein, and DNA, from the donor cell to the destination cells, where they have been reported to have diverse effects. Exosome-mediated cell-to-cell communication has been shown to influence the development of atherosclerosis; therefore, it is essential to investigate the mechanism of exosome-mediated intercellular crosstalk in this disease, which may have diagnostic and therapeutic applications. This thesis aims to characterize exosomes formed by the interacting of vascular smooth muscle cells (VSMC) and endothelial cells (EC) in different co-culture models. Novel miRNAs in exosomes formed in such models hold promise as new biomarkers for the early detection of atherosclerosis. Our study utilized three culture models: a direct co-culture model, an indirect culture, and an isolated exosome treatment model. We characterized the exosomes using nanoparticle tracking analysis (NTA) and Western blot experiments. Then we isolated miRNA, from these exosomes and sequenced their RNA to identify potential miRNA markers. The results pointed to hsa-miR-335-5p as a potential candidate. We plan further research to explore the functional role of hsa-miR-335-5p in atherosclerosis.

Keywords: Atherosclerosis, exosome, miRNA, vascular smooth muscle cell (VSMC), endothelial cell (EC).

ÖZETÇE

in vitro Vasküler Düz Kas Hücresi ve Endotel Hücre Kültür Modellerinde Oluşturulan Eksozomlarda Potansiyel Aterojenik miRNA Belirteçlerinin Tanımlanması

Fatıma Hacer Kurtoğlu Babayev

Yüksek Lisans, Hücresel ve Moleküler Tıp

4 Ağustos, 2023

Tüm dünyadaki ölümlerin yaklaşık %20'si kardiyovasküler hastalıklara atfedilebilir ve ateroskleroz bu ölümlerin önde gelen nedenidir. Ateroskleroz, kan damarında lipid birikimine, endotel ve damar düz kas hücrelerinde moleküler değişikliklere yol açan, yağlı çizgi oluşumuna ve plak büyümesi ile damar daralmasına neden olan enflamatuar bir hastalıktır. Aterosklerozda lezyon damar duvarında yavaşça gelişir ve yırtılması ani bir kalp krizi veya felce neden olabilir. Bu komplikasyonları önlemek için erken tanı şarttır. Hücreler arası iletişim kardiyovasküler hastalıkların erken evrelerinde önemlidir ve eksozomlar bu tür iletişimde çok önemli bir rol oynar. Eksozomlar, proteinleri ve genetik materyali; mRNA, küçük RNA (miRNA ve lncRNA), protein ve DNA'yı donör hücreden hedef hücrelere taşıyan hücreler tarafından üretilen ve çeşitli etkilere sahip oldukları bildirilen veziküllerdir. Eksozom aracılı hücreden hücreye iletişimin ateroskleroz gelişimini etkilediği gösterilmiştir; bu nedenle, bu hastalıkta tanı ve tedavi uygulamalarına sahip olabilecek eksozom aracılı hücreler arası çapraz iletişim mekanizmasının araştırılması önemlidir. Bu tez, vasküler düz kas hücreleri (VSMC) ve endotel hücrelerinin (EC) farklı ko-kültür modellerinde etkileşime girmesiyle oluşan eksozomları karakterize etmeyi amaçlamaktadır. Bu tür modellerde oluşan eksozomlardaki yeni miRNA'lar, aterosklerozun erken teşhisi için yeni biyobelirteçler olarak umut vaat etmektedir. Çalışmamızda üç kültür modeli kullanılmıştır: doğrudan bir birlikte kültür modeli, dolaylı bir kültür ve izole bir ekzozom tedavi modeli. Eksozomları nanopartikül izleme analizi (NTA) ve Western blot deneyleri kullanarak karakterize ettik. Daha sonra bu eksozomlardan miRNA izole ettik ve potansiyel miRNA belirteçlerini tanımlamak için RNA'larını sıraladık. Sonuçlar potansiyel bir aday olarak hsa-miR-335-5p'ye işaret etti. Aterosklerozda hsa-miR-335-5p'nin işlevsel rolünü keşfetmek için daha fazla araştırma planlıyoruz.

Anahtar kelimeler: Ateroskleroz, eksozom, miRNA, vasküler düz kas hücresi (VDKH), endotel hücre (EH).

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Identification of Potential Atherogenic miRNA Markers in Exosomes formed in an *in vitro* Vascular Smooth Muscle Cell and Endothelial Cell Co-culture Model

1. INTRODUCTION:

1.1 What is Atherosclerosis?

Cardiovascular disease, of which atherosclerosis is the primary contributor, accounts for approximately 20% of global deaths. This chronic inflammatory disease leads to the accumulation of lipids on blood vessel walls, resulting in thickening and obstruction. Atherosclerosis progresses slowly within the vessel wall, potentially culminating in sudden heart attacks or strokes. [1] Early diagnosis is crucial in preventing such complications, as the lesions within the vessels are reversible [2] but challenging to detect using conventional imaging methods. Therefore, identifying novel markers capable of indicating early disease development is imperative. Intercellular communication is crucial in the early stages of cardiovascular diseases, with exosomes serving as essential mediators of this communication.

Endothelial cells (ECs) form the inner layer of the vascular lumen and exist in all blood vessels. [3] Tunica intima is the middle layer of the blood vessels and consists of smooth muscle cells (SMCs). [4] Smooth muscle cells generate multiple layers with elastic lamellae. [5] Arterioles contain fewer smooth muscle layers, and pericytes (PCs) rather than SMCs cover the capillaries. Veins are more elastic and thinner compared to arteries. Tunica adventitia is the outer layer that involves fibroblast, progenitor cells and extracellular matrix (ECM). It is found in arteries and veins. (**Figure 1**)

Blood vessels form in a complex structure that can meet the needs of their surrounding tissues. Cardiovascular disorders may result from flaws in the vasculature's fundamental design. [6] Arterioles branch off from arteries, which are the vessels that deliver blood from the heart to the pulmonary or systemic circulation. Capillaries, help tissues exchange nutrients, waste products, carbon dioxide, and oxygen. Blood is drawn from capillaries by venules, which then carry it to veins and deliver it to the heart. [7]

Gene,protein expression and cellular signaling must be rigorously managed for the development and maintenance of the blood vessel wall. These processes carefully drive vascular cell quiescence, proliferation, apoptosis, differentiation, and extracellular matrix (ECM) synthesis. All these steps are fundamental to vascular homeostasis and morphogenesis. [8-10]

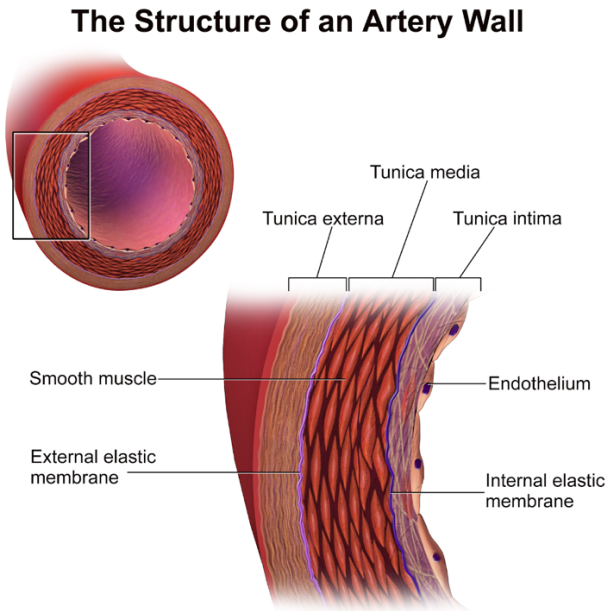

***Figure 1**: (Adapted from Wikipedia)*

The Ecs comprising the tunica intima's monolayer lining the entire vasculature are pivotal to these processes. (**Figure 1**) These cells are identified through markers such as vascular endothelial growth factor receptors, platelet endothelial cell adhesion molecule 1, and vascular endothelial-cadherin biomolecules. Ecs have crucial roles in forming a tight barrier between the blood and the vascular tissue, regulation of blood flow, synthesis/degradation of ECM components, and inflammation/coagulation to barrier function. Other crucial roles of Ecs in barrier are the regulation of paracellular permeability, control of macromolecules transport, prevention of cell leakage of blood components into the surrounding tissue and transcellular transport. [3,11] Given their vast functional repertoire, ECs' normal functioning is vital for normal vascular development and homeostasis. EC dysfunction have been related to various pathologies, such as atherosclerosis, hypertension, inflammatory syndromes, cancer, and stroke. [12-15]

1.1.1 Smooth Muscle Cells

The main cell type of the tunica medium are smooth muscle cells (SMCs). They modulate vascular tone and blood flow through cell contraction and relaxation. [16] Contractile and cytoskeletal proteins are the ones responsible for these modifications. The α-smooth muscle actin (SMA) emerges as the first SMC marker, ultimately becoming the most abundant protein in SMCs during embryogenesis. SM22α (transgelin) is also expressed in early-developing SMCs, and which stabilizes actin filaments. Smooth muscle myosin heavy chain and smoothelin are specific to mature SMCs while SMA and SM22α are expressed in several cell types. [4,16,17]

Cells undergo radial patterning sequentially, layer-by-layer from the inside outward in arteries with several smooth muscle layers with regard to shape and marker expression. [18] Transforming growth factor (TGF) β is also very important for SMC differentiation. [19] When co-cultured with ECs, undifferentiated embryonic mesenchymal cells show TGF-dependent extension and SMC marker expression. SMCs that have reached maturity produce less ECM and migrate less. Adult SMCs can dynamically change their phenotype in response to injury or disease, altering shape, gene expression, and proliferation and migration rates. [20,21]

Smooth muscle cell phenotypic plasticity refers to the ability of smooth muscle cells (SMCs) to change their phenotype in response to various environmental cues or stimuli. [22] This plasticity plays a crucial role in the development and progression of atherosclerosis, a chronic inflammatory disease characterized by the accumulation of lipid-rich plaques in the arterial walls.[22-25] Several genes have been implicated in regulating smooth muscle cell phenotypic plasticity and its role in atherosclerosis. The following is a list of the most prominent genes that define the characteristics of SMCs:

ACTA2 (Alpha Smooth Muscle Actin): ACTA2 is a gene that codes for alpha smooth muscle actin, a contractile protein found predominantly in smooth muscle cells. It is involved in the regulation of smooth muscle cell contraction and cytoskeletal organization. In atherosclerosis, the expression of ACTA2 is often downregulated in SMCs undergoing phenotypic switching, leading to a loss of contractile phenotype and acquisition of a synthetic phenotype associated with increased proliferation and migration. [26]

SM22alpha (Transgelin): SM22alpha is a gene that encodes a cytoskeletal protein called transgelin. It is involved in regulating actin dynamics and cell motility. SM22alpha expression is typically high in contractile smooth muscle cells and low in synthetic SMCs. Decreased expression of SM22alpha has been observed in SMCs within atherosclerotic plaques, indicating a shift towards a synthetic phenotype. [27,28]

MYH11 (Myosin Heavy Chain 11): MYH11 is a gene that codes 13iRbase smooth muscle-specific isoform of myosin heavy chain, a motor protein essential for muscle contraction. It is predominantly expressed in contractile SMCs. In atherosclerosis, the downregulation of MYH11 is associated with a loss of contractile phenotype and increased SMC proliferation and migration. [29]

KLF4 (Krüppel-Like Factor 4): KLF4 is a transcription factor that regulates various cellular processes, including cell differentiation, proliferation, and inflammation. In smooth muscle cells, KLF4 acts as a modulator of phenotypic switching. It can suppress the expression of contractile genes and promote the expression of synthetic genes, contributing to the phenotypic transition of SMCs in atherosclerosis. [25,30]

OPN (Osteopontin): OPN is a matricellular protein that is involved in multiple biological processes, including cell adhesion, migration, and tissue remodeling. OPN expression is upregulated in atherosclerotic lesions and has been implicated in promoting SMC phenotypic switching towards a synthetic phenotype. It may trigger SMC migration and growth, which could help atherosclerotic plaques form and advance. Overall, the

dysregulation of these genes and the resulting phenotypic plasticity of smooth muscle cells contribute to the pathological changes observed in atherosclerosis, such as plaque formation, vascular remodeling, and inflammation. [4,31,32]

1.2 Pathogenesis of Atherosclerosis

The pathogenesis of atherosclerosis involves a series of interconnected events that contribute to the disease's development and progression. Central to this process is the dysfunction of endothelial cells within the arterial walls, which plays a crucial role in atherosclerotic cardiovascular disease (ACVD). [33]

Endothelial cell dysfunction (ECD) is the initial finding that is observable in the vessel wall leading to the creation of atherosclerosis. [34] Due to increased permeability of the endothelium, lipoprotein particles can accumulate, change, and become trapped in the subendothelial region. After being drawn to the intima, circulating monocytes change into macrophages, internalize changed lipoproteins, and transform into foam cells, which are a defining hallmark of early fatty streak lesions. Additionally, the activated endothelium and macrophages release chemokines and growth factors (and exosomes) that stimulate the proliferation of neighboring smooth muscle cells (SMCs) and the synthesis of extracellular matrix components, leading to the development of a fibromuscular (muscle and fibrous tissues) plaque. [33-35] Vascular smooth muscle cells (VSMCs) undergo phenotypic changes during atherosclerosis progression. VSMC loses its contractile properties and gains a proliferative phenotype due to the transition from contractile to synthetic phenotype.

The initiation of atherosclerosis involves the infiltration of small dense low-density lipoprotein (sd-LDL) into endothelial cells lining the blood vessels. This triggers an immune response to restore homeostasis in the face of sd-LDL accumulation. Immune cells, including cytokines, lymphocytes, and monocytes, play a vital role in this response. While these cells internalize sd-LDL, they are vulnerable to oxidative stress, forming a necrotic core beneath the endothelial cell surface. [36]

As the necrotic core expands, it enlarges endothelial cells, marking the thin cap atheroma stage. This phase represents a critical juncture in atherosclerosis progression, emphasizing the significant role of sd-LDL and the immune response in this cardiovascular disease. [37]

VSMC-specific marker expression changes throughout the phenotypic shift, including the loss of -SMA, SM22, and MYH11. Inflammation, growth factors, and lipid accumulation drive this shift. The synthetic VSMCs contribute to plaque formation, but they can also weaken the fibrous cap and promote plaque instability through increased matrix degradation and phenotypic modulation. [38] A fibrous cap covering a necrotic core made up of oxidized lipoproteins, cholesterol crystals, and cellular debris forms on top of the growing lesion's structural remodeling throughout time. [39] Inflammatory cells, including macrophages, T cells, natural killer T cells, and dendritic cells, are concentrated in high numbers along the borders of the plaque. These cells alter the extracellular

matrix through proteolysis, which makes the plaque more unstable. [40] This instability can cause atherothrombotic blockage in susceptible plaques by causing plaque rupture and releasing thrombogenic materials from the necrotic core. [41] Alternatively, superficial intimal erosions, caused by endothelial cell apoptosis and localized denudation, can also lead to thrombus formation. These erosions generally develop in regions of disrupted blood flow, and they are connected to lesions that have a dearth of macrophages but an abundance of smooth muscle cells and proteoglycans. [42] In contrast, stable plaques have an extensive fibrous cap, a decreased lipid content, and an infiltration of inflammatory cells. Although they can impede the vascular lumen and result in ischemia symptoms, they seldom trigger atherothrombotic events. [43]

Elevated concentrations of heparin sulfate contribute to enhanced lipoprotein adherence, leading to their accumulation at arterial bifurcations due to hemodynamic strain. [44] The persistent presence of these low-density lipoprotein (LDL) particles in the bloodstream and arterial walls increases their oxidative state, activating T-cells and triggering cytokine release. These cytokines modulate gene expression pathways in endothelial and smooth muscle cells (SMCs), initiating a cascade of events that culminate in atherosclerosis. [45] Monocytes differentiate into macrophages and invade the inner surface of the arterial wall, representing a pivotal event in atherosclerosis onset. Macrophages loaded with oxidized LDL (Ox-LDL) can transform into foam cells, which, upon apoptosis, contribute to the formation of the necrotic core in advanced atherosclerotic plaques. [46] (**Figure 2**)

During the fibrous atheroma stage, cytokine release by smooth muscle cells and endothelial cells activates the migration of smooth muscle cells toward the luminal side. Subsequently, these cells undergo apoptosis, generating necrotic debris that exacerbates inflammation and promotes lesion progression. Simultaneously, a central region of the atherosclerotic plaque forms beneath a fibrous tissue layer consisting of smooth muscle cells (SMCs) and T-lymphocytes. This plaque core disrupts the intimal architecture, impeding blood flow. If the protective fibrous cap surrounding the plaque becomes compromised or ruptures, released cytokines can induce arterial clotting, increasing the risk of heart attack or stroke. [47] (**Figure 3**)

The advanced stage of atherosclerosis, known as thin cap atheroma, is typically observed in individuals in their late fifties. It is characterized by the gradual thinning and weakening of the fibrous cap due to proteolytic enzymes. This condition significantly increases the susceptibility of the plaque to rupture, heightening the risk of thrombotic events such as heart attack or stroke. [48]

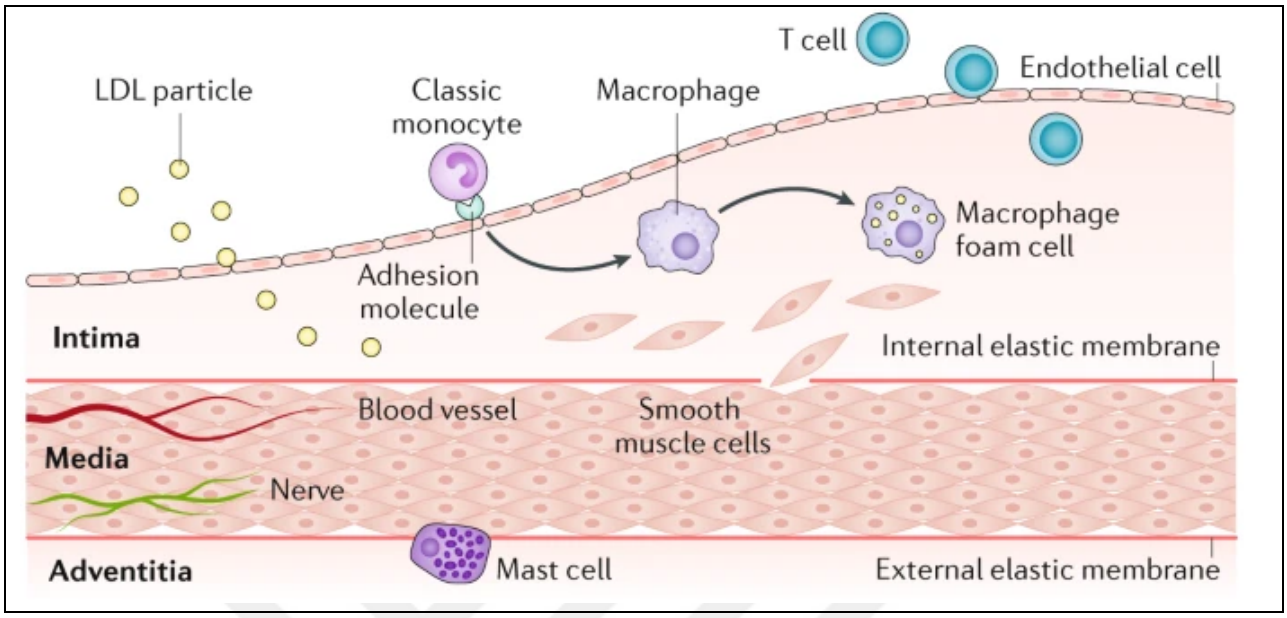

***Figure 2**: Initiation and progression of atherosclerosis.* [49]

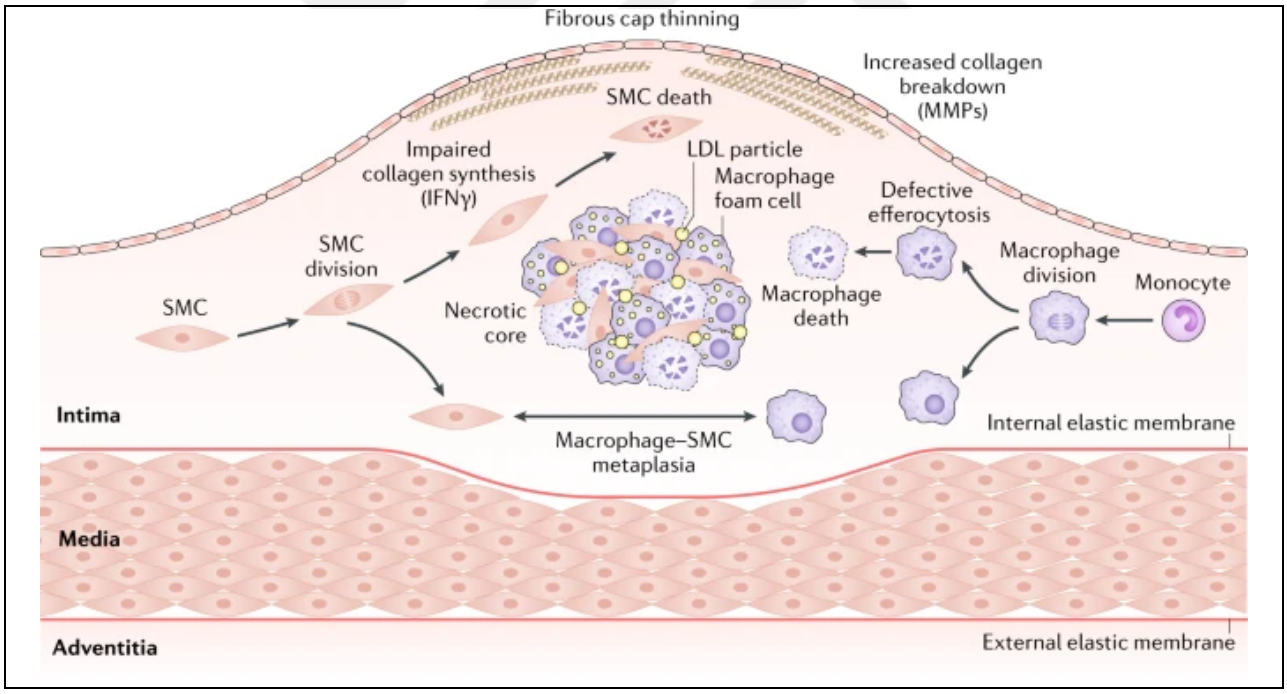

***Figure 3**: The progression of atherosclerotic lesions: cellular birth and death.* [49]

1.3 Normal Endothelial Cell Function

Fluid mechanical stimuli control specific endothelium genes involved in critical processes such as hemostasis, thrombosis, growth regulation, and proinflammatory activation. The force produced per square centimeter when blood flow acts tangentially on an endothelial surface is known as shear stress. Hemodynamic shear forces acting in the direction of blood flow at the vessel luminal surface are very sensitive to the endothelium. [50] Shear stress response elements are found in the promoters of certain genes, which modulate their transcription. Comparative studies on undisturbed laminar shear stresses revealed sustained upregulation of important endothelial genes with beneficial effects in the vascular system. Because atheroprotective genes are coordinatedly expressed in response to stable shear loads, some areas of the artery vasculature are still resistant to atherosclerotic lesions. [51]

Nitric oxide (NO) has important effects on blood vessels. It causes vasodilation, inhibits platelet aggregation, prevents adhesion of neutrophils and platelets to endothelial cells, preventing smooth muscle cell migration and proliferation, regulating programmed cell death (apoptosis) and maintaining endothelial cell barrier function. It is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) and is released by endothelial cells lining the blood vessels. It is a critical factor in the maintenance of vascular tone, blood pressure, and overall cardiovascular health. [34,52-56]

1.3.1 Endothelial Cells and Nitric Oxide (NO)

NO is also crucial in maintaining endothelial health. It inhibits the adhesion and aggregation of platelets and the adhesion of leukocytes to the endothelium, which are early steps in the development of atherosclerosis. It also prevents the expression of pro-inflammatory genes and proteins in endothelial cells, reducing inflammation within the vessel walls. The “endothelial dysfunction” in atherosclerosis is characterized by a decreased production or availability of NO. This dysfunction contributes to impaired vasodilation, increased platelet aggregation, leukocyte adhesion, and inflammation, accelerating the atherosclerotic process. Its actions on smooth muscle cells and endothelial cells are essential for maintaining vascular homeostasis. In the context of atherosclerosis, the proper functioning of NO can prevent or slow the progression of the disease by inhibiting various pathological processes, such as the proliferation of smooth muscle cells, platelet aggregation, leukocyte adhesion, and inflammation. [56-58]

1.4 Endothelial Cell Dysfunction in Atherosclerosis

Understanding endothelial cell dysfunction (ECD) in atherosclerosis is important. The idea of endothelial dysfunction emerged when researchers became aware of the diverse roles played by endothelial cells and their dynamic phenotypic regulation in atherosclerosis. Multiple groups investigated the pathophysiological stimuli of ECD, leading to detailed characterizations of its molecular manifestations. [59]

ICAM and **VCAM** proteins are cell adhesion molecules expressed on the surface of endothelial cells. They facilitate the attachment and recruitment of immune cells, particularly leukocytes, to

the site of vascular inflammation. In atherosclerosis, the expression of ICAM-1 and VCAM-1 is upregulated in response to proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). The increased expression of ICAM-1 and VCAM-1 promotes the adhesion of circulating leukocytes, particularly monocytes and T cells, to the activated endothelial cells. These adhesion events are crucial for the recruitment of immune cells into the arterial wall and the initiation of the inflammatory response characteristic of atherosclerosis. Once adhered, leukocytes migrate into the subendothelial space, where they contribute to the formation of atherosclerotic plaques. These immune cells release inflammatory mediators, such as cytokines and chemokines, which further propagate endothelial dysfunction and recruit additional leukocytes. The continued presence of leukocytes and their interaction with the endothelial cells contribute to the chronic inflammation and progression of atherosclerosis. [60]

The endothelial lining of small blood vessels plays a significant role in acute inflammatory responses. Exposure to agents like histamine leads to changes in microvascular tone, permeability, and the movement of leukocytes across the endothelium (diapedesis). These immediate responses, known as endothelial stimulation or type I activation, are rapid and temporary. However, endothelial cells can undergo significant functional changes in response to bacterial products, such as endotoxins, as well as other pathogen-associated molecular patterns, modified lipoproteins, and certain cytokines like interleukin-1, tumor necrosis factor, and IFN-γ. This secondary activation, referred to as type II activation, results in the production of specific proteins regulated by transcription factors like nuclear factor-κB (NF-κB). These proteins include class II major histocompatibility antigens, endothelial-leukocyte adhesion molecules, procoagulant molecules, and chemokines released into the surrounding environment. This activation program confers a localized, proinflammatory endothelial phenotype that is crucial in both acute and chronic inflammatory responses and related diseases. [61,62]

NF-κB (nuclear factor-kappa B) is a transcription factor that plays a critical role in regulating gene expression involved in immune responses, inflammation, cell survival, and other biological processes. In the context of atherosclerosis, NF-κB activation and subsequent gene expression changes are implicated in the pathogenesis of the disease. The activation of NF-κB occurs in endothelial cells, smooth muscle cells, and immune cells present in atherosclerotic lesions. NF-κB activation in endothelial cells leads to the expression of adhesion molecules (such as ICAM-1 and VCAM-1) and proinflammatory cytokines, which promote the adhesion of immune cells and perpetuate the inflammatory response within the arterial wall. Furthermore, NF-κB activation in smooth muscle cells contributes to the proliferation, migration, and phenotypic changes observed in atherosclerosis progression. [63,64]

KLF2 (Krüppel-like factor 2) is a transcription factor primarily found in endothelial cells. It helps maintain vascular health by suppressing inflammation, reducing adhesion molecule expression, and promoting the production of nitric oxide, which has beneficial effects on blood vessels. KLF2 plays a crucial role in preventing atherosclerosis and preserving endothelial function. Previous studies have observed KLF2 in the endothelium of regions in human arteries that are resistant to

atherosclerosis. [65,66] Introducing KLF2 into human endothelial cells enhances the expression of numerous genes associated with atheroprotective hemodynamic effects. KLF2 expression in endothelial cells promotes an anti-inflammatory and antithrombotic state by counteracting the NF-κB pathway. Additionally, it controls metabolism, the release of microRNAs through endothelial microvesicles, and endothelial barrier performance. Nitric oxide (NO) and C-type natriuretic peptide are lacking in dysfuntional endothelium, whereas endothelial KLF2 expression increases their synthesis. Importantly, compared to mice with normal KLF2 levels, mice without KLF2 have higher atherosclerotic plaque development. [65,67]

Flow-mediated expression of the transcription factor KLF2 in endothelial cells depends on the activation of a signaling pathway involving multiple components. This pathway includes mitogen-activated protein kinase (MAPK), extracellular-regulated kinase kinase kinase-3 (MEKK3), mitogen/extracellular signal-regulated kinase 5 (MEK5), extracellular signal-regulated protein kinase 5 (ERK5), and myocyte-specific enhancer binding factor-2 (MEF2). The activation of ERK5 and MEK5 leads to upregulation of KLF2 and promotes endothelial vasoprotection. Interestingly, this vasoprotective effect can occur both in a KLF2-dependent and KLF2-independent manner. This suggests that these kinases may have additional targets that mediate the beneficial effects of flow on endothelial function. One such target is the transcription factor KLF4, which is also upregulated by atheroprotective flow through the involvement of the same signaling pathway, including MEK5/ERK5/MEF2. The expression of KLF4 promotes the expression of genes associated with vascular protection and has been implicated in atheroprotection in experimental models of atherosclerosis using approaches that selectively increase or decrease its expression in endothelial cells. [65,68,69]

In addition to transcriptional control, endothelial gene expression is influenced by atheroprotective and atheroprone flow via miRNAs and epigenetic changes. miRNAs influence post-transcriptional gene expression and the control of atherosclerosis-prone genes to contribute to the pathogenesis of atherosclerosis. These will be discussed further below. Studies have shown that atheroprotective flow upregulates miR19-a, miR-23b, and miR101, suppressing endothelial cell proliferation. [70-72] The miR143-145 cluster secretion is induced by atherosclerotic flow and they control vascular smooth muscle cell'' turnover and phenotype to reduce atherosclerotic plaques. [73] Epigenetic modifications, such as alterations in DNA methylation patterns, have also been observed in endothelial cells under atheroprone flow conditions. These findings open up new avenues for targeted pharmacological interventions to modulate flow-mediated gene regulation and reduce proinflammatory endothelial cell activation. [74]

Studies investigating multiple atherosclerotic lesions within a single subject have revealed their autonomous behavior, with lesions progressing, regressing, or remaining stable independently. [36,75,76] Local factors play a crucial role in determining the pathobiology of these lesions. Endothelial shear stresses have been identified as potential predictors of the subsequent behavior of a lesion. [77]

Monocytes and macrophages are crucial components of the immune system that help protect the body from infections and assist in the healing process. They are types of white blood cells, which are the cells of the immune system involved in protecting the body against both infectious diseases and foreign invaders. Monocytes are a type of leukocyte, or white blood cell, that circulate in the blood. They make up about 2-10% of the total population of white blood cells in the bloodstream. Monocytes play a significant role in the body's immune response to infections. They are produced in the bone marrow and then released into the bloodstream. When the body experiences an infection, inflammation, or tissue damage, monocytes migrate from the bloodstream to the affected tissue. Once there, they can further differentiate into macrophages or dendritic cells, depending on the signals they receive from the surrounding environment. [78] Macrophages are a type of white blood cell that reside within tissues. They are derived from monocytes and play a central role in initiating the immune response. Macrophages are often referred to as the 'big eaters' of the immune system because of their role in phagocytosis, which is the process of engulfing and digesting cellular debris, foreign substances, microbes, and cancer cells. In addition to their role in phagocytosis, macrophages also help to stimulate other immune cells to respond to pathogens by presenting them with antigens (parts of the pathogen). They are involved in both the innate immune response (the body's first line of defense against infection) and the adaptive immune response (where the immune system adapts to fight specific pathogens). Macrophages also play a critical role in wound healing and tissue repair. They can release cytokines, growth factors, and other signaling molecules that help in tissue remodeling and repair. [79,80]

In conclusion, while both monocytes and macrophages play essential roles in the immune response, the primary difference between them is their location and level of differentiation. Monocytes are circulating cells that can differentiate into macrophages or dendritic cells when they migrate into tissues, whereas macrophages are fully differentiated cells that reside within tissues and have critical roles in phagocytosis, antigen presentation, and tissue repair. [79]

1.4.1 In Vitro Methods to Analyze Atherosclerosis

In vitro models have been developed to recreate an atherosclerotic environment outside the human body. These models typically involve culturing endothelial cells under conditions that mimic aspects of atherosclerosis, such as exposure to oxidized lipids or inflammatory cytokines, allowing researchers to study the cellular and molecular changes associated with the disease. [81]

In the context of our study, the co-culture of vascular smooth muscle cells (**VSMC**) and endothelial cells (**EC**) is meant to simulate the *in vivo* environment of a blood vessel. [81,82] Under normal physiological conditions, endothelial cells form the inner lining of the vessel, providing a barrier between the blood and the surrounding tissues, while VSMCs form the middle layer of the vessel, controlling its diameter and thereby regulating blood pressure and flow. [82,83] Both endothelial cells and VSMCs play crucial roles in the initiation and progression of atherosclerosis disease.

Endothelial dysfunction (often due to inflammatory and oxidative stress) and VSMC proliferation and migration contribute significantly to plaque formation and progression. [81]

Tumor Necrosis Factor-alpha (**TNF-α)** and Lipopolysaccharide (**LPS**) are agents often used *in vitro* to induce inflammatory responses and mimic the atherosclerotic environment. TNF-α is a pro-inflammatory cytokine that plays a critical role in promoting the inflammatory response associated with atherosclerosis. [84] It can induce endothelial cells to express adhesion molecules and chemokines that attract leukocytes, contribute to thrombus formation, and cause VSMC proliferation and migration, processes that are pivotal in the formation and progression of atherosclerotic plaques. [81,85-87]

LPS, an endotoxin found in the outer membrane of Gram-negative bacteria, can trigger an innate immune response, leading to inflammation. In the context of atherosclerosis, LPS can induce endothelial dysfunction, promote the expression of pro-inflammatory cytokines, and stimulate the migration and proliferation of VSMCs. [88]

Therefore, by co-culturing VSMCs and endothelial cells and treating them with TNF-α and LPS, we have aimed to recreate aspects of the inflammatory environment characteristic of atherosclerosis, providing a valuable model for studying the disease and testing potential treatments.

1.5 What are exosomes?

Extracellular vesicles (EVs) are small membranous structures that are released by cells into their surrounding environment. They are involved in intercellular communication and play important roles in various physiological and pathological processes. They are involved in cell-to-cell communication, immune responses, tissue repair, and the spread of diseases, including cancer, infectious diseases, and neurodegenerative disorders. [89] Exosomes are lipid bilayer membranous vesicles that carry biological fluid that contains proteins and genetic material. [90] mRNA (miRNA and lncRNA), protein, and DNA are packaged into exosomes, a naturally occurring transport system, from donor to destination cells. (**Figure 4**) Exosomes can perform vital functions such as maintaining cellular homeostasis, removing cellular waste, and fostering intercellular and interorgan communication. Exosomes circulate across all body fluids and communicate molecular information in endocrine, autocrine, and paracrine ways. Exosomes are composed of a wide range of high-order oligomeric protein complexes that are membrane-associated, exhibit great molecular diversity, and are formed by budding at both the plasma membrane and the membrane of the endosome. Once released, exosomes perform various functions, including remodeling the extracellular matrix, transferring signals and chemicals to neighboring cells, and playing a vital part in intercellular communication [91-96]. Additionally, they participate in a number of inflammatory signaling pathways that result in atherosclerosis. [90,97,98] Exosome-derived components may have clinical utility when used as biomarkers for atherosclerosis management and cardiovascular disease risk assessment. [90]

1.6 EV Classification, Biogenesis and Functions

1.6.1 EV classification

EVs can be classified into different subtypes based on their biogenesis and size: apoptotic bodies (> 1000 nm), microvesicles (MVs) (100-1000 nm), and exosomes (30-200 nm). Although all eVs have complex protein, nucleic acid, lipid, and metabolite compositions, the overlaps in sizes and markers between diverse eVs might make the separation more difficult. [99]

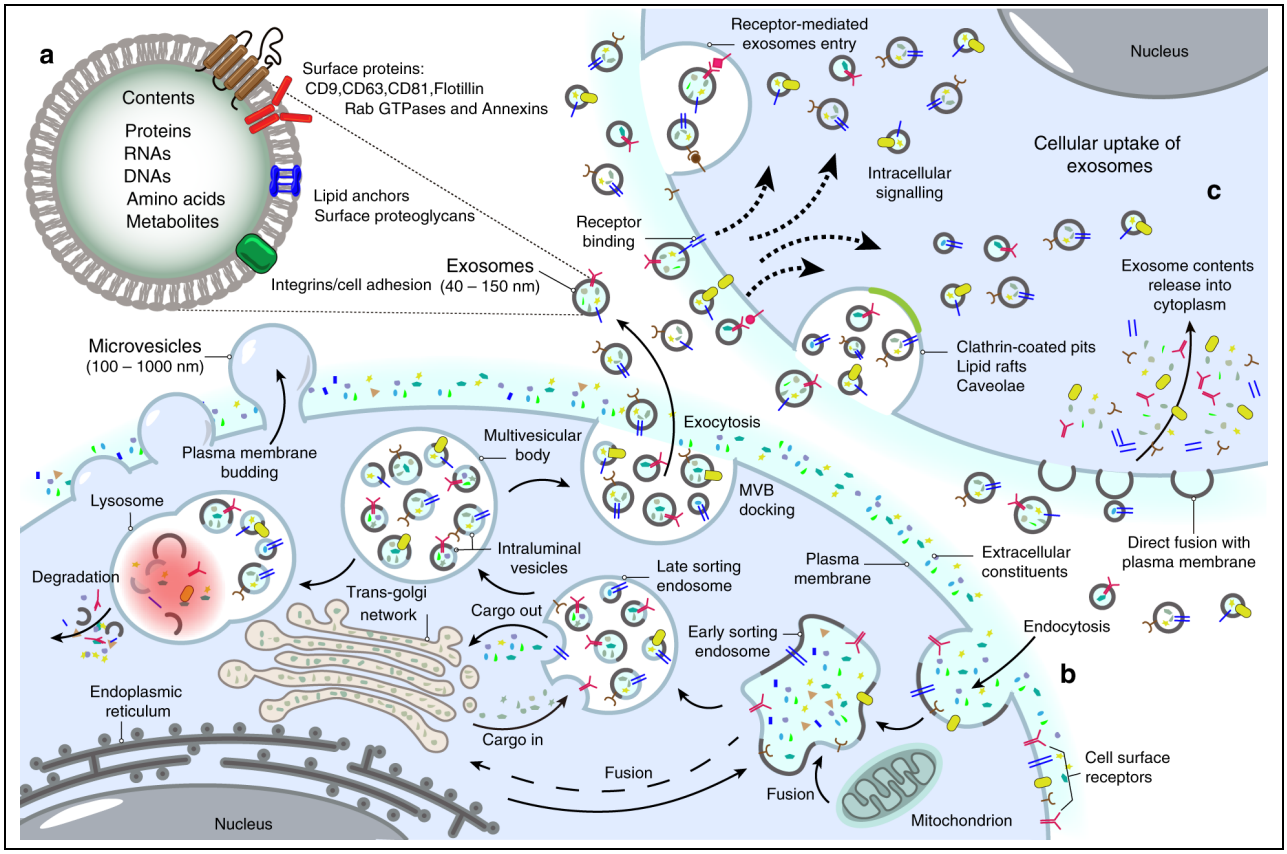

Figure 4: [100]

1.6.2 Structure of exosome membrane: The structure of an exosome is defined by its lipid bilayer membrane, which encloses a small amount of cytosol from the parent cell. This membrane protects the contents of the exosome from degradation and allows it to travel to distant sites within the body. (**Figure 4**) In solution, exosomes have a spheroid form, but when artificial drying is used during production, they take on a bi-concave or cup-shaped appearance. **Tetraspanin** family members (**CD9, CD63**, and **CD81**), endosomal sorting complex needed for transport (**ESCRT**) proteins (Alix, TSG101), integrins, heat shock proteins (Hsp), actin, and flotillins are the primary

membrane-bound and cytosolic proteins included in exosomes. [101] All exosomes have a number of similar proteins, including cytoskeletal proteins, CD63, ESCRT, and heat shock proteins. MHC Class I and II proteins, however, are unique to the type of donor cell. (**Figure 5,6**)

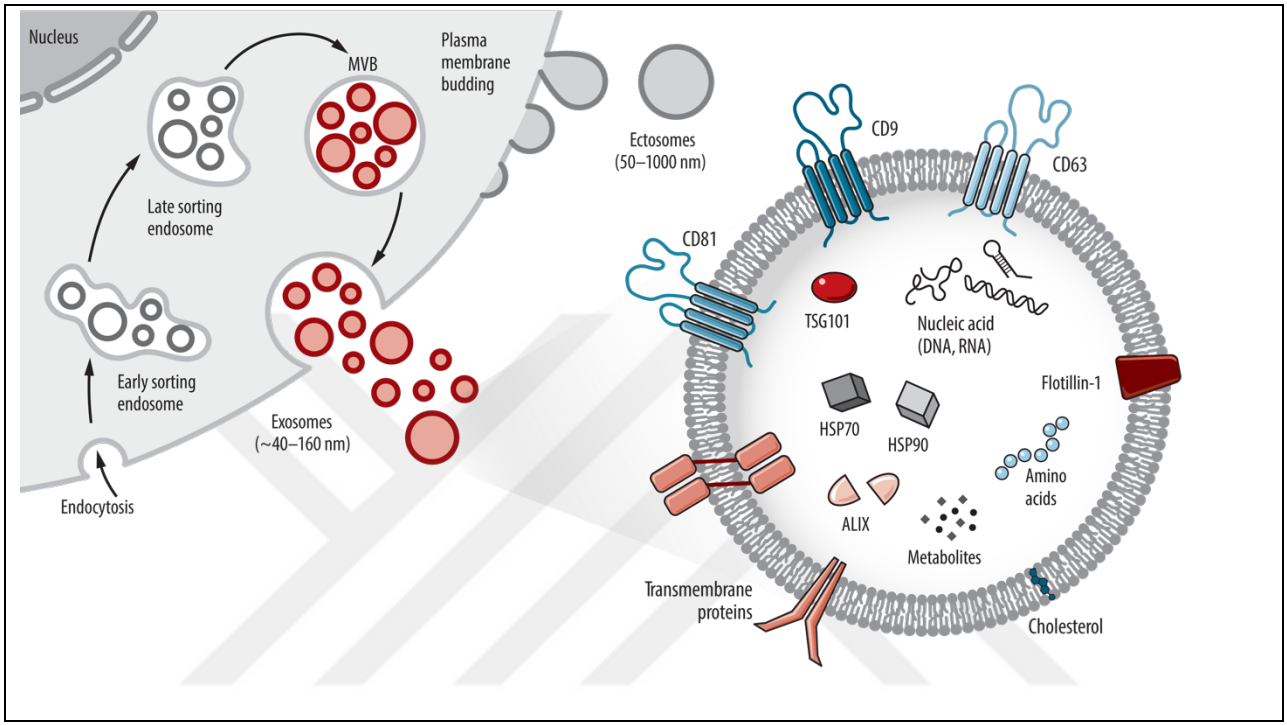

***Figure 5**: Structure and content of an exosome.* [102]

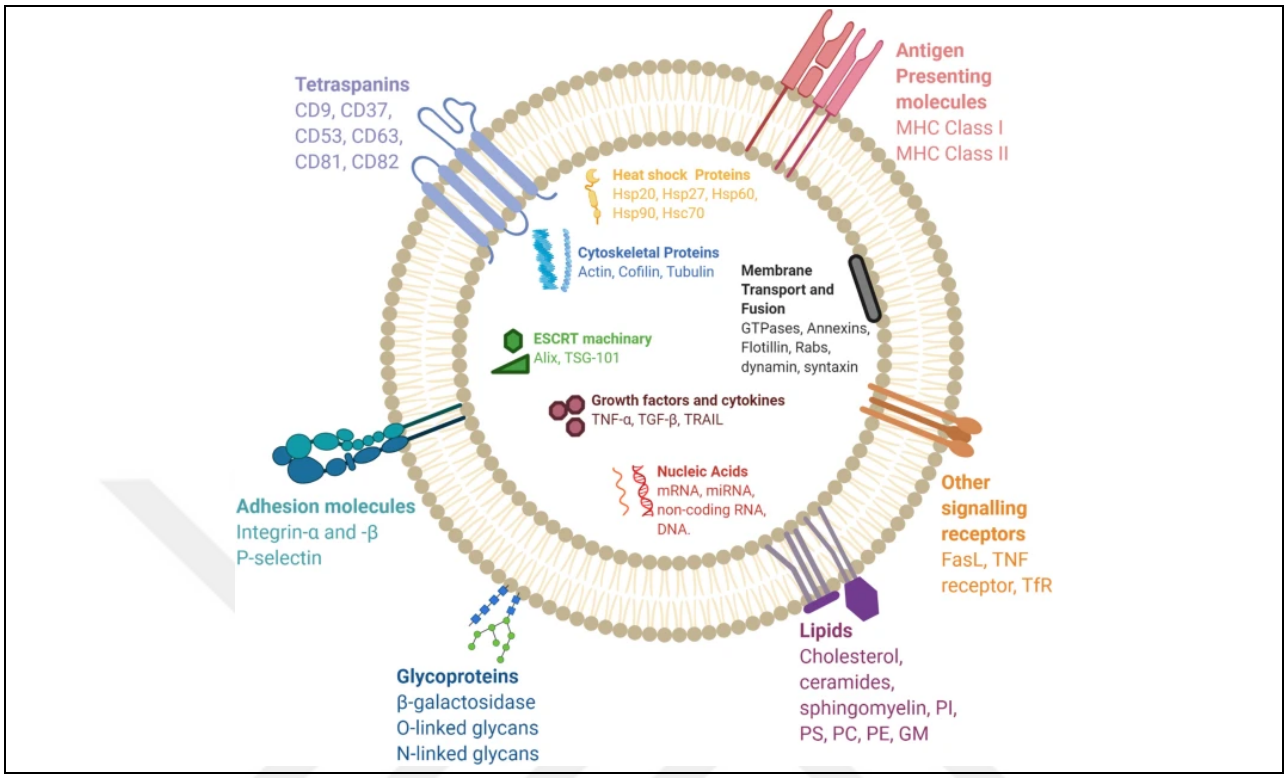

***Figure 6**: Composition of exosomes.* [103]

1.6.3 Biological composition of exosomes

Exosomes are membrane-bound carriers. Proteins, nucleic acids, and metabolites can be found in their cargo, reflecting the donor cell's make-up and physiological condition. (**Figure 6**) Nucleic acids such as DNA, mRNA, miRNA, and various forms of non-coding RNAs that exosomes can transport are integral to numerous biological functions like cell secretion, blood cell production, and blood vessel formation, and they significantly contribute to cell-to-cell communication via exosomes. Exosomes also carry other kinds of RNA such as ribosomal RNA (rRNA), long non-coding RNA (lncRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA. Each of these plays a role in various biological processes. Given their role in these vital processes, many studies are investigating their use as non-invasive diagnostic and prognostic tools for diseases. [104-106]

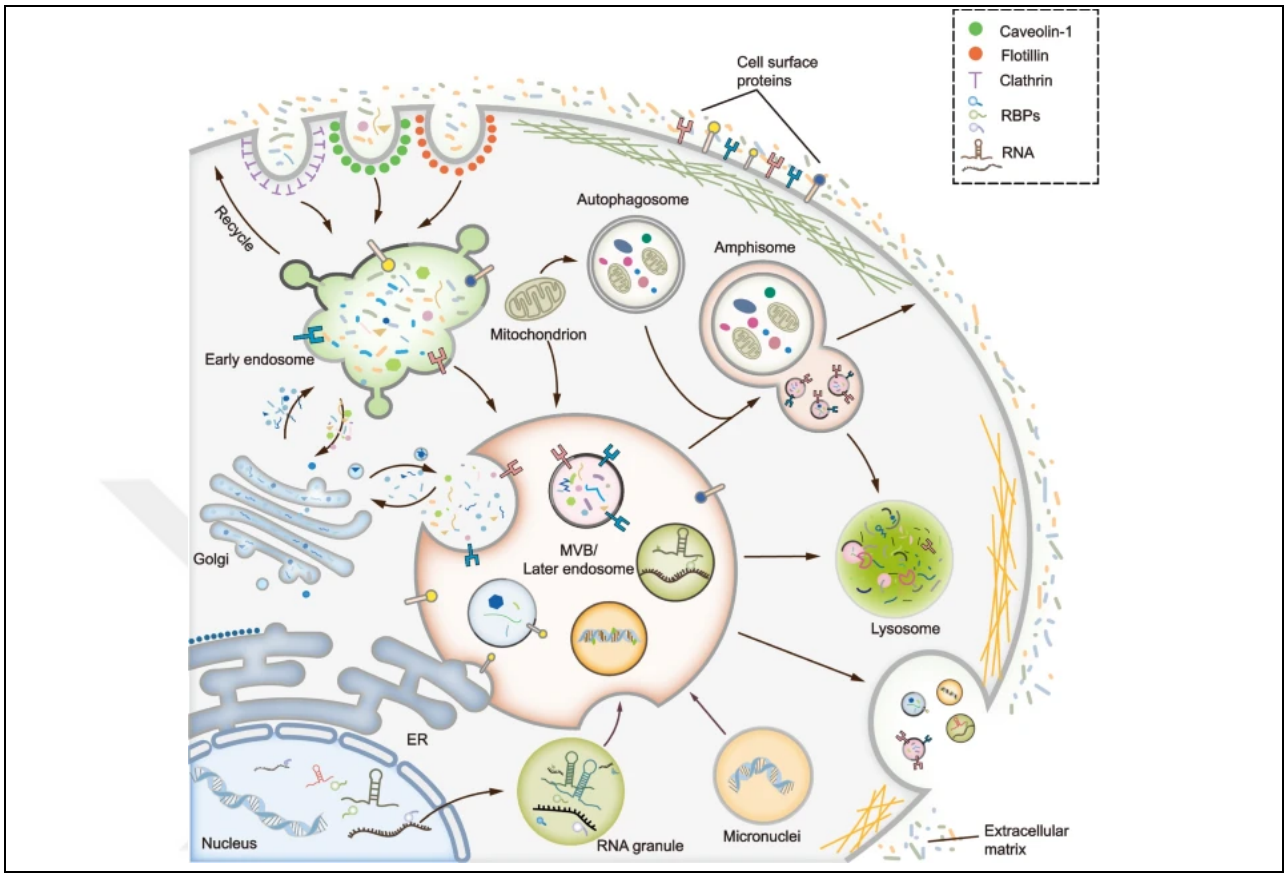

***Figure** 7: Exosome biogenesis.*[107]

1.6.4 Exosome biogenesis in multivesicular bodies

Cargo sorting to Multivesicular Bodies (MVBs), MVB formation and maturation, MVB transport, and MVB fusion with the plasma membrane are the four main processes in exosome biogenesis. (**Figure 7, 8**)

Exosomes are heterogeneous due to the many mechanisms that are involved in each step of exosome synthesis. These mechanisms are highly varied depending on the cargo, cell type, and microenvironment. Several mechanisms can be used by the same MVB.

Multivesicular bodies (MVBs) are a type of endosome, characterized by the presence of multiple small vesicles, known as intralumenal vesicles (ILVs), contained within their lumen. These ILVs are formed by the inward budding of the MVB membrane, and can carry a variety of cargo, including proteins, lipids, and RNA.

1.6.4.1 MBVs formation

Exosome biogenesis is centered on MVB formation. Key actions in this process include ILV production and membrane budding in particular. [108] There are several mechanisms to drive the

budding of limiting membrane and generation of ILVs. These mechanisms consist of endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways.

1.6.4.2 Cargo sorting to MVBs

For the secretion of exosomal cargo, sorting to MVBs is crucial. Exosomes also include a range of proteins and nucleic acids, including as DNA, PIWI-interacting RNAs (piRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), microRNAs (miRNAs), lncRNAs, and messenger RNAs (mRNAs). [102] Exosomal content profiles dynamically fluctuate and influence the functional characteristics of exosomes based on the state of the source cells. [109,110] Exosome cargo packaging is therefore extremely selective and strictly managed. (**Figure 8**)

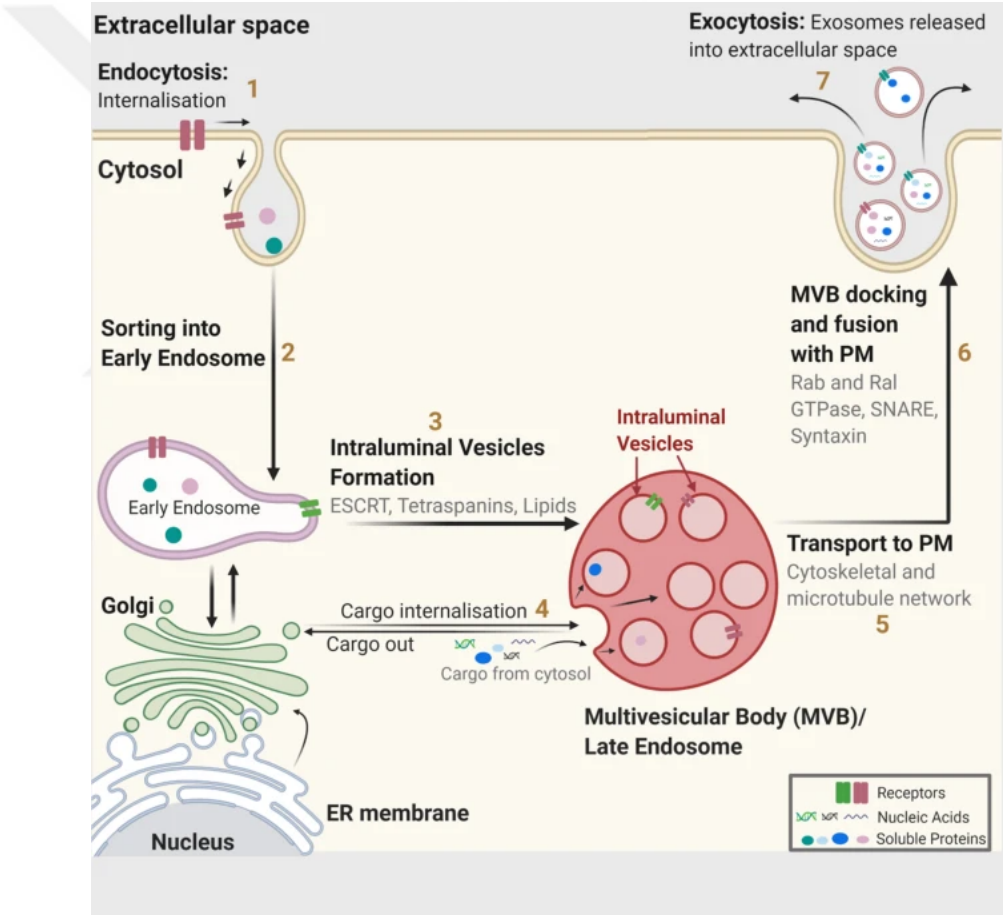

***Figure 8**: Multivesicular body (MVB) formation [103]*

(1) internalized cargoes are (2) sorted into early endosomes, (3) which then mature into late endosomes or multivesicular bodies. Late endosomes/multivesicular bodies are specialized endosomal compartments rich in intraluminal vesicles (ILVs), (4) Cargoes are also delivered from the trans-Golgi network and possibly from cytosol. (5) Multivesicular bodies containing exosome cargoes get (5) transported to the plasma membrane, (6) fuse with the cell surface and (7) the ILVs then get secreted as exosomes.

1.6.4.3 Maturation of MVBs

MVBs are characterized as containing intralumenal vesicles. [111] They undergo maturation and can fuse with other organelles, such as the endoplasmic reticulum (ER), trans-Golgi network (TGN), mitochondria, recycling endosome, and RNA granules. [112-116] Amphisome is a unique kind of mature MVBs which is combined autophagosomes with MVBs under particular conditions. Amphisomes can also fuse with a lysosome to degrade or fuse with the plasma membrane, to secrete exosomes. [117] MVBs can be diverse based on their origin, maturation state, or route. They can be degraded by fusion with lysosomes (degradative MVBs or dMVBs) or fuse with the plasma membrane for secretion (secretory MVBs or sMBVs). [107]

1.6.4.4 Transport of MVBs

After sMVBs have reached maturity, a number of processes work in concert or sequentially to regulate their antegrade migration, tethering/docking, and fusing with the plasma membrane. (**Figure 9**)

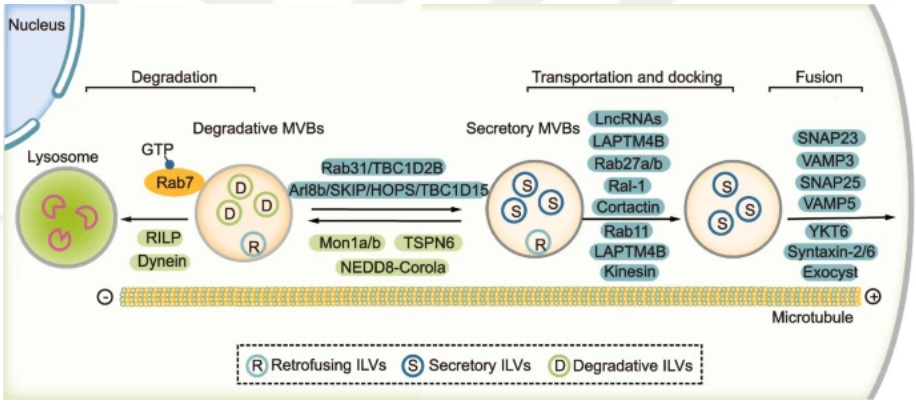

***Figure 9**: Transport of MVBs*[107]

1.6.4.5 MVB fusion with the plasma membrane

When MVBs fuse with the plasma membrane, a process regulated by various proteins including the Rab GTPases (notably Rab27 and Rab35), the MVB's ILVs are released into the extracellular environment. These released vesicles are known as exosomes. [107]

Once MVB reaches the plasma membrane, SNARE proteins facilitate their fusion. This fusion process is dependent on the cell's physiological state and can be increased under stress conditions or during immune responses. [107]

1.6.4.6 Relation Between Autophagy and Exosomes

Autophagy is a catabolic process where the cell degrades and recycles its own components. [118] It involves the formation of a double-membraned vesicle called an autophagosome, which engulfs damaged proteins and organelles. The autophagosome then fuses with a lysosome, an organelle containing enzymes that degrade the components enclosed in the autophagosome, thus recycling

them for future use. Autophagy is vital for cellular health, survival during nutrient starvation, development, differentiation, and the prevention of diseases such as cancer and neurodegenerative disorders. [119,120] Several studies have shown that impairment of the autophagy pathway can lead to an increased release of exosomes. [121,122] In particular, it's suggested that when the autophagy-lysosome pathway is overloaded or blocked, cells may resort to exosome-mediated disposal of unwanted or excessive cellular components. [123] Additionally, some components related to the autophagy machinery have been found in exosomes, suggesting a shared pathway between autophagy and exosome formation. [121,124,125] For example, the protein LC3, an essential protein in the autophagy pathway, has been found on the surface of some exosomes, suggesting an overlap in their biogenesis. [126] Furthermore, autophagy-related proteins, such as Atg5 and Atg16L1, have been found to play a role in exosome biogenesis. [127] The development of autophagosomes, double-membrane vesicles that ingest cytoplasmic components for destruction, is correlated with the presence of these proteins. Autophagy-related proteins may influence exosome secretion by regulating the trafficking and fusion of MVBs with autophagosomes. [127]

Overall, exosome biogenesis is a complex and regulated process involving the ESCRT machinery, complex lipids, tetraspanins, Rab GTPases, and autophagy-related proteins. These molecules interact to maintain correct exosome synthesis, sorting, and release, essential for intercellular communication and various biological activities.

1.6.5 Transport and biodistribution

Exosomes released by different types of cells and are present in biological fluids. They participate in systemic and local cell-to-cell communication. Breast milk, blood, serum, urine, saliva, amniotic fluid, and synovial fluid have all been found to contain exosomes. ***[128]*** They can go through numerous absorption and release cycles, which enables them to penetrate various tissues. [103]

The size of exosomes also influences their transport and biodistribution.

It is essential to target recipient cells specifically in order to distribute exosome contents and allow them to operate. ***[129]*** The exosomes' surface makeup serves as a conduit for this targeting. Additionally discovered as surface cues that direct exosome targeting to particular tissues or cell types are integrins and CD63. ***[130]***

In summary, the origin of exosomes, the route of administration, and the composition of exosomes all play significant roles in determining their biodistribution and targeting capabilities.

1.6.6 Interaction with the recipient cell

Exosomes can either initiate signaling by directly engaging with extracellular receptors when they reach the target cell, be absorbed by direct fusion with the plasma membrane, or become internalized. [103] (**Figure 10**)

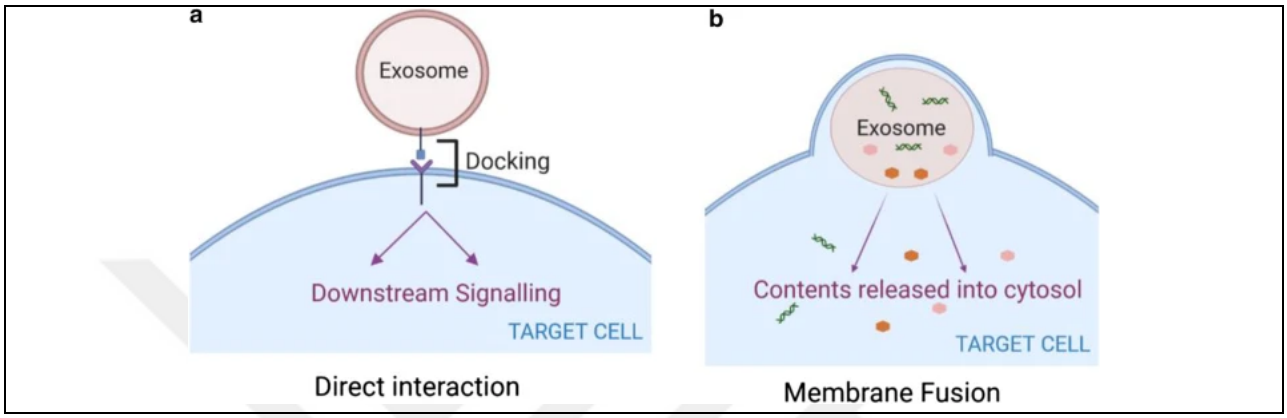

***Figure 10**: Exosome signaling by direct interaction or membrane fusion.* [103]

1.6.6.1 Direct interaction

By attaching their transmembrane ligands to surface receptors, exosomes can interact directly with recipient cells, starting signaling cascades that activate the target cells. (**Figure 10**) Exosomes present in umbilical cord blood, contain proteins including MHC-I, MHC-II, CD34, and CD80 plus tumor antigens. These can stimulate the growth of T cells and cause them to become anti-tumor active. [131] Furthermore, tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL), which can bind to TNF receptors on tumor cells and activate caspases and promote apoptosis, are carried on exosomes released by dendritic cells. [131]

1.6.6.2 Fusion with the plasma membrane

Exosomes have the capacity to merge with the plasma membrane of their target cells and release their contents into the cytoplasm. (**Figure 10**) The exosome and plasma membrane's hydrophobic lipid bilayers form a stalk during this phase, which is followed by expansion to create a single, unified structure. Families of SNAREs and Rab proteins mediate this fusion. Interaction, attachment, and membrane fusion with the target cell are also mediated by integrins, adhesion molecules, and lipid raft-like regions on the exosome surface. [132]

1.6.6.3 Internalisation

Exosomes are first internalized by the recipient cell before releasing their payload. Rapid and temperature-sensitive, this absorption mechanism is slowed down by low temperatures. [103] Exosome internalization involves the common endocytic pathways:

- **Clathrin-mediated endocytosis** is a common pathway for exosome uptake in various cell types. It involves the assembly of clathrin-coated vesicles and their subsequent fusion with endosomes. This process relies on factors essential for clathrin-mediated endocytosis and is influenced by the cargo and composition of exosomes. Clathrin-mediated endocytosis is considered one of the primary mechanisms for exosome internalization. [103] (**Figure 11**)

  Lipid raft-associated membrane invagination is a major mechanism for exosome uptake. Sphingolipids, GPI-anchored proteins, and cholesterol-rich microdomains called lipid rafts are found in cell membranes. Chemicals that interfere with the transport of cholesterol, such as methy–-cyclodextrin, decrease the uptake of exosomes by breast cancer cells. [133] Inhibition of sphingolipid synthesis impairs exosome uptake by dendritic cells. [103] Pre-treating cells with filipin, which binds to cholesterol, reduces exosome uptake. [133] Annexin AnxA2 and flotillin are proteins that promote lipid raft-mediated endocytosis of exosomes. [134] (**Figure 11**)
-
- **Caveolin-dependent endocytosis** is a potential exosome uptake pathway, but there are conflicting reports. Caveolins are integral membrane proteins that create flask-shaped invaginations called caveolae. Large vesicles called caveosomes, laden with sphingolipids and cholesterol, can be internally absorbed with the help of caveolae. The three primary structural proteins in caveolae are caveolin-1, 2 and 3. Although it inhibits it in fibroblasts and glioma cells, caveolin-1 stimulates exosome uptake in epithelial cells. Further research is necessary since dynamin-2 and other common actors in clathrin- and caveolin-mediated endocytosis are shared. Specific clathrin inhibitors can be used to distinguish between different routes. [135] (**Figure 11**)

  Phagocytosis is a biological mechanism that can ingest microscopic particles like exosomes as well as larger ones like bacteria and dead cells. It involves a sequential mechanism in which external particles are engulfed by the cell membrane, generating phagosomes, which then transport the absorbed cargo to lysosomes. Phospholipase C (PLC) and phosphatidylinositol-3-kinase (PI3K) are necessary for the closure of phagosomes. Given their reliance on PI3K and actin cytoskeleton activity, immune cells like macrophages and dendritic cells naturally adopt this pathway most frequently for exosome uptake. [103,136] (**Figure 11**)
-

  Macropinocytosis is an actin-driven process where the plasma membrane forms inward invaginations called macropinosomes, allowing nonspecific uptake of molecules. It depends on growth factors, cholesterol, and cellular proteins like Rac1 and dynamin. Macropinosomes mature and can be either degraded or recycled. Exosome uptake can occur through30iRbase30ocytosiss in various cell types, including HeLa cells and certain

microglial and epithelial cells. It has also been observed in Ras-expressing carcinoma cells and can be induced by growth factor stimulation. [137,138] (**Figure 11**)

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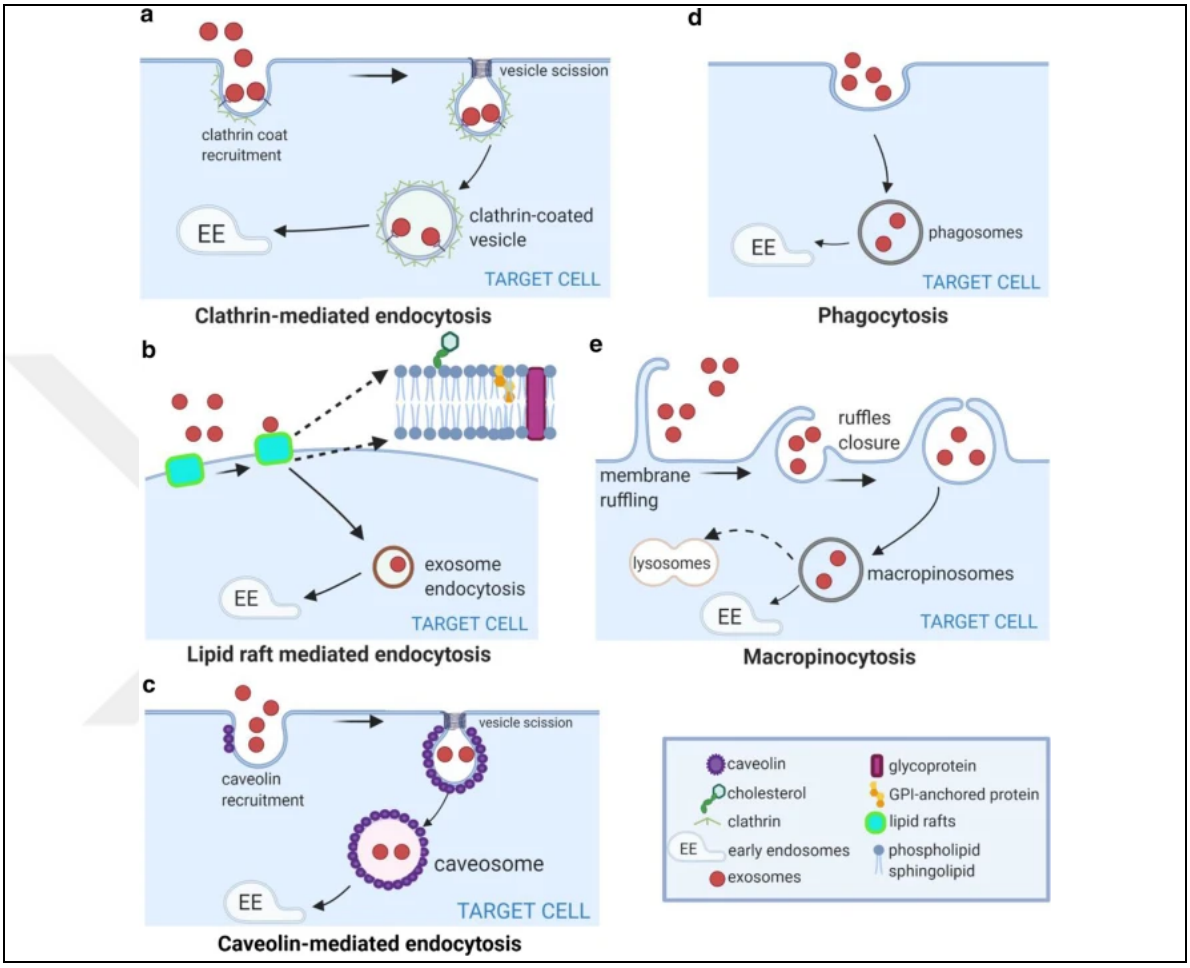


***Figure 11**: Exosome internalization.* [103]

These various pathways can coexist and may be dependent on factors such as cell type, cargo composition, and the specific context of the interaction. For example, exosome uptake occurs simultaneously in lipid rafts, clathrin-mediated endocytosis, phagocytosis, and31iRbase31ocytosiss in ovarian tumor and melanoma cells. [139] The uptake of exosomes by different cell types can also involve multiple mechanisms, such as the combination of31iRbase31ocytosiss and clathrin-mediated endocytosis observed in certain cases.

It is important to note that the understanding of exosome uptake mechanisms is still evolving, and further research is needed to fully elucidate the specifics of each pathway and their significance in different cellular contexts.

The precise targeting of recipient cells is largely determined by the composition of the exosome surface, which is fundamental for the successful delivery of its contents and fulfilment of its function. [103] Cellular markers such as mannose and sialic acid-enriched glycoproteins and integrins present on exosomes facilitate specific cellular interactions. [140,141] Cancer cells, for example, utilise this mechanism to manipulate specific cell types. In addition, complex lipids are influential in directing exosome targeting and different lipid species have been shown to favour specific cell types depending on their enrichment.

Therefore, numerous factors, including the origin of the cell, the delivery method and the composition of the exosome, largely determine its biodistribution. CD63 positive exosomes secreted by neuroblastoma cells specifically target neuronal dendrites, whereas exosomes that do not harbour this marker target all neurons and glial cells. [142] In addition, exosomes are known to target metastatic cancer cells via various integrins. [141]

1.7 Exosomal Cargo Sorting

Exosomes carry a diverse range of biological molecules, which include proteins, lipids, and nucleic acids. This cargo can be a snapshot of the state of the cell of origin, and exosomes can transfer their cargo to recipient cells to modify their state or behavior.

Exosomes can carry a variety of **proteins**, including membrane proteins, cytosolic proteins, and cytoskeletal proteins. The specific proteins they carry often depend on the cell of origin. For instance, they typically carry proteins involved in their biogenesis (e.g., Alix and TSG101, which are components of the endosomal sorting complexes required for transport, or ESCRT) and specific tetraspanins (e.g., CD63, CD81, CD9). Additionally, they can carry cell-specific proteins that can give us a clue about their origin.

Exosomes carry a distinct set of **lipids** compared to the cells from which they originate. These include cholesterol, sphingomyelin, ceramide, and phosphatidylserine. These lipids play crucial roles in the biogenesis, cargo sorting, and the uptake of exosomes by recipient cells. [143]

Exosomes can carry a variety of **nucleic acids**, including mRNAs, microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and even DNA. These nucleic acids can be functional in recipient cells. For instance, mRNAs can be translated into proteins, and miRNAs can regulate gene expression in the recipient cells. This suggests a role for exosomes in genetic exchange between cells. [144]

Recent research has also shown that exosomes can carry **metabolites**, including amino acids, lipids, and TCA cycle intermediates. These metabolites can potentially influence the metabolic state of recipient cells. [145]

The composition of exosome cargo is not random. Instead, it appears that cells can selectively sort cargo into exosomes, although the mechanisms of this sorting process are not fully understood. [146,147] Importantly, the cargo of exosomes can reflect the state of the cell of origin, such as whether the cell is stressed or whether it is cancerous. [148] Thus, studying the cargo of exosomes offers a way to probe the state of the cells in our body, and it has the potential for use in diagnostics and therapeutics.

1.8 Methods for Exosome Analysis and Characterization

Western blot analysis and nano tracking analysis are two commonly used techniques for the analysis of exosomes.

In exosome analysis, **western blotting** is used to identify and quantify specific proteins (**CD63, CD81**) present in exosome. The process involves several steps: Firstly, exosomes are isolated from the biological sample using ultracentrifugation. Then, the isolated exosomes are lysed to release their protein content. This can be achieved by using lysis buffers or detergent-based methods. Secondly, the proteins in the exosome lysate are separated based on their size using gel electrophoresis. The proteins are then transferred to a membrane, such as a nitrocellulose or PVDF membrane. The transferred proteins on the membrane are probed with specific primary antibodies that recognize the target proteins of interest. After that, secondary antibodies labeled with enzymes or fluorescent dyes are applied to bind to the primary antibodies. This enables the visualization of the protein bands using enzymatic reactions or fluorescence detection methods. By comparing the protein bands on the western blot with known standards or controls, researchers can identify and quantify specific proteins within the exosome sample. [149]

Nano tracking analysis is a technique used to measure the size distribution and concentration of nanoparticles, including exosomes. NTA employs a combination of light scattering and Brownian motion analysis to characterize and track the movement of particles in a liquid suspension. The process involves several steps: Exosome samples are diluted appropriately and loaded into a sample chamber. Then, a laser beam is directed through the sample, and the scattered light from the particles, including exosomes, is captured by a microscope. Software algorithms track the Brownian motion of individual particles, allowing for the determination of their size and concentration. The analysis provides information on the size distribution and concentration of exosomes in the sample. NTA provides quantitative data on exosome size distribution and concentration, allowing researchers to characterize and compare different exosome samples. [149]

In summary, Western blot analysis is used to detect specific proteins in exosome samples, while nano tracking analysis is a technique to measure the size distribution and concentration of exosomes. Both methods provide valuable information for exosome analysis and characterization.

1.9 Small RNAs

Small RNAs are a class of short, non-coding RNA molecules that play a crucial role in the regulation of various cellular processes. They are typically less than 200 nucleotides in length.

There are several types of small RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs). [150,151]

MicroRNAs (miRNAs): These small RNAs, about 22 nucleotides long, are involved in post-transcriptional regulation of gene expression. They function by binding to the 3' untranslated region (3' UTR) of specific messenger RNAs (mRNAs), which can result in either the degradation of the mRNA or inhibition of its translation, thereby decreasing the expression of the associated gene. [152]

Small Interfering RNAs (siRNAs): These are typically 20-25 nucleotides long and involved in the RNA interference (RNAi) pathway. They are usually produced from double-stranded RNA precursors and function by guiding the RNA-induced silencing complex (RISC) to degrade complementary mRNA molecules, thus preventing their translation. [153]

PIWI-interacting RNAs (piRNAs): Ranging from 21 to 35 nucleotides, piRNAs direct the PIWI proteins to cleave the target RNA, encourage the construction of heterochromatin, and methylate DNA. [151] piRNAs interact with PIWI proteins and play a significant role in the silencing of transposons to maintain genome integrity, especially in germline cells.

Small RNAs are involved in numerous biological processes, such as development, cell differentiation, proliferation, apoptosis, and response to stress. [151] In addition, dysregulation of small RNAs is associated with various diseases, including cancer, cardiovascular disease, and neurological disorders. They also have potential as biomarkers and therapeutic targets in various diseases. [154]

1.9.1 What is miRNA?

miRNAs are small (18-22 nucleotide) RNAs that serve as post-transcriptional regulators of protein-encoding genes so, they are selectively attached to mRNAs and induce mRNA degradation or translation inhibition [155]. They play a vital function in gene expression regulation by altering the expression of target genes[156]. The primary mechanism by which microRNAs interact with their target mRNAs is by complementary base-pairing to sites in the mRNA's 3′-untranslated region, which are found in multiple copies. (**Figure 12**)

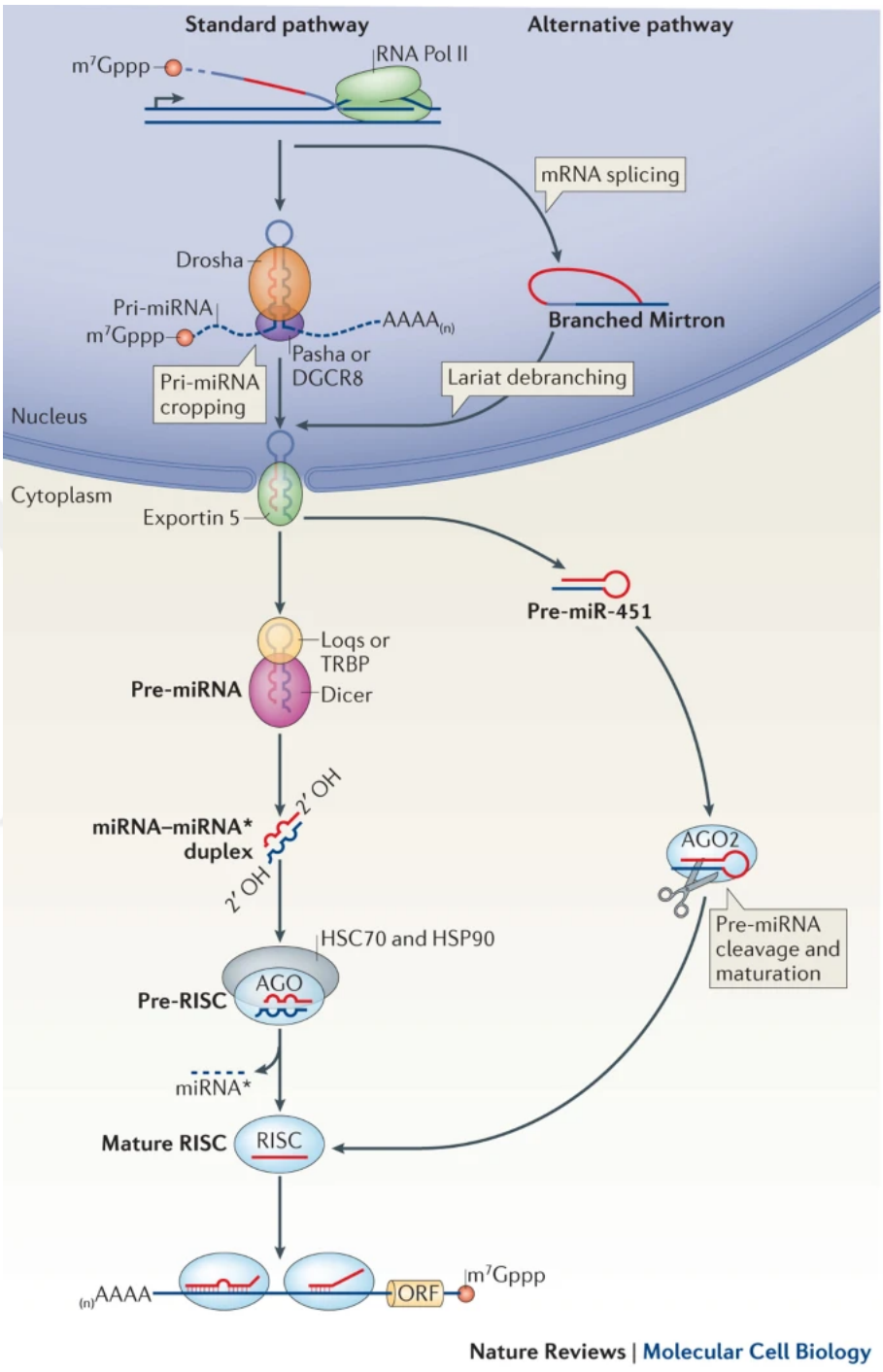


***Figure 12**: miRNA biogenesis*[157]

By accelerating the natural process of mRNA degradation, a miRNA can lower the intracellular concentration of its target mRNAs. It can also prevent the translation or decoding of the target mRNA. The main reason for the occurrence of these two mechanisms is due to the difference between miRNA-mRNA pairs. [158]

miRNAs are also involved in diverse biological processes, including cell differentiation, proliferation, apoptosis, and metabolism. Reports indicate that miRNAs have roles in different mechanisms of disease[159-162].

miRNAs can also be detected in body fluids such as blood, making them attractive candidates as biomarkers. [163] Shortly after the discovery of miRNAs[164], the application of miRNAs as disease biomarkers was first investigated. [165] Chronic lymphocytic leukemia patients have changed miRNA expression patterns, as demonstrated by Calin et al. in 2002. [166] Following this, miRNA expression was discovered to alter during carcinogenesis [167], and it was later discovered that it could be used to accurately categorize a variety of human malignancies. [168] Since then, research has looked into the potential of miRNAs as biomarkers from human blood plasma for disorders such as diabetes [169,170], Alzheimer's disease[171], and a variety of other non-infectious diseases. Moreover, miRNAs might increase the clinical applicability of existing protein- or metabolite-based diagnostics. For instance, a recent study found that the widely used carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) tests were outperformed by the combination of miR-29a and miR-335 with matrix metalloprotease protein-2 (MMP2) in the diagnosis of breast cancer. [172]

Several recent reports have described changes in circulating miRNAs in response to infectious diseases. This supports the use of miRNAs as a potential diagnostic tool against infections. MicroRNAs have been identified as potential biomarkers against to various pathogens such as HIV [173], Ebola [174], tuberculosis [175] and malaria [176]. [165]

One of the intriguing aspects of miRNA biology is their extracellular existence, specifically their presence in extracellular vesicles like exosomes, and the role they play in intercellular communication. The incorporation of miRNAs into exosomes and their subsequent uptake by recipient cells provide a novel mechanism for genetic exchange between cells.

The pathogenesis of atherosclerosis involves a complex interplay between lipid metabolism, immune response, and vascular wall cell function. Is in this complex disease process that the role of miRNAs has been increasingly recognized.

The miRNAs packaged into exosomes are protected from degradation and can be taken up by recipient cells, acting in a paracrine or endocrine manner. [177] In the context of atherosclerosis, exosomal miRNAs could provide a snapshot of the ongoing pathological processes within the atherosclerotic plaques. Some studies have reported altered levels of certain exosomal miRNAs, such as miR-126 [163], miR-155 [178], and miR-92a [179], in the blood of patients with atherosclerosis, suggesting their potential use as diagnostic or prognostic markers.

Research has identified different miRNAs, often called "atheromiRs" involved in the various stages of atherosclerosis, such as endothelial dysfunction, inflammation, foam cell formation,

plaque stability, and thrombosis. For example, miR-33 contributes to atherosclerotic lesion development [180], while miR-21 is implicated in modulating vascular inflammation [181] and neointimal lesion formation [182]. Therefore, miRNAs constitute a novel layer of regulation in atherosclerosis, holding the potential to revolutionize our understanding of this disease.

In conclusion, exploring the role of exosome-derived miRNAs in atherosclerosis may unlock a new realm of possibilities in disease understanding, diagnosis, and treatment. These small RNA molecules may serve as critical biomarkers for atherosclerosis, providing insights into disease progression and response to therapy.

1.10 Exosome Functions in Atherosclerosis Development

Thrombosis, endothelial cell death, vascular inflammation, and the accumulation of monocytes and macrophages are only a few of the multifactorial conditions that are connected to atherosclerosis. Exosomes from endothelial cells, smooth muscle cells, and macrophages have been related to the pathological processes associated with atherosclerosis, such as inflammation, vascular damage, calcification, apoptosis, thrombosis, and coagulation.

1.10.1 The Roles of Monocytes/Macrophages Derived Exosomes in Atherosclerosis

During early atherosclerosis, inflammation triggers the movement of monocytes into arteries. Here, they transform into macrophages, a different type of cell crucial in building atherosclerotic plaques. These monocytes and macrophages release exosomes that affect inflammation, immune response, and blood vessel inflammation, making them key players in atherosclerosis.

1.10.1.1 Monocyte Exosomes Promote Vascular Inflammation, Endothelial Cell Apoptosis and Thrombosis

Friedrich and colleagues [183] found that exosomes from starved human monocytes increased immune cell infiltration and plaque formation in genetically modified mice on high-fat diets. This exacerbated vascular inflammation, a key aspect of atherosclerosis. In lab experiments, they saw these exosomes trigger harmful cellular responses and migration in immune cells. When in contact with endothelial cells, these exosomes increase expression of an adherence molecule, worsening inflammation.

Chen and colleagues [184] noted that exosomes from altered fat particle-treated monocytes carry specific RNA, GAS5 lncRNA that can promote cell death in vascular cells. In contrast, exosomes from cells lacking this RNA reduced cell death.

Maria and colleagues [185] discovered that exosomes can also enhance by preventing caveolin-1 protein expression, endothelial cells can produce less NO. This suggests multiple ways exosomes can impact endothelial cell behavior and worsen atherosclerosis.

1.10.1.2 Macrophage Exosomes Affect Endothelial Cells and Contribute to Atherosclerosis

Macrophage exosomes play several roles in atherosclerosis. They have been found to hinder the movement of endothelial cells, which are cells lining the blood vessels. They also enhance the stickiness of vascular cells, causing more monocytes to adhere to and infiltrate the vessel wall. [186]

Furthermore, macrophage exosomes are involved in the formation of foam cells, a hallmark of early atherosclerosis. They help monocytes take up harmful fats and transform into these foam cells. [187] Moreover, these exosomes promote the movement and adherence of endothelial cells, contributing to plaque development. [188]

In the final stages of atherosclerosis, macrophages contribute to the hardening and narrowing of blood vessels, leading to a heart attack or stroke. They release exosomes that induce microcalcification, making the vessels more brittle. Research also indicates that these exosomes might exacerbate inflammation and plaque progression through the actions of certain molecules, like miR-146a. [189]

In summary, monocyte and macrophage exosomes play a key role in atherosclerosis, impacting inflammation, EC behavior, recruitment of monocytes, formation of foam cells, and calcification of vessels.

1.10.2 The Influence of Endothelial Cell Exosomes in Atherosclerosis

Research indicates that exosomes from endothelial cells, containing a complex array of proteins and mRNA, mediate the interaction between various cells, playing a key role in atherosclerosis development. [190-192]

1.10.2.1 How Endothelial Cell Exosomes Contribute to Vessel Stability

Exosomes' content can influence inflammation, survival of cells and even cancer spread. Therefore, exosomes have a part in regulating blood vessel formation and health. For instance, the Kruppel-like factor 2 (KLF2), a protective factor against atherosclerosis, encourages the release of exosomes rich in miR-143/145 from endothelial cells. [73] These exosomes assist in maintaining the normal function of the smooth muscle cells in our blood vessels. Other studies have found that exosomes carrying certain molecular elements, like miR-214 and miR-126-3p, can also support blood vessel formation. [193-195]

Several complex biological signals control blood vessel health and growth. One important pathway involves angiopoietin-2 (Ang2), a protein mainly produced by endothelial cells, which supports vascular health. Researchers have shown that Ang2 is secreted via exosomes, with its release influenced by various biological signaling pathways. [196,197]

1.10.2.2 How Endothelial Cell Exosomes Contribute to Vascular Inflammation

Certain molecules, such as ox-LDL, homocysteine (Hcy), and heat shock protein 70 (HSP70), are linked to atherosclerosis and heart disease. Ox-LDL and Hcy induce the release of exosomes containing HSP70 from artery endothelial cells, according to studies. [192,198,199] These exosomes can attract circulating white blood cells the ability to attach to vascular endothelial cells, contributing to inflammation in the blood vessels.

Researchers also found that certain non-coding RNAs and enzymes can contribute to inflammation in atherosclerosis. [200,201] Additionally, exosomes from inflamed vascular cells contain certain inflammatory molecules and can alter the behavior of recipient cells to either promote or suppress inflammation. [202,203]

Endothelial cells can release exosomes rich in miR-155 following stimulation by ox-LDL, which can then drive anti-inflammatory cells towards a more inflammatory state. Other molecules, like KLF2, can counter this effect, reducing inflammation. [93,190,204]

Certain processes like neutrophil extracellular traps (NETs) also play a role in atherosclerosis. [205] Exosomes from patients with atherosclerosis can trigger NETs release from white blood cells, worsening the disease. [192]

1.10.3 The Roles of Vascular Smooth Muscle Cell Exosomes in Atherosclerosis

The transformation of SMCs and damage to the lining of blood vessels encourage the development of atherosclerotic plaques. Microscopic vesicles called exosomes, originating from vascular SMCs, carry a molecule called miR-155 from the SMCs to the blood vessel lining, disrupting their tight structure and integrity. [206] This causes increased permeability of the blood vessels, promoting atherosclerosis. Once miR-155 has been delivered, it's overproduced within the endothelial cells, inhibiting their growth, movement, and repair abilities, further increasing vascular permeability. [93,192]

Alexander and his team [207] studied this process using human vascular SMCs grown *in vitro*, which had undergone calcification as an experimental model of atherosclerosis. These SMCs released exosomes rich in calcium and proteins found outside cells. When exposed to high calcium concentrations, these SMCs produced an enzyme called sphingomyelin diesterase 3 and released exosomes high in calcium. [208,209] After exposure to inflammation-inducing molecules, these SMCs released exosomes that accelerated calcification of the blood vessel walls.

In studies involving mice with diabetes, a condition linked to atherosclerosis, researchers found that exosomes from SMCs carrying miR-221/222 were more likely to cause atherosclerosis than in non-diabetic mice. When these diabetic SMC exosomes were introduced into a certain breed of mice with a genetic predisposition to atherosclerosis, the atherosclerotic lesions worsened. [210]

Lastly, rupture of atherosclerotic plaque can cause thrombosis, a significant cause of vascular blockages leading to heart attacks, unstable chest pain, and stroke. [211] The exposure of a molecule called tissue factor (TF) after plaque rupture initiates the clotting process. Research from Kapustin and others [210] has shown that growth-stimulating molecules like PDGF and inflammatory molecules like TNFα could stimulate the release of exosomes from vascular SMCs. This suggesting TF is secreted from small vesicles produced by vascular SMCs under the influence of PDGF and TNFα. As these exosomes can bind to clotting factors, they may contribute to blood clot formation linked to atherosclerosis.

1.11 The Interaction of Endothelial Cells and Vascular Smooth Muscle Cells in Atherosclerosis

Endothelial cells and vascular smooth muscle cell interaction is essential in atherosclerosis progression. EC-VSMC communication has been linked to atherosclerosis. There are research several reports on the interaction between VMSC and EC and their role in atherosclerosis progression.

- Exosomes produced by eCs control VSMC phenotypic alterations, which are crucial in the development of atherosclerosis. [4,212,213] LncRNA LINC01005 is substantially expressed in ox-LDL-treated HUVECs, and as a result, it is also significantly expressed in the exosomes that are produced. This lncRNA stimulates cell migration and proliferation, which causes VSMCs to exhibit a synthetic phenotype. [214] In addition, LINC01005 controls KLF4 gene expression via sponging miR-128-3p, which is a target of KLF4. KLF4 regulates contractile genes negatively; therefore, when LINC01005 modifies the miR-128-3p/KLF4 axis in ox-LDL-treated HUVEC-derived exosomes, it decreases contractile markers like SM22 and -SMA and increases synthetic SMC markers like OPN, which indicate the synthetic phenotype of VSMCs and subsequently cause atherosclerosis. [215,216]
- It has been observed that exosomes expressing miR-155 can be transferred from smooth muscle cells to vascular endothelial cells, causing endothelial damage and ultimately promoting atherosclerosis. [217] By weakening EC barrier function, exosomes produced by ox-LDL-treated VSMCs aid in the development of atherosclerosis. Krüppel-like factor 5 (KLF5), according to earlier research, causes atherosclerosis by encouraging VSMC migration and proliferation. [218,219] Ox-LDL was reported to upregulate KLF5 in VSMCs by Zheng et al. .[217] MiR-155 was concentrated in exosomes generated by ox-LDL-treated VSMCs, where miR-155 levels are increased by KLF5. In ox-LDL-treated VSMC-derived exosomes, miR-155 lowered the expression of tight junction proteins in eCs such as -catenin, vascular endothelial-cadherin, zonula occludens-1, and claudin 1, leading to disruption of the EC barrier. As a result, miR-155-mediated modulation of EC barrier function by VSMC-derived exosomes under atherogenic conditions speeds up the development of atherosclerosis. [190]

- In another study, human aortic smooth muscle cells (HASMCs) were co-cultured with HUVECs, which led to the transfer of miR-143/145 to the smooth muscle cells and the decrease of several target mRNAs. [73] Most significantly, miR-143/145 translocation from smooth muscle cells to endothelial cells appears to be atheroprotective *in vivo*. When eVs from KLF2-transduced mouse endothelium cells were injected into ApoE-/- mice, the size of the aortic lesion was reduced by two times compared to controls. [73]
- Atherosclerosis has been caused by THP-1 monocyte-derived exosomes treated with oxy-LDL. Hu et al. found that exosomes from ox-LDL-treated THP-1 cells had higher expression levels of the long non-coding RNA (lncRNA) LIPCAR. [220] LIPCAR enhances VSMC proliferation and migration by regulating CDK2 and PCNA, while inhibiting EC proliferation by inducing apoptosis. In fact, atherosclerotic patient plasma has significant levels of LIPCAR, which is frequently linked to cardiovascular illnesses. [221,222]

1.12 The Hypothesis of the Thesis

The effect of endothelial exosomes induced by stimuli on smooth muscle cells have been partially described in the literature. These published articles discuss the transmission of exosomal content from endothelial cells to smooth muscles via exosomes and the potential for altering the phenotype of these muscle cells. [213,217,223,224] Furthermore, existing knowledge suggests a phenotypic change in vascular smooth muscle cells, notably under the influence of inflammatory signals such as TNF/LPS. This phenotypic modulation is postulated to be linked with specific microRNAs (miRNAs), which are short non-coding RNA molecules involved in post-transcriptional regulation of gene expression. However, the miRNAs being studied in the context of vascular smooth muscle cells genes, are limited to miR-155[217], miR-143/145 [219,225], and miR-221[226], miR-26a[227], miR-29b[228],miR-125b[229], miR-21[230].

Despite the progress in this field, our research is set to provide data based on physiopathological conditions present in the vessel wall by mimicking the EC-VSMC interactions *in vitro.* In this model, I planned to follow the effects of low concentrations of TNF and LPS, at levels present in the blood of patients under in low grade inflammation conditions. Under these co-culture conditions, I aimed to delineate novel miRNAs that are packaged into formed exosomes through detailed sequencing methods.

Using the *in vitro* co-culture models, our goal is to delineate other potential miRNAs that might be implicated in endothelial-to-smooth muscle signaling and contribute to the nuanced understanding of the progression of atherosclerosis. =tekrar gibi oldu

2. MATERIALS AND METHODS:

2.1 Reagents

VSMC an HUVEC cells were used. HUVECs were isolated from human umblical cord. VSMC were commercially available. Human aortic vascular smooth muscle cells (VSMC) were obtained from ATCC and used in passages 5-12.

Cell Culture: EMB-2 medium (LONZA), FBS(LONZA), Endothelial growth factor (EGF) (LONZA), BBE (Bovine Brain Extract) (LONZA), Fibroblast growth factors (FGF) (LONZA), Hydrocortisone, %1 penicillin-streptomycin, SMBM medium (LONZA), insulin, Glutamine (LONZA), TNF (ThermoFisher), LPS (Sigma), dPBS (LONZA), BCA reagent (Pierce ThermoFisher).

Western Blot, NTA: Sample buffer (Lammeli + SDS buffer) (Bio-Rad), 4-20% ready-made gel (Bio-Rad), 10x tris-glycine-SDS buffer (Bio-Rad), PVDF methanol activated membrane, Bovine serum albumin (BSA), 0.01% NAN3, PBS, Tween20, CD63 and CD81 primary antibody, anti-mouse horseradish peroxidase (HRP) secondary antibody, Enhanced chemiluminescence (ECL from Pierce Thermofisher).

RNA Isolation, miRNA Isolation, cDNA, PCR: Quick-RNA Miniprep Plus kit (Zymo), miRNeasy micro kit (Qiagen), iScript cDNA synthesis kit (Bio-Rrad), miScript RT II kit, miRCURY LNA II RT kit, QuantiNova SYBR Green mix (Qiagen), miScript Primer Assays, miScript SYBR Green PCR kit, miRCURY LNA miRNA SYBR Green PCR kit.

2.2 Cell culture

cHUVEC and VSMC were used between passages 19-25 and 7-8, respectively. cHUVEC were cultured in EBM-2 (LONZA) with 5% FBS and supplemented with EGF, BBE, FGF, Hydrocortisone and 1% penicillin-streptomycin. While VSMC were cultured in SMBM (LONZA) with 2% FBS and 1% penicillin-streptomycin, and supplemented with insulin, EGF, FGF and 2 Mm glutamine under sterile conditions. Media was changed every 2 days (48 hours). For the experiment in which the supernatants are collected, media was used where FBS was replaced by exosome-depleted FBS having the same concentration (v/v).

In my project, I am utilizing different cell culture models to simulate the complex environment of atherosclerosis *in vitro*.

Three different cell culture method was used to mimic the atherosclerotic environment; transwell/direct co-culture method, indirect culture method and isolated exosome treatment method.

The first model is the **direct co-culture using a transwell system**, which involves placing VSMCs and eCs in separate compartments of a special insert. This setup allows the two cell types to

communicate and exchange signaling molecules, closely mimicking the interactions that occur between VSMCs and eCs in the blood vessel wall during atherosclerosis. (**Figure 13**)

The second model is the **indirect culture** approach, where VSMCs and eCs are cultured separately but their culture media is switched, such that one cell receives the other's supernatant. This model allows for the paracrine signaling between the two cell types, as they secrete factors into the shared media, creating a microenvironment that resembles the conditions found in atherosclerotic vessels. (**Figure 14**)

Lastly, the **isolated exosome treatment model** involves isolating exosomes released by ECs, and treating the VSMCs with these exosomes. This method, adding exosomes isolated from a cell supernatant onto another cell, is widely used in the literature. [231-235] Exosomes carry various bioactive molecules, including proteins, lipids, and nucleic acids, which can play a role in atherosclerosis development. By subjecting VSMCs and ECs to exosome treatment, I can investigate the specific effects and signaling pathways involved in atherosclerosis progression. (**Figure 15**)

By employing these diverse cell culture models, I aim to capture different aspects of the atherosclerotic environment, enabling a comprehensive understanding of the role of exosomes and the communication between EC and VSMC, and the contribution of this interaction to atherosclerosis.

Exosome-depleted FBS: Fetal Bovine Serum (FBS) is rich with vesicles of varying sizes including large microvesicles and exosomes. In order to eliminate the effect of exosomes coming from FBS on cultured cells in our models, we isolated exosomes from the FBS and used it as "exosome-depleted FBS". [236,237] Making sure that the effect we are getting in our models' results are due to the exosomes secreted by the cells only. Exosome-depleted FBS was obtained by differential centrifugation steps, where the last centrifugation step was using ultracentrifuge (Coulter Beckman, Optima XPN-80 Ultracentrifuge, Rotor Type 70.1 Ti) at a speed of 100,000xg for 16 hours (4^0C) and the supernatant was collected and kept.

2.2.1 Cell Characterization by Immunofluoresence

- **Sample Preparation:** Human aortic smooth muscle cells (VSMCs) are seeded in a T75 plate with 80% confluency and then trypsinized and inactivated using Vascular Smooth Muscle Cells Special Media Immunofluorescence2% FBS. The cell suspension is then removed, centrifuged, and resuspended. Cell counts are performed using Trypan Blue, a dye that enters dead cells and colors them blue.
- **Coverslip Preparation:** 14 mm coverslips are coated with sterile 2% gelatin, which helps cells adhere more effectively, mimicking the extracellular matrix. The coated slips are then left to dry in the sterile cabin under UV light and stored at 4 ^{0}C until use. Cells are then seeded onto each gelatin-coated coverslip and incubated at 37 ^{0}C for 24 hours to allow cells to attach and reach the proper confluency.

- **Incubation with molecules:** Certain coverslips were treated with TNF or LPS, while some were not treated and marked as controls.
- **Cell Fixation:** At the end of the incubation, the cells are washed with 1X PBS and fixed with 100% cold methanol, which permeabilizes the membrane and preserves epitopes without modifications. After this, cells are removed from the laminar flow, washed with 1x PBST, and the coverslips with fixed cells are placed in a petri dish covered with paraffin.
- **Cell Permeabilization:** Cells are further permeabilized with 0.25% Triton X-100 for 10 min, a popular detergent for improving the penetration of the antibody and washed with PBST.
- **Blocking and Immunostaining:** Cells are incubated with 2% BSA in PBST for 30 min to block unspecific binding of antibodies. Actin is then stained using Mouse Alpha-Smooth Muscle Actin Monoclonal Antibody (ThermoFisher Scientific, cat:MA5-11547), which are diluted in 2% BSA in PBST. After incubation at 4 ^{0}C overnight, cells are washed again with PBST.
- **Secondary Antibody Staining:** Goat anti-Mouse IgG Alexa 594 (ThermoFisher Scientific, lot: 1966996, ref:R37121) are used as secondary antibody against the primary antibody. It is also diluted in 2% BSA in PBST and added to the cells, which are incubated at room temperature in the dark for 1 hour.
- **Final Steps:** After secondary antibody incubation and further washing with PBST, DAPI mouting buffer (a DNA-specific probe) (Abcam, cat: ab104139) is added to the slides. The DAPI enables the staining of the nuclei in each cell. The coverslips are flipped onto the slides (cells facing DAPI) and sealed with nail polish to prevent drying. The slides are then stored in the dark at 4 ^{0}C until visualization. The imaging using a fluorescent microscope was done within a week to prevent signal fading.

2.2.2 Transwell/Direct Co-culture:

The co-culture of HUVEC (used between passages 19-25) and VSMC (used between passages 7 and 8) was performed using a Transwell system with sterile SARSTEDT TC-insert 6well, pore 0.4 μm PET, transparent membrane. [73] The HUVEC were planted at a seeding density of 120.000 in the lower wells (6-well plate) and VSMC were planted at a seeding density of 90.000 cells in the upper wells. (Figure 2) After 24h incubation, co-cultured cells were treated with TNF or LPS, in addition to a non-treated control group for 72 hours. In different experiments, 4 or 5 wells were used for each treatment/control. After 72h of incubation, **cells** and **conditioned media** were collected from wells and inserts. (**Figure 13**)

RNA isolation from cells; Total RNA isolation was performed from VSMC and EC that were grown in the inserts or the bottom of the plates. Their total RNA was converted to cDNA. Then these cDNA samples were used to measure the expression of genes by performing quantitative real-time PCR.

Exosome isolation from conditioned media; Exosomes were isolated by using several centrifugation steps (the last step was performed by ultracentrifuge) (Explained in detail below). Isolated exosomes were characterized and used for miRNA isolation.

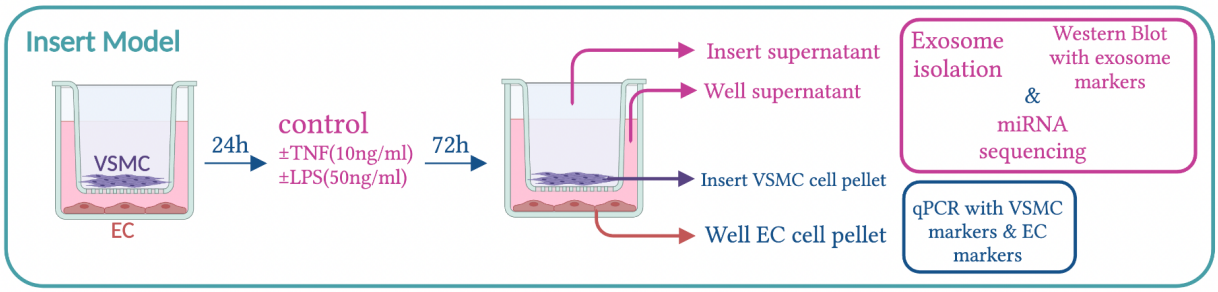


Figure 13: Transwell/direct method

2.2.3 Indirect method

VSMC and EC were seeded at the seeding density of 120.000 cells into the wells separately. They were incubated for 24h with appropriate conditions, then the cells were either treated with TNF or LPS, in addition to a non-treated control group for 72 hours. Cells were collected separately to make qPCR. (**Figure 14**)

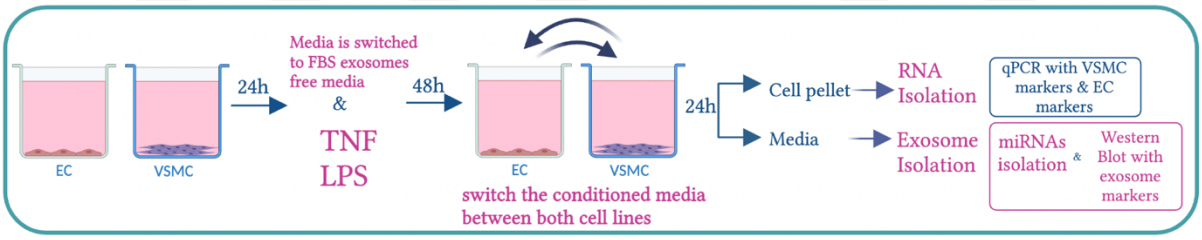


Figure 14: Indirect method

2.2.4 VSMC treated by EC isolated exosome method

Most experiments in this thesis have been made using this co-culture model. Therefore, for this model, large numbers of cells were grown in multiple T75 to obtain large exosome yields (**Figure 15**).

12 T75 flasks HUVEC (Passage 33) were propagated until confluency. 4 T75 was kept as control where media was changed to conditioned exosomes-free FBS. The other 8 flasks also changed to media with exosome-free FBS free in EBM2, 4 flasks were treated with TNF (10ng/ml) the other 4 with LPS (50ng/ml). All 12 flasks were incubated for 72 hours. Exosomes were isolated through ultracentrifugation as described below and characterized through western blot and NTA. BCA assay was performed to see exosome concentration and according to BCA results, concentration of exosome, that used for treatment, was calculated.

6 T75 of VSMC (P7) were propagated until confluency. 2 T75 were kept as control where media was changed to conditioned exosomes free FBS. The other 4 flasks also changed to conditioned media exosomes FBS free in SMBM, 2 flasks were treated with 231 µg Endothelial cells isolated exosomes CTRL, 2 flasks where treated with 231 µg Endothelial cells isolated exosomes TNF, 2 flasks where treated with 231 µg Endothelial cells isolated exosomes LPS, all incubated for 72 hours.

The amount of exosomes added was calculated from experiments performed in our laboratory as part of a TÜBİTAK 1001 project. In this project, we have determined that 20 ug exosomes (as measured by BCA) added to 100000 VSMC have effects on the cell phenotype, without a toxic effect.

Following a 72 hour incubation, the supernatant was collected and exosomes were isolated as described in detail below.

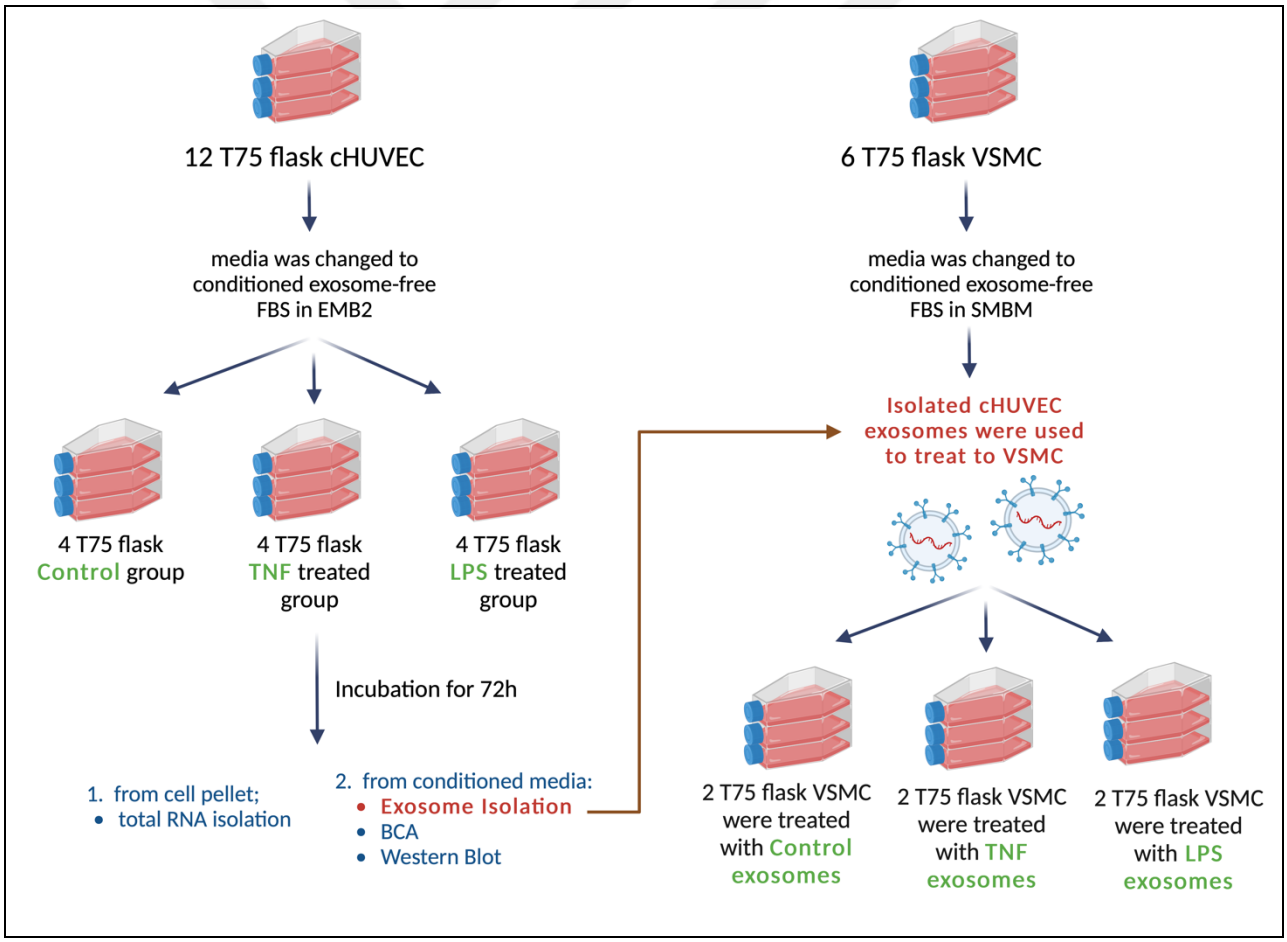


***Figure 15**: Exosome Treatment Experiment.*

2.3 Total RNA isolation from cells

At the completion of the co-culture experiments, the EC or VSMC were collected and pelted by trypsinization and the collected cells (pellet) were used for total RNA isolation using Quick-RNA Miniprep Plus Kit (ZYMO).

Nanodrop was used to measure total RNA. 1µL RNA samples were used to measure its concentration. In Nanodrop measurements, the 280/260 value and 280/230 value are used to assess the purity of nucleic acid samples. They are ratios calculated from the absorbance readings at specific wavelengths.

280/260 value: This ratio compares the absorbance at 280 nm (A280) to the absorbance at 260 nm (A260). It is commonly used to estimate the purity of DNA and RNA samples. A pure nucleic acid sample will have an A260 reading indicating its concentration, while the A280 reading provides information about the presence of contaminants such as proteins or other organic molecules. A higher 280/260 ratio (~2) indicates a purer nucleic acid sample.

280/230 value: This ratio compares the absorbance at 280 nm (A280) to the absorbance at 230 nm (A230). The 280/230 ratio is used to assess the presence of contaminants such as salts, phenol, or other organic molecules that can interfere with nucleic acid measurements. A higher 280/230 ratio (>1.8) suggests a lower level of contamination in the sample.

2.4 Exosome Isolation:

For exosome isolation, supernatants from cultured cells were isolated, and differentiation centrifugation steps were applied. [238-240] Supernatants were kept throughout the first 4 centrifugation steps.

- First-step supernatants were centrifuged at 1500xg for 5 minutes (4^0C).
- The second step; supernatants of the first step at 3000xg for 10 minutes (4^0C).
- Third step supernatants of the second step at 10.000xg for 30 minutes (4^0C).
- The last step to isolate exosomes from collected supernatants by ultracentrifuge (Coulter Beckman, Optima XPN-80 Ultracentrifuge, Rotor Type 70.1 Ti) at speed of 100.000xg for 2 hours, (**Figure 16**) The supernatants were discarded and the pelleted exosomes were resuspended in 400-500 µl dPBS (LONZA).

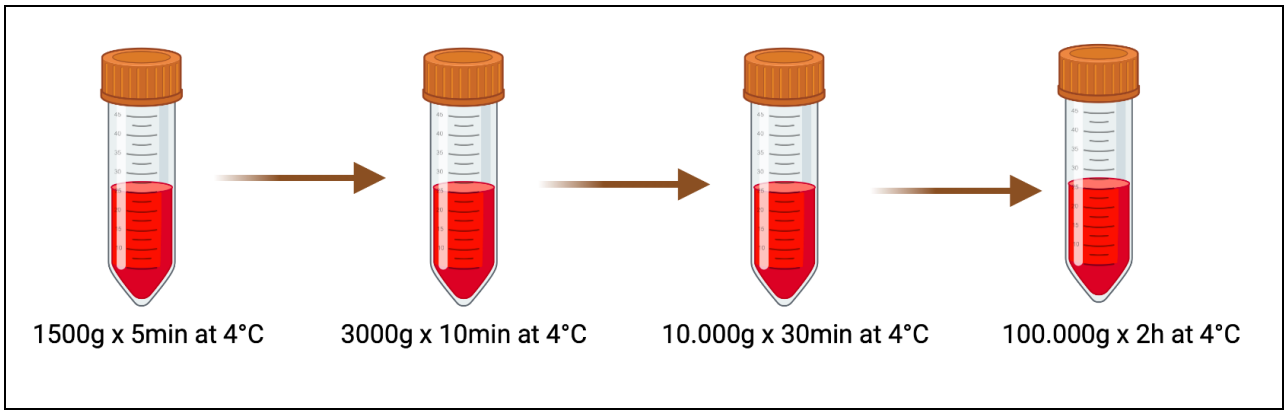


Figure 16: *Centrifugation Steps for Exosome Isolation*

2.5 Exosomal protein quantification through BCA assay:

Isolated exosomes were diluted 5 times and BCA assay was applied to quantify the protein concentration. BCA reagent used (Pierce Thermofisher) incubated for 30 minutes at 37^0C.

2.6 Exosomes characterization through Western Blot

10 µg of exosomal proteins mixed with sample buffer (Bio-Rad) and incubated at 95°C for 5 min, then loaded into 4-20% ready-made gel (Bio-Rad). 10x tris-glycine-SDS buffer (Bio-Rad) diluted to 1X and used as a running buffer. Proteins were transferred to PVDF methanol activated membrane through wet transfer. These were subsequently blocked with 5% BSA in PBS-T for 1 hour at room temperature. Then membrane was washed between each step 3 times each for 10 minutes at RT in PBS-T. CD63 and CD81 primary antibodies were used at dilution of 1:1000 diluted in 2%BSA in PBST and 0.01% NAN3. Secondary antibody used anti-mouse HRP diluted at 1:5000 in 2% BSA in PBST. Primary Antibodies were incubated separately overnight at 4^0C. Secondary antibody was left for 2 hours at RT. Images were obtained after ECL addition (Pierce ThermoFisher). Images were obtained using Chemidoc Bio-Rad.

2.7 Exosome characterization through nano tracking analysis (NTA)

Isolated exosomes were analyzed using the Nanosight NS300 instrument. The analysis settings were optimized and kept constant between samples, and each video (5 videos per sample) was analyzed to give the mean, mode, median, and estimated concentration for each particle size. All experiments were carried out at 1:500 dilutions, yielding particle concentrations in the region between $1x10^8$ and $1x10^{10}$ particles per ml. Twice-filtered distilled water was used as a diluent.

2.8 miRNA isolation from exosomes

miRNA extracted from isolated 100 µL exosomes by using miRNeasy Micro Kit (Qiagen) (Lot:175012576, Mat:1071023). The manufacturing protocol of the kit has been followed. Isolated miRNA was measured with 3 different methods; Agilent 2100 Bioanalyzer using Agilent RNA

Pico 6000 kit (Part no:5067-1513) , Agilent Tapestation 4150 Agilent High Sensitive RNA ScreenTape(Part no: 5067-5579), and Qubit 3.0 using RNA HS Assay kit (cat:Q32852).

The Agilent 2100 Bioanalyzer measures the concentration based on electrophoretic mobility, relying on the migration pattern of the RNA molecules. It also shows the purity of the miRNA sample. The results of miRNA samples are different from the results of total RNA samples in the measurement of the Agilent 2100 Bioanalyzer. 18S and 28S rRNAs are observed in the total RNA samples, but have been removed from the miRNA samples. (**Figure 17**)

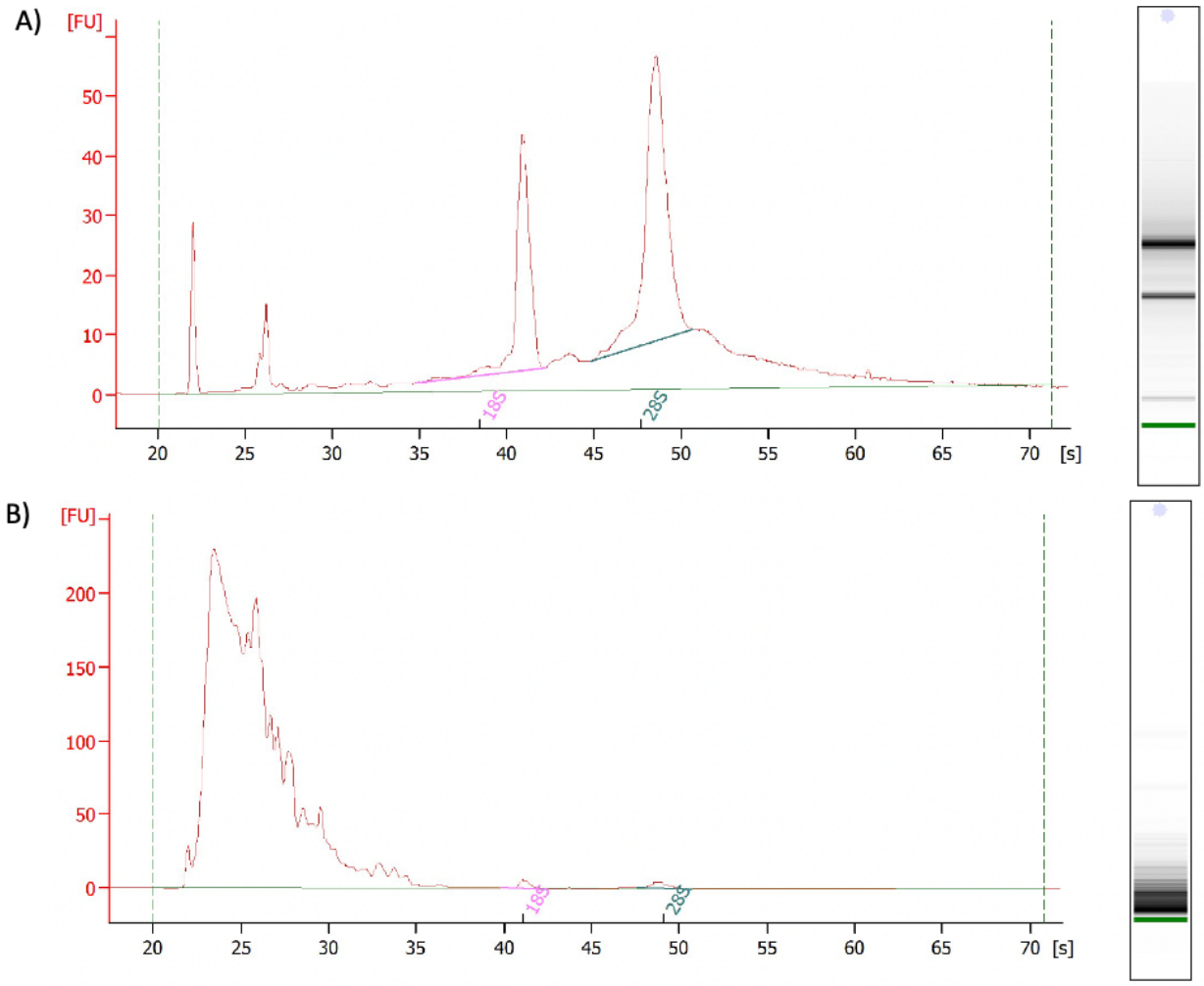


***Figure 17**: Representative Bioanalyzer results from total RNA sample and miRNA sample.*

A) representative total RNA (mRNA) result B) representative miRNA result

2.9 cDNA synthesis

For total RNA:

Total RNAs isolated from the cells were converted to cDNA using the iScript cDNA synthesis kit from Bio-Rad (Cat:1708891), which is optimized for gene expression analysis using real-time qPCR. 1µg cDNA was converted from each RNA samples into a 20 µl reaction.

For miRNA:

miRNA extracted from exosomes, converted to cDNA using miScript RT II kit (cat:218161) and miRCURY LNA II kit from Qiagen. (cat:339340)

2.10 Quantitave Polymerase Chain Reaction (qPCR)

For total RNA:

The converted cDNAs were used for qPCR analysis using the iScript SYBR Green PCR kit, and the Roche LightCycler 480 machine. VSMC and EC-specific primers were used (Table 1).

As previously mentioned in cDNA part in above, 1µg cDNA was converted from RNA samples. Then 1:10 dilution was made and from this dilution, 4µl diluted samples were added into the 25µl PCR reaction. Each reaction was made in triplicate.

For miRNA:

The converted cDNA from miRNA was used for qPCR analysis using miRNA-specific primers called miScript Primer Assays, miScript SYBR Green PCR kit, miRCURY LNA RT Kit (Ref:339340), miRCURY LNA miRNA SYBR Green PCR kit (Ref: 339345), and Roche Light Cycler 480 machine.

Gene	**Gene name**	**Primer sequence (F,forward; R,reverse)**	**Product size**
ACTA2[241]	Alpha smooth muscle actin	F: CGA TGC TCC CAG GGC TGT TT R: TTC GTC ACC CAC GTA GCT GTC TTT	100
SM22α[242]	Smooth muscle 22 alpha	F: AAC AGC CTG TAC CCT GAT GG R: CGG TAG TGC CCA TCA TTC TT	212

MHY11[29]	Myosin heavy chain 11	F: TGG AAC TTC ATC GAC TTT GGG R: ACA GCT TCT CCA CGA AAG AC	145
OPN[243]	Osteopontin	F: CGA GGT GAT AGT GTG GTT TAT GG R: GCA CCA TTC AAC TCC TCG CTT TC	128
KLF4[215]	Krüppel-like factor 4	F: CCC AAT TAC CCA TCC TTC CT R: CGT CCC AGT CAC AGT GGT AA	259
ICAM-1[244]	Intercellular adhesion molecule 1	F: AGC GGC TGA CGT GTG CAG TAA T R: TCT GAG ACC TCT GGC TTC GTC A	115
VCAM-1[244]	Vascular cell adhesion molecule 1	F: GAT TCT GTG CCC ACA GTA AGG C R: TGG TCA CAG AGC CAC CTT CTT G	118

Table 1: Primer tables for VSMC (ACTA2, SM22α. MHY11, OPN, KLF4) and EC (ICAM-1, VCAM-1.)

The ΔΔCt (Delta-Delta Ct) method is commonly used in quantitative polymerase chain reaction (PCR) to analyze gene expression changes between different samples or experimental conditions. [245] It allows for relative quantification of gene expression levels by comparing the Ct (cycle threshold) values of the target gene with that of a reference gene. In our experiment reference gene is GAPDH. [245]

Ct determination: Perform the PCR amplification for both the target gene and the reference gene in all the samples being analyzed. The Ct value represents the cycle number at which the fluorescence signal reaches a predefined threshold level, indicating the detection of the amplified product.

Ct normalization: Calculate the ΔCt values by subtracting the Ct value of the reference gene from the Ct value of the target gene for each sample. This normalization step helps account for variations in sample input and PCR efficiency.

$$\Delta Ct = Ct(\text{target gene}) - Ct(\text{reference gene})$$

ΔΔCt calculation: Choose a reference sample or condition (control) and calculate the ΔΔCt value by subtracting the ΔCt value of the reference sample from the ΔCt value of each of the other samples.

$$\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference sample})$$

Fold change calculation: The ΔΔCt value represents the difference in gene expression between the target sample and the reference sample. To determine the fold change in gene expression, calculate $2^{(-\Delta\Delta Ct)}$, which gives the relative expression level of the target gene in the target sample compared to the reference sample.

$$\text{Fold change} = 2^{(-\Delta\Delta Ct)}$$

The ΔΔCt method assumes that the amplification efficiencies of the target gene and reference gene are approximately equal. It provides a relative quantification of gene expression, indicating whether the target gene is upregulated (fold change > 1), downregulated (fold change < 1), or unchanged (fold change = 1) in the target sample compared to the reference sample or condition. It's important to note that proper experimental design, appropriate selection of reference genes, and validation of PCR efficiency are crucial for accurate and reliable ΔΔCt analysis.

2.11 Library preparation

miRNA library was made by using TAKARA SMARTer smRNA-Seq kit. The manufacturing protocol of the kit has been followed. (**Figure 18,19**)

TAKARA SMARTer smRNA-Seq Kit for Illumina Components (cat:635032)

TAKARA Index Primer Set HT for Illumina_12 (cat:634871)

TAKARA SeqAmp DNA Polymerase

MN Nucleospin Gel and PCR Clean-up Kit (lot:2208-001)

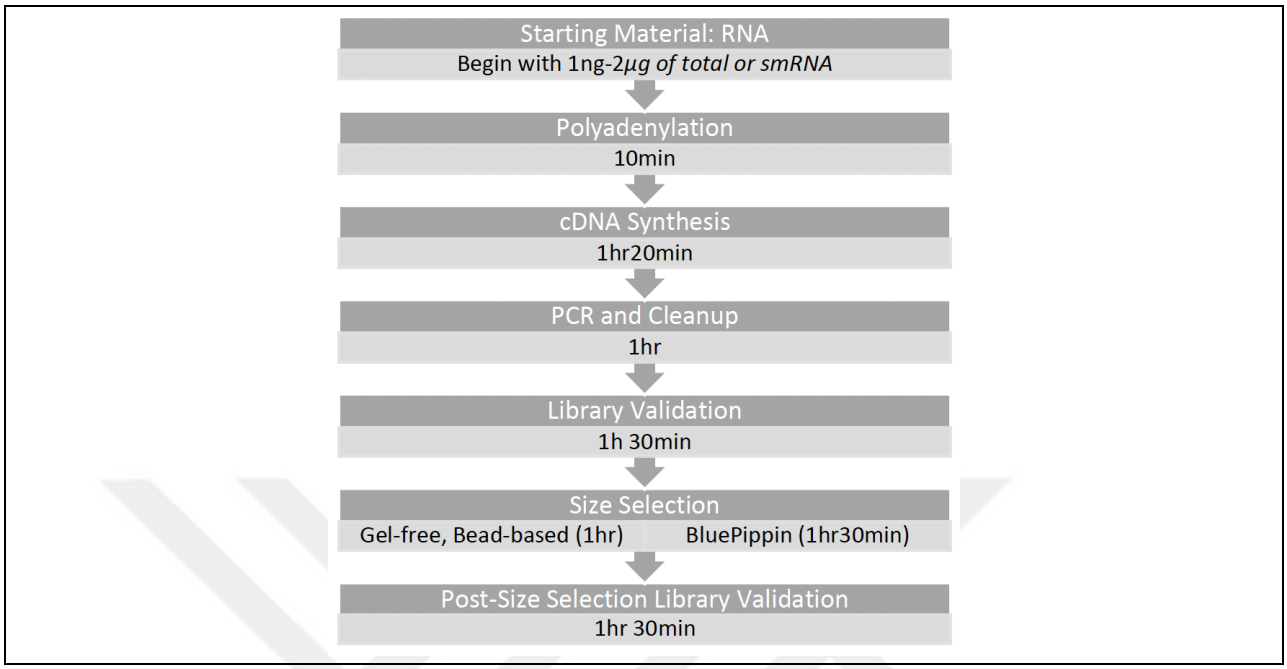


Figure 18: *Library Preparation Steps (this table was taken from the TAKARA SMARTer smRNA-Seq Kit Handbook)*

Size selection

In the size selection steps, we used AMPure XP beads. To bind bigger DNA fragments, AMPure XP beads are first added to the sample at a low concentration. The beads that contain the larger fragments are eliminated in this stage. Then, more beads are added to the supernatant to increase the PEG and NaCl concentrations, causing smaller fragment sizes to bind. [246]

Measurement of samples after library preparation

cDNAs were measured before and after treatment with the magnetic beads. These measurement steps were performing by using Qubit 3.0 dsDNA HS Assay kit (ref:Q32851)

Key features that make "TAKARA SMARTer smRNA-Seq kit" for small RNAs

The key features of the TAKARA53iRbaseR RNA kit that make it suitable for small RNA sequencing are:

- **Size selection**: This kit allows for efficient size selection of small RNAs. Small RNAs have specific size ranges, and the kit enables the isolation and enrichment of these small RNA molecules for sequencing.
- **Adapter ligation**: The kit provides optimized protocols for adapter ligation, which is a crucial step in preparing small RNA libraries for sequencing. Adapter ligation enables the small RNAs to be linked to the sequencing adapters, which are necessary for the subsequent steps of sequencing.

- **Bias reductio**n: Small RNA sequencing requires special considerations to minimize bias and ensure accurate representation of different small RNA species. The kit incorporates steps to reduce bias during library preparation and sequencing.
- **Data analysi**s: Small RNA sequencing data analysis differs from the analysis of data obtained through regular sequencing. Small RNA sequencing data requires specific bioinformatics tools and pipelines to accurately identify and quantify the small RNA species and their expression levels.

In summary, the TAKARA54iRbaseR sm RNA kit is tailored for small RNA sequencing and includes features like size selection, optimized adapter ligation, and bias reduction to ensure accurate representation of small RNA molecules. It is distinct from regular sequencing kits, which are designed for different types of genomic or transcriptomic sequencing and lack the specialized steps for handling small RNA molecules.

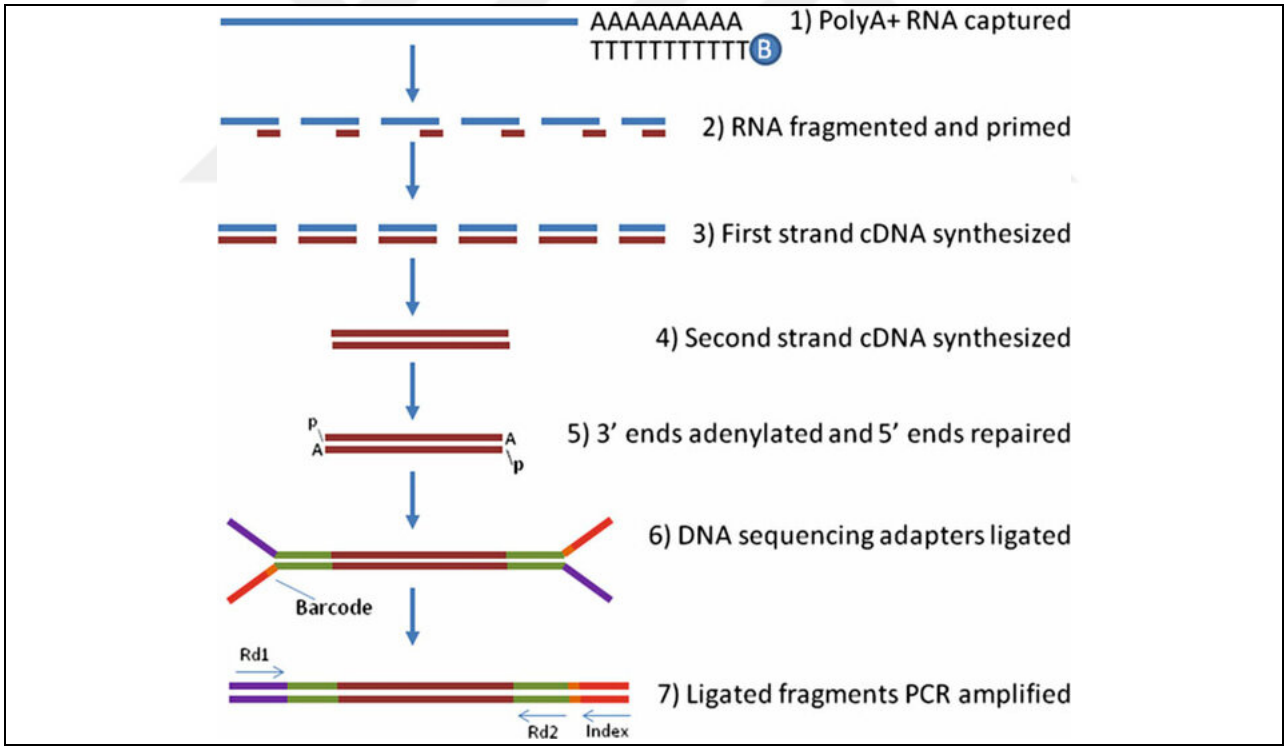


***Figure 19**: Library preparation steps before sequencing.*[247]

2.12 Library Sequencing:

In our study, we outsourced the New-Generation Sequencing (NGS) of miRNA samples to a service provider. As a research team, we sent our samples to an external company to perform the

sequencing and obtain the necessary data. This approach allowed us to leverage the expertise and resources of the service provider, ensuring high-quality and accurate sequencing results for our miRNA samples. "**Illumina NovaSeq 6000**" machine was used for sequencing. (**Figure 20**)

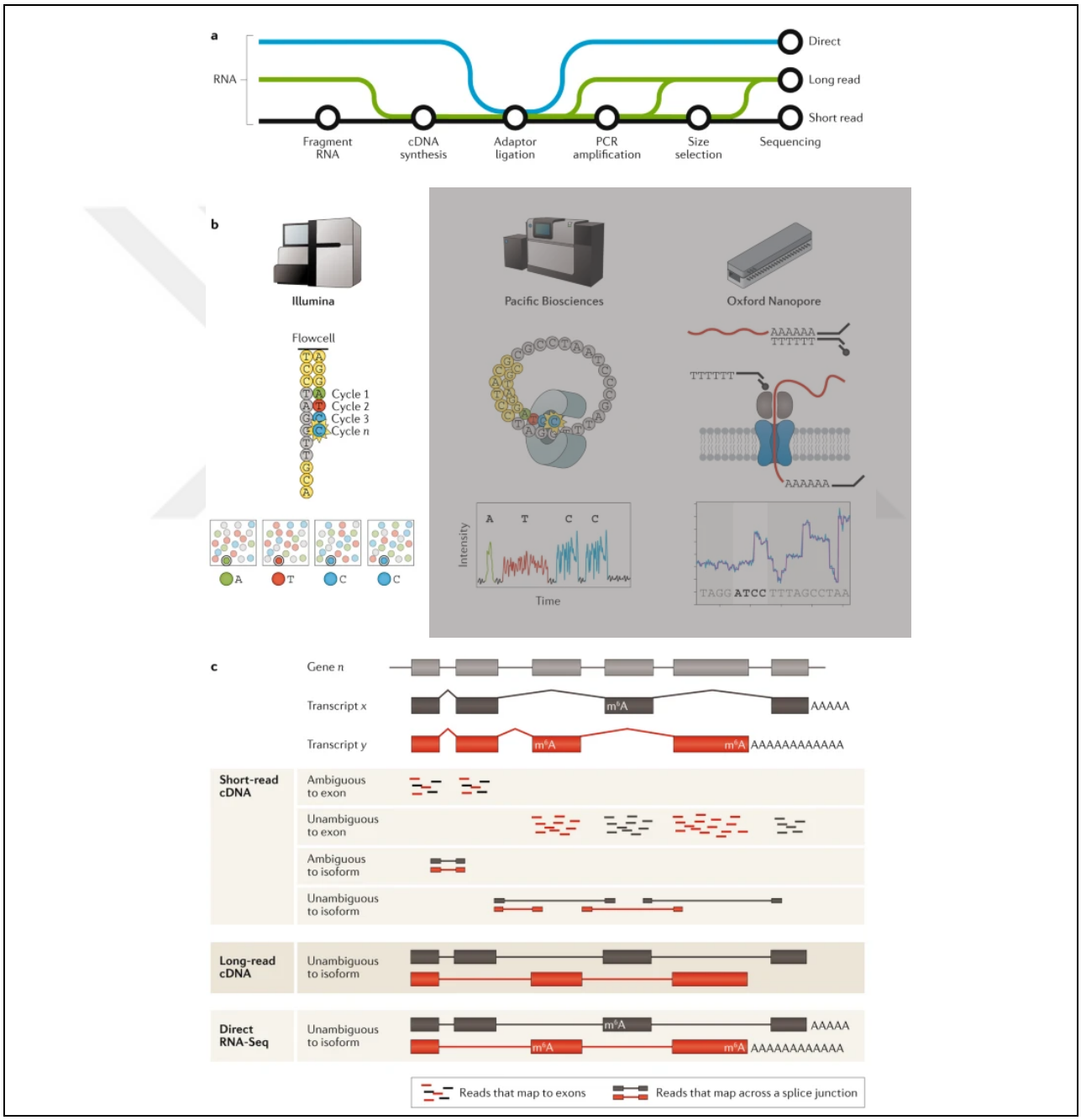


***Figure 20**: Overview of library preparation and sequencing steps.*[248]

***a)** The methods used to prepare libraries for various RNA-sequencing (RNA-seq) techniques are shown in the figure. These techniques can be classified as short-read sequencing (black), long-read cDNA sequencing (green), or long-read direct RNA-seq*

(blue). ***b)*** *An overview of the three primary RNA-seq sequencing technologies is presented. The Illumina workflow (left panel) shows the clustering of individual cDNA molecules on a flowcell for sequencing by synthesis employing 3 blocked fluorescently tagged nucleotides following library preparation. The developing DNA strand is scanned during each cycle of sequencing to determine which of the four fluorophores has been integrated, and readings of 50–500 bp can be produced.* ***c)*** *Short-read, long-read, and direct RNA-seq analysis comparison. Transcripts x and y are examples of alternatively spliced isoforms that are created from more than 90% of human genes (gene n).*

2.13 Bioinformatics analyzes:

RNA-seq data alignment and feature counting

For mRNA sequencing data, we aligned the reads to the reference genome (GRCH38) with Subread, a high-performance read aligner. We chose Subread with optimates parameters for small RNAs (>15nt) due to its high alignment accuracy and efficiency. [249] The gene expression counts were generated using the FeatureCounts function from the Subread package. This function counts mapped reads for genomic features such as genes, exons, promoters, gene bodies, genomic bins, and chromosomal locations. We used the most recent Ensembl annotation file to assign reads to genes. In parallel, for the microRNA sequencing data, we aligned the reads to the56iRbasee reference database (miRbase v22) using the Subread aligner. [250,251] FeatureCounts was again utilized to enumerate the read counts aligning to each annotated microRNA.

Identification of Differentially Expressed Genes and miRNAs

To identify differentially expressed mRNAs and miRNAs, we utilized the DESeq2 package from Bioconductor. DESeq2 uses a model based on the negative binomial distribution to estimate variance and test for differential expression, providing a robust framework for handling biological variability. [252] The raw count data from FeatureCounts was used as the input. We considered genes and miRNAs with an adjusted p-value (using Benjamini-Hochberg procedure) less than 0.05 and a |log2 fold change| > 1 as differentially expressed.

3. RESULTS:

3.1 Cell Culture

VSMC and EC were cultured in a T75 flasks under sterile conditions. (**Figure 21**). Cells were grown with no mycoplasma contamination.

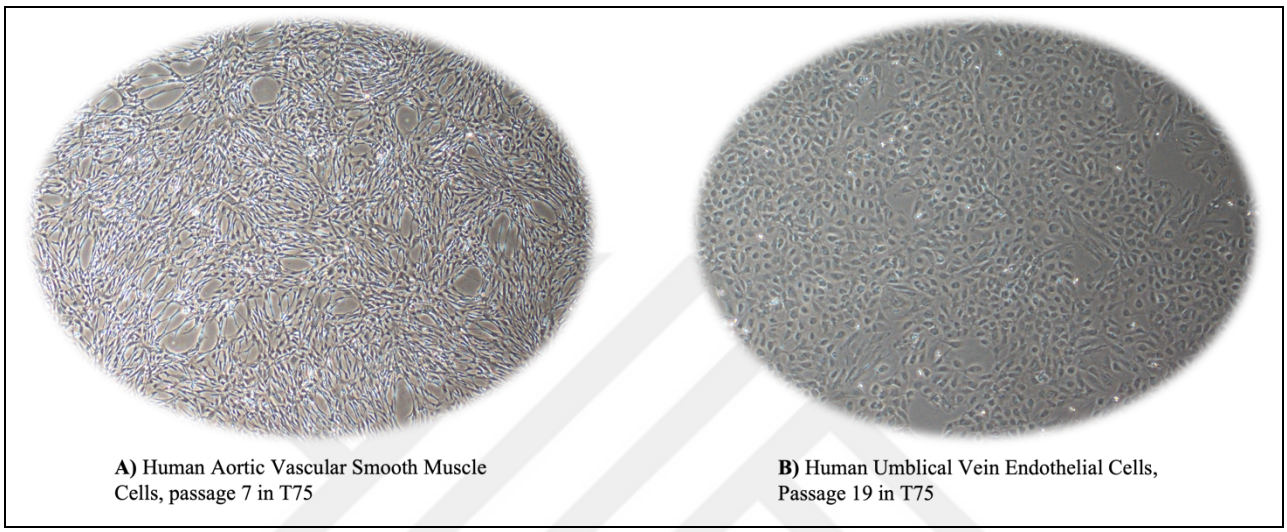


***Figure 21**: Vascular smooth muscle cells (VSMCs) and Endothelial Cells (eCs) images from T75 flask.*

3.2 Cell-cell Communication Models for Atherosclerosis

Three different culture methods were performed by using VSMCs and eCs; "**Direct/Transwell co-culture**", "**Indirect culture**" and "**Isolated exosome method**". We compared the efficiency of these methods and their effects on cell-cell communication. We aimed to measure the effect of low-grade inflammation in these settings. For this, we used two molecules that would generate n inflammatory response **TNF (10ng/ml)** and **LPS (50ng/ml)**.

In these 3 different methods, phenotypic changes were observed in both cells types. The effect of the direct/transwell culture model on the change of phenotype was compared with the effect of the indirect and isolated exosome-treated models on the change of phenotype. In Table 2, there are representative data of total RNA concentration from 3 different culture types. VSMC seeding density was 450.000 cells, EC seeding density was 600.000 cells for Direct co-culture and Indirect culture methods. But in Isolated exosome method, VSMC and EC seeded density were $1x10^6$. To make the comparison, results from isolated exosomes models were normalized according to the same number of cells. In **Table 2**, there are total concentration of the RNA from 3 different methods.

	Direct/transwell co-culture	Indirect cell culture	Isolated exosome treatment
VSMC_Control	1419	864	308,7
VSMC_TNF	1177	742	177,6
VSMC_LPS	1170	851	105,4
EC_Control	497	502,9	622,6
EC_TNF	486,3	541	842,3
EC_LPS	466,3	492,9	404,3

Table 2: Representative data for isolated RNA amount in different cell culture models. (ng/uL) (Measured by Nanodrop 2000C). For VSMC, cell number was normalized to 450.000; for EC, cell number was normalized to 600.000 .

3.3 Phenotypic Changes of Cells by using Immunofluorescence

Immunofluorescence analysis was employed to visualize and quantify the alterations in actin expression, an indicative marker of Vascular Smooth Muscle Cells (VSMCs) phenotypic modulation, under various conditions. The VSMCs were divided into three groups: control, TNF-treated, and LPS-treated. (**Figure 22**) In experiments made in parallel to co-culture experiments, the cells were treated with **TNF (10ng/ml)** and **LPS (50ng/ml) for 72 hours**.

In the control group of VSMC, the cells demonstrated robust actin expression, representative of their contractile phenotype. Conversely, both the TNF and LPS-treated groups showed a significant reduction in actin expression. The observed changes corroborate well with the role of TNF and LPS as potent inflammatory stimuli, often implicated in the onset and progression of vascular pathologies where VSMC phenotypic modulation is a critical element.

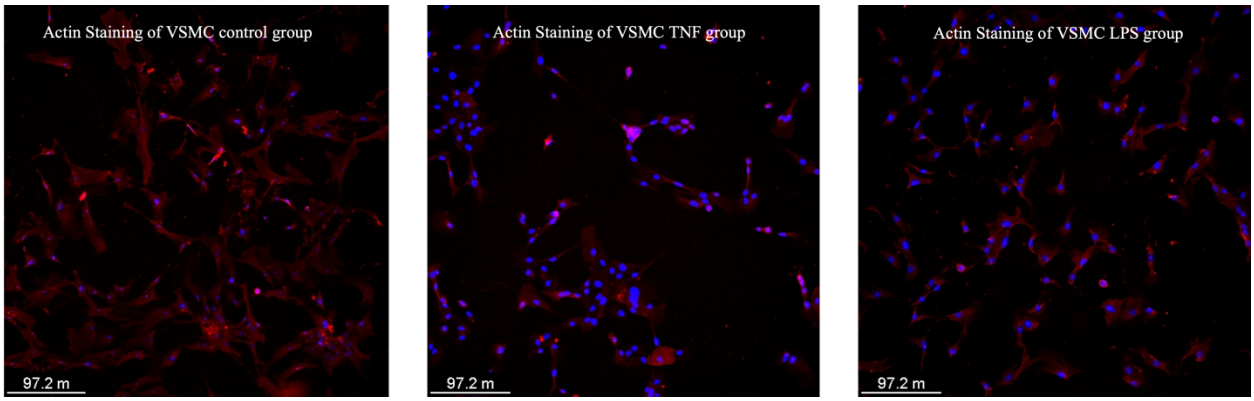


Figure 22: Immunoflouresence Actin staining for Vascular Smooth Muscle Cells

3.4 qPCR with VSMC and EC markers

TNF treatment was performed for eCs and LPS treatment was performed for VSMCs. We wanted to measure the phenotypic changes in cells in co-culture and compare the treated groups with controls.

qPCR was performed to see the phenotypic changes in the transcriptional level of cells in these treatment conditions. We focused on several relevant genes for VSMCs and eCs;

VSMC: ACTA2, SM22α, MHY11 and OPN genes are a contractile-phenotypic marker of VSMC. According to these genes' expressions, we can conclude that VSMC has undergone phenotypic changing in TNF and LPS conditions, VMSC were lost their contractile phenotype. OPN used as a synthetic-phenotypic marker of VSMC. According to OPN gene expression, we can conclude that VSMC has synthetic phenotypic changes following TNF and LPS treatment. (n=4 for direct co-culture, n=1 for indirect and exosome treatment experiment.) (**Figure 23**)

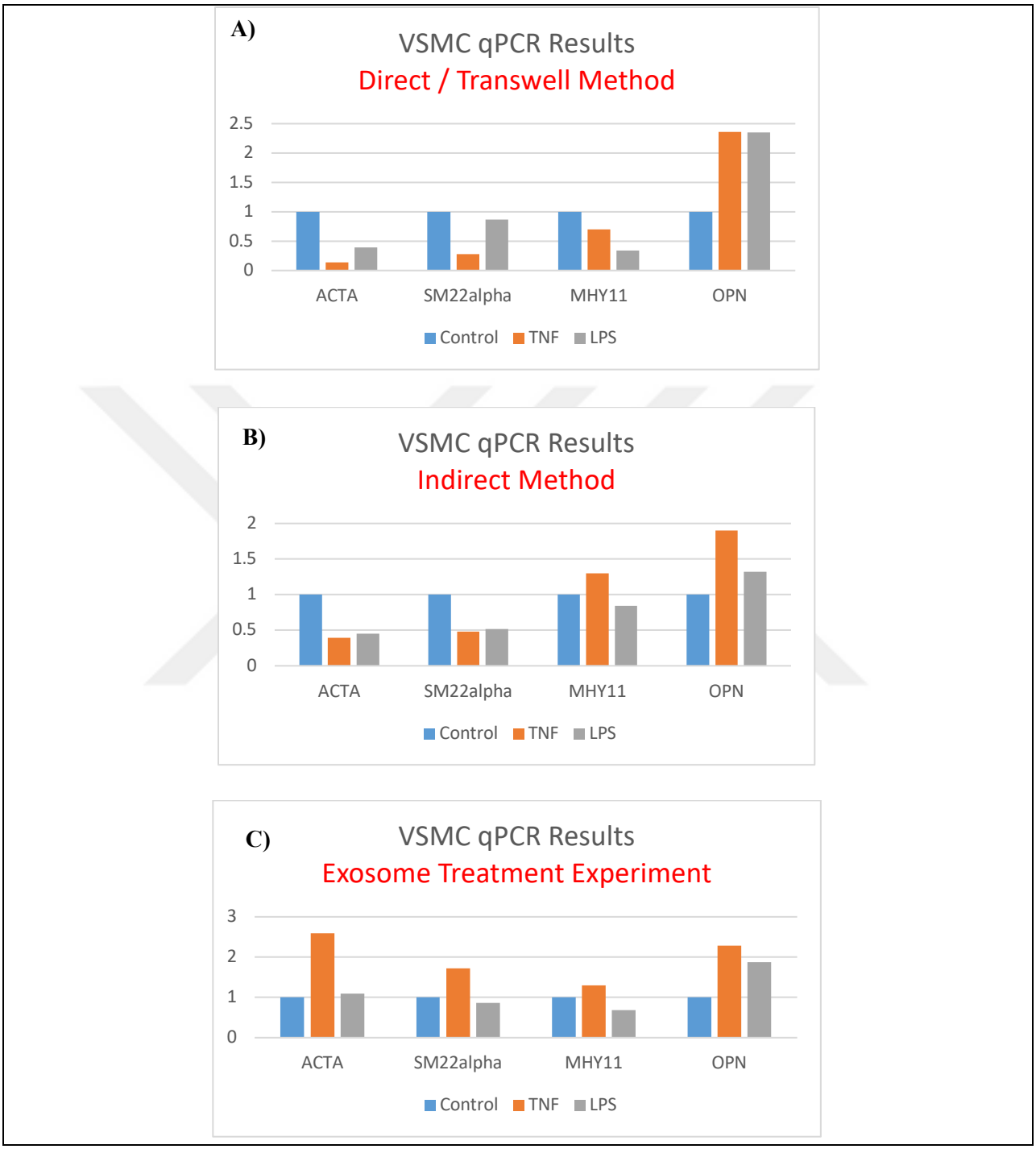


***Figure 23**: VSMC qPCR results with contractile (ACTA2, SM22α, MHY11) and synthetic (OPN) phenotypic markers for three different cell culture models. A) qPCR result for VSMC from direct/transwell method B) qPCR result for VSMC from indirect method C) qPCR results for VSMC from EC exosome treatment for VSMC*

EC: VCAM-1 and ICAM-1 primers were used for EC. Their expression was decreasing in the TNF and LPS+ treatment conditions as we expected. Our qPRC results demonstrate the viability of our atherosclerosis model. (n=4 for indirect model, n=1 for indirect model, n=1 for exosome treatment model). (**Figure 24**)

Figure 24:EC qPCR results with ICAM-1 and VCAM-1. A) qPCR result for EC from direct/transwell method. B) qPCR result for EC from indirect method. C) qPCR result for EC from exosome treatment experiment.

3.5 Exosome Isolation and Characterization

Exosome isolation was performed from the cell culture supernatant of all 3 different culture methods. BCA was performed to measure the protein concentration.

Characterization of exosomes was performed by using two different methods: Western Blot (WB) and Nano Tracking Analysis (NTA). CD63 and CD81 antibodies were used in Western blot analysis. In the NTA assay, we analyzed the size distribution of particles found in our sample. The NTA results give information on the size distribution of the particles and its range being between 30 and 200 nm indicates that the particles that have been isolated are exosomes.

Western Blot results,

We quantified the exosome protein concentration using the BCA assay. This assay allowed us to accurately measure the protein content within the isolated exosomes. According to the results of the BCA analysis, it was observed that samples treated with TNF and LPS a higher production of exosomes compared to the control group (n=4). According to the BCA concentration, 10 µg of exosomes was loaded into Western blot gel.

We used Western blot analysis to characterize the isolated exosomes. By employing specific exosomal marker antibodies, CD63 and CD81[253,254], we were able to detect their respective proteins in our samples. CD63 band molecular weight was detected at 55 kDa, and CD81 band molecular weight was detected at 25 kDa. The presence of CD63 and CD81 proteins at the expected molecular weights confirmed the successful identification of exosomes. (**Figure 25,26**)

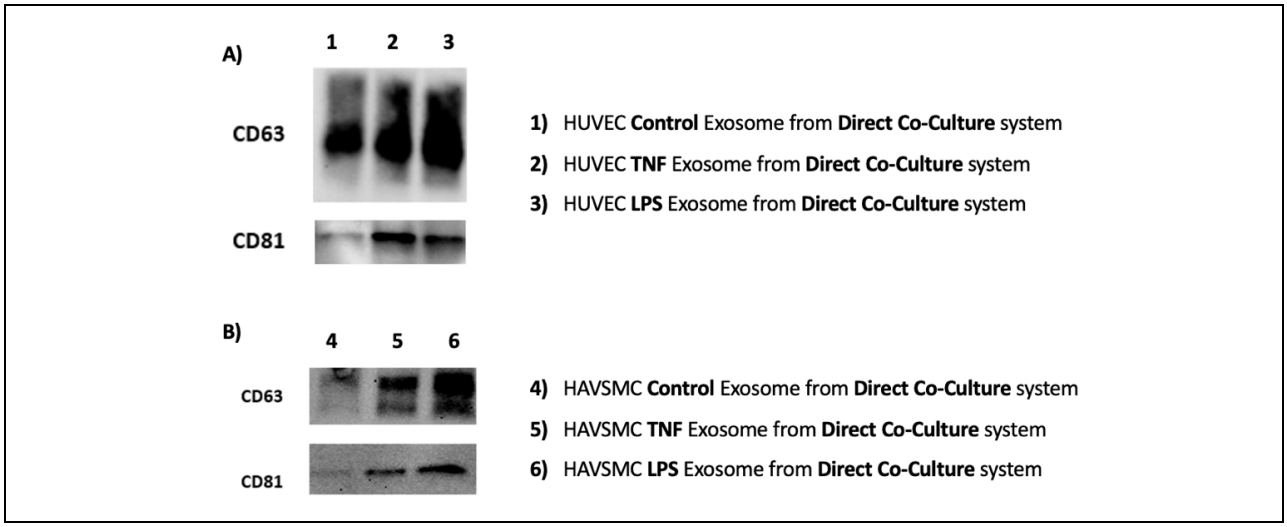


*Figure 25: HUVEC and VSMC **direct (trans-well) experiment** western blot results with CD63 and CD81.*

A)HUVEC exosomes western blot B) VSMC exosomes western blot

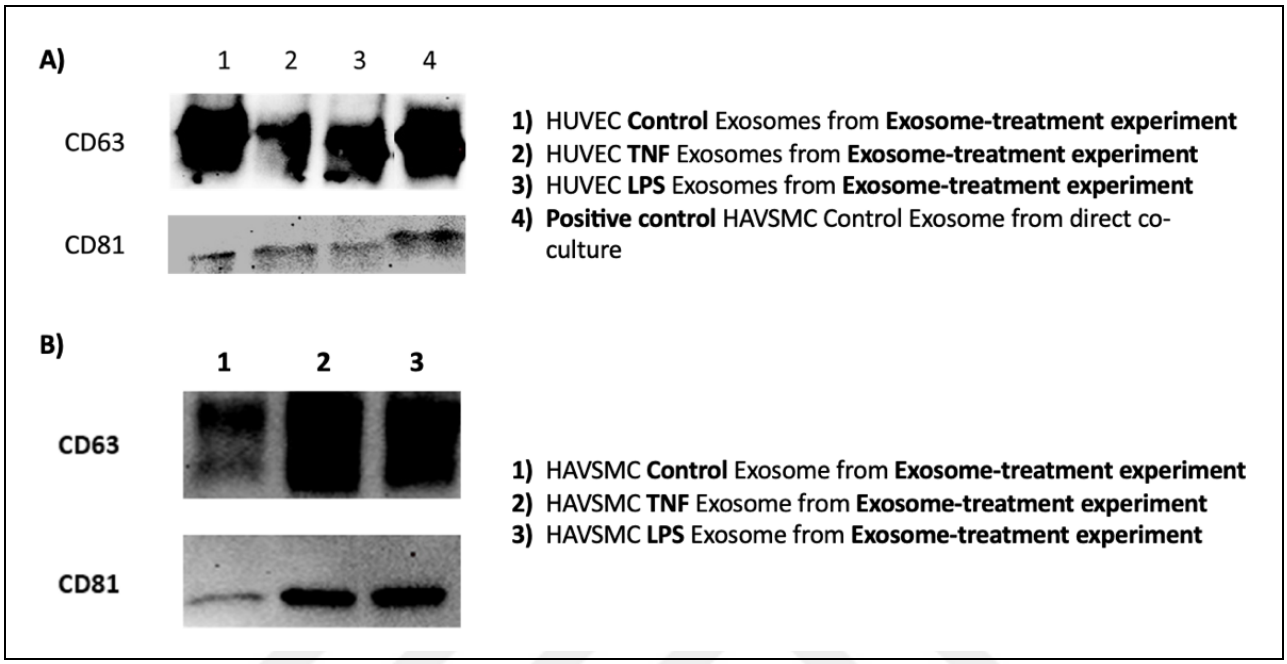


*Figure 26: Western blot results from **isolated exosomes experiment** (VSMC treated with Isolated HUVEC exosomes experiment). A) Isolated HUVEC exosomes western blot results (10ug protein was loaded into the membrane.) B) VSMC (treated with HUVEC exosome) exosomes western blot result (10ug protein was loaded into the membrane.)*

NTA results,

Our nano-tracking analysis revealed a finding regarding the size distribution of the analyzed samples. The obtained graphs clearly demonstrate that the majority of the particles fall within the range of 30-200 nm [255-257], which aligns perfectly with the typical size range of exosomes. This observation strongly suggests that the analyzed samples predominantly consist of exosomes, as their size distribution closely matches the established characteristics of these extracellular vesicles. (**Figure 27, 28, 29**)

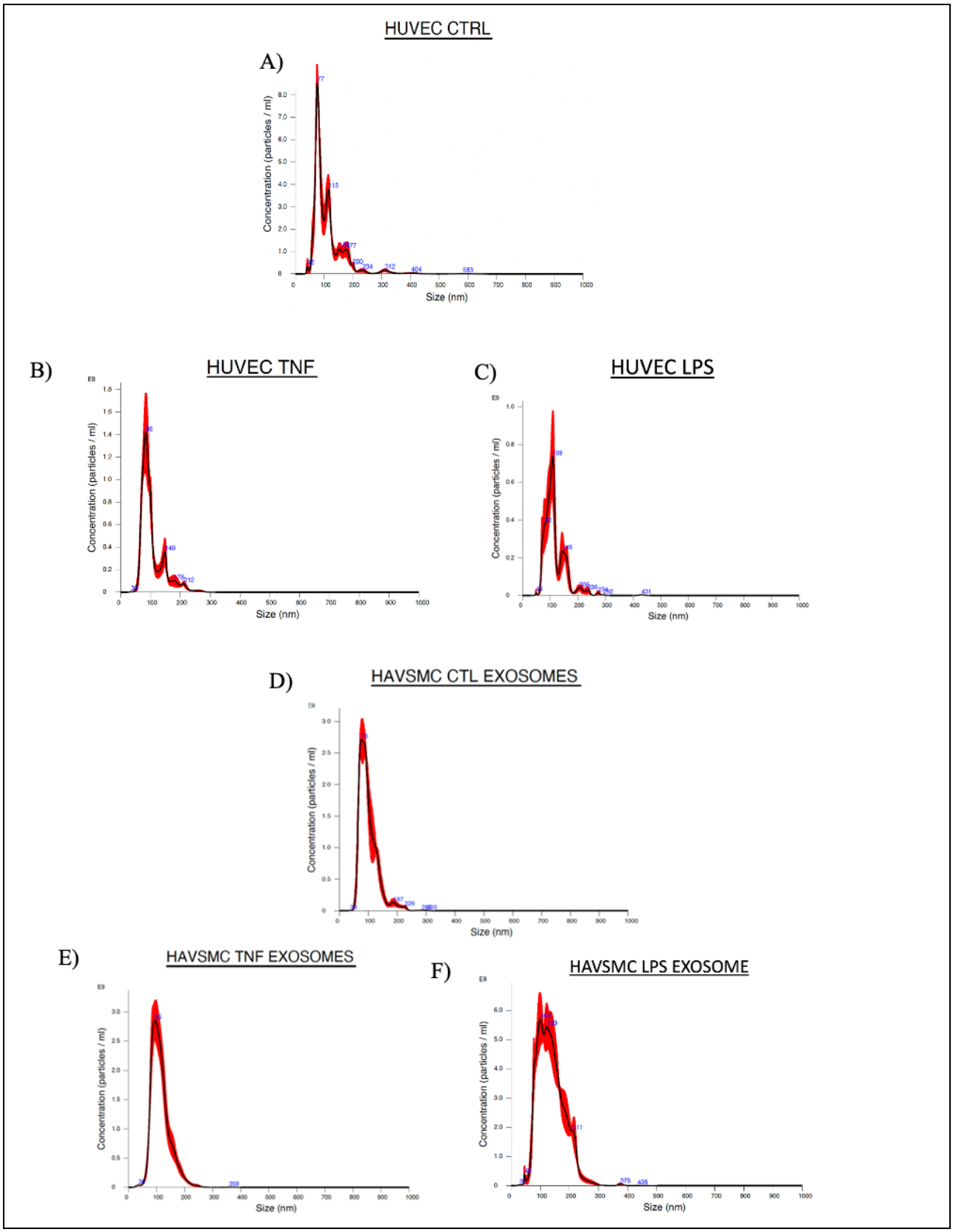


***Figure 27**: Nano tracking analysis (NTA) results from **direct co-culture (Transwell)** experiment. N=4*

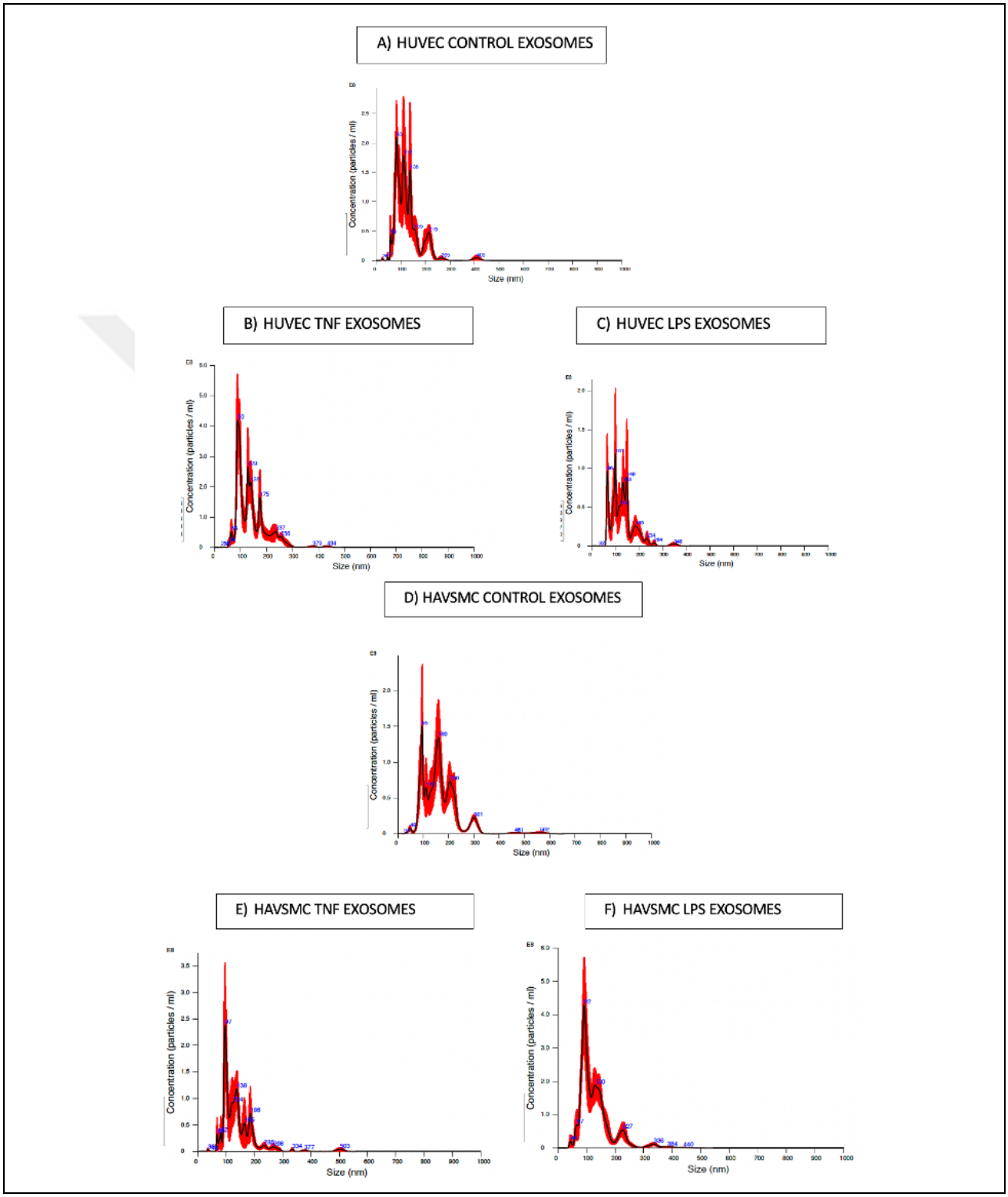


***Figure 28**: Nano tracking analysis (NTA) results from **indirect culture** model. n=1*

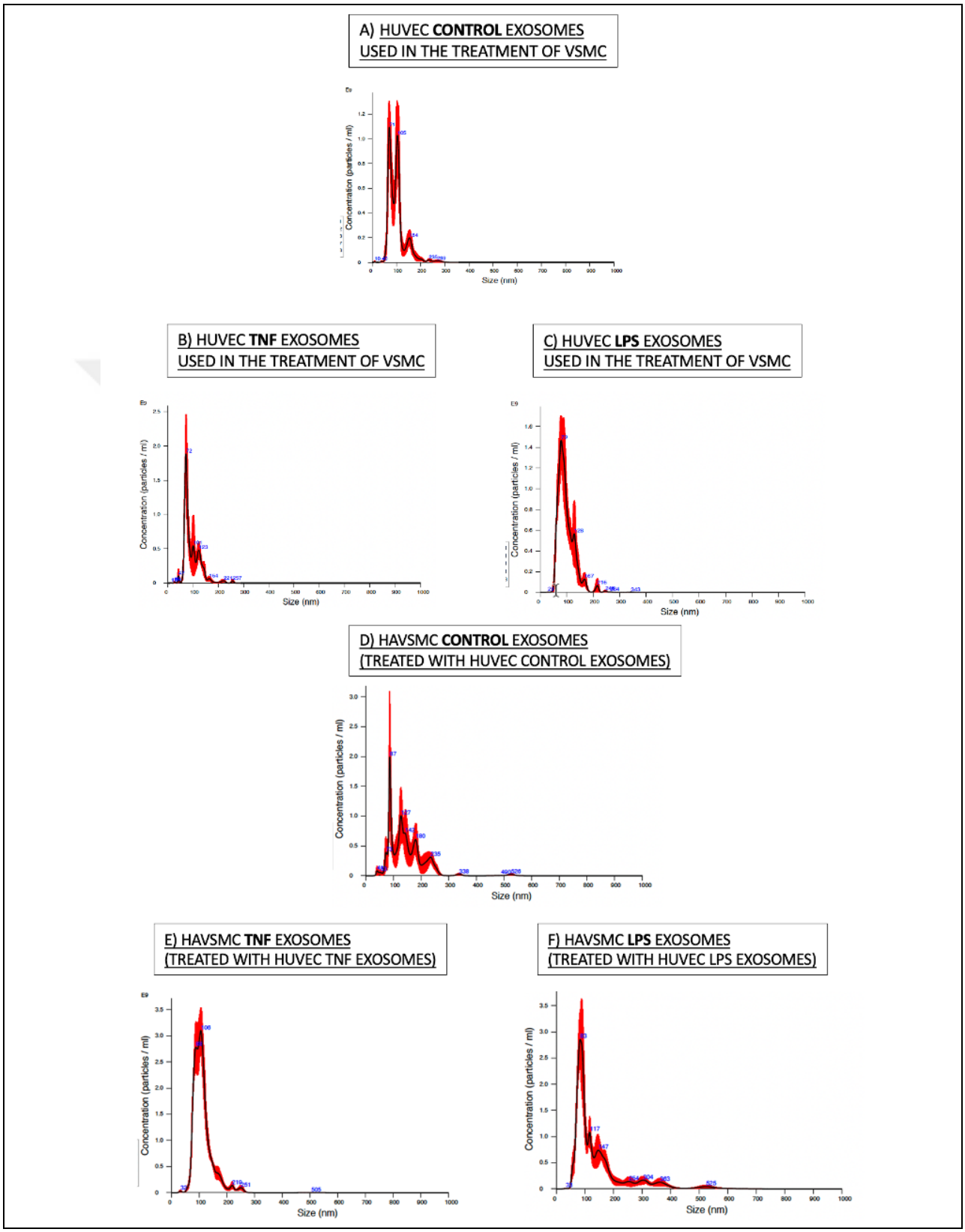


Figure 29:*Nano tracking analysis (NTA) results from* ***VSMC treated with EC exosome*** *experiment. N=1*

3.6 Exosomal miRNA

Some miRNAs were selected for miRNA sequencing.

For the sequencing; Direct/transwell co-culture experiment model was the one we focused on in this thesis. Therefore, we chose 6 samples from direct co-culture experiments for the sequencing. 3 of them were EC, and the other 3 were VSMC samples.

Another 11 miRNA isolations were made from other exosome samples. The sequencing from these samples provided very low reads, therefore, they were not included in the thesis.

These 6 miRNA samples were measured with Nanodrop 2000c, Bioanalyzer 2100, and Tapestation 4150. Each measurement method gave different results for miRNA concentration. (**Table 3**)

miRNA samples	***Nanodrop 2000c ng/µL***	***Bioanalyzer 2100 ng/µL***	***Tapestation 4150 ng/µL***
VSMC_Control	14,1	9	0,3
VSMC_TNF	8,1	16,7	0,5
VSMC_LPS	9,8	10,5	0,3
EC_Control	34,4	87,3	1,6
EC_TNF	29,6	94,3	2,5
EC_LPS	21,1	52,5	1,5

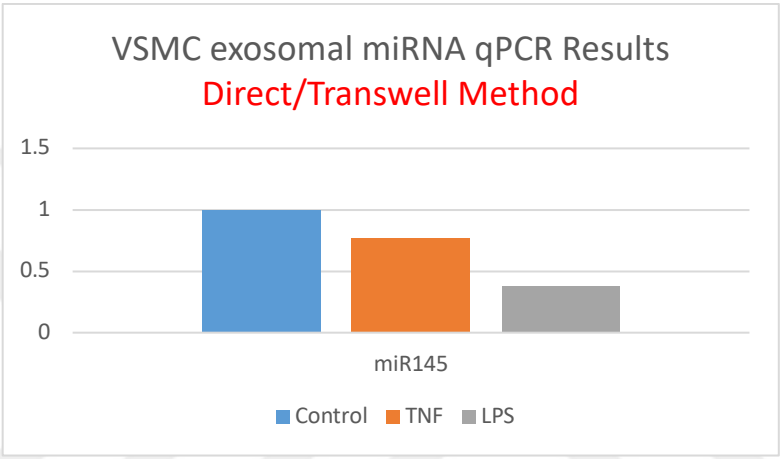
Table 3*: miRNA concentration (ng/□L) of 6 candidates of miRNA sequencing from direct/transwell co culture method. (Measured by Nanodrop 2000c, Agilent Bioanalyzer 2100 machine RNA Pico 6000 kit and Tapestation 4150 machine HS RNA kit.)*

According to literature, bioanalyzer was the most common method to measure miRNA. [258,259] Because of this reason, we decided to continue with bioanalyzer results to start for miRNA sequencing.

VSMCs exosomal miRNA qPCR

miR-145 is the novel miRNAs that are related to atherosclerosis. The expression of miR-145 is significantly downregulated in dedifferentiated VSMCs and in balloon-injured arteries. Some authors identified that miR-145, VSMC phenotypic marker and modulator, has the power to regulate the growth of vascular neointimal lesions. [260,261]

We wanted to see their expression on qPCR.



3.7 Library Preparation for RNA sequencing

Before start to miRNA sequencing with Novaseq, a miRNA library preparation was performed. 6 samples were selected from direct/transwell method. There were three VSMC and three EC samples.

Library preparation consists of 6 steps. In the 2nd step, miRNAs were converted to cDNAs. In 3rd, 4th and 5th steps (PCR, library validation and size selection steps) were performed on cDNAs. (Figure 18)

In the size selection steps, we used AMPure XP beads. cDNAs were measured before and after treating with the magnetic beads. In **Table 4**, there are cDNA concentrations of 6 samples before and after the size selection.

Experiment Type	*miRNA Samples*	*miRNA input amount in the beginning(ng)*	*Qubit measurement (ng/uL)* *Before beads*	*Qubit measurement (ng/uL)* *After beads*
Direct/Transwell model	E_C	340	1.38	0.138
Direct/Transwell model	E_T	340	0.73	0.113
Direct/Transwell model	E_L	340	2.04	0.452
Direct/Transwell model	V_C	60	0.59	0.075
Direct/Transwell model	V_T	60	0.73	0.127
Direct/Transwell model	V_L	60	1.1	0.194

***Table 4**: cDNA concentrations of samples used for the library preparation*

3.8 Bioinformatic Analysis of miRNA Sequencing

In our study, we outsourced the New-Generation Sequencing (NGS) of miRNA samples to a service provider. As a research team, we collaborated with an external company to perform the sequencing and obtain the necessary data. "**Illumina NovaSeq 6000**" machine was used for sequencing. Raw data that obtained from sequencing, was used to make bioinformatic analysis of miRNA.

After the bioinformatic analysis of miRNA sequencing, we obtained reads for different miRNAs. (**Table 5**) In the results section of miRNA sequencing, I present the findings from the miRNA sequencing of six samples. These groups comprised of exosome from vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), for each cell, there were samples from the control group, tumor necrosis factor (TNF) treated group, and lipopolysaccharide (LPS) treated group. (**Table 5**)

miRNA name	E_C (direct/ transwell model)	E_T (direct/ transwell model)	E_L (direct/ transwell model)	V_C (direct/ transwell model)	V_T (direct/ transwell model)	V_L (direct/ transwell model)
hsa-miR-335-5p	114	8	433	160	188	391
hsa-miR-1248	13	3	23	0	0	0
hsa-miR-1282	5	1	5	13	8	4
hss-let7a-5p	1	17	2	17	17	1

Table 5*: Number of reads for miRNA sequencing. Top four miRNA having the highest reads are shown.*

The most significant finding was the striking differential expression of miR-335-5p in these groups. This microRNA was notably upregulated in both the TNF and LPS-treated VSMCs (**Figure 30**) and ECs LPS-treated group but downregulated in ECs TNF-treated group compared to their respective control groups. (**Figure 31**)

In **Figure 30** and **Figure 31**, there are representative common miRNA into 3 different group (Control,TNF,LPS). Green color is represented to upregulated miRNA, red is represented downregulated miRNA and black is represented the one didn't change in the treatment condition.

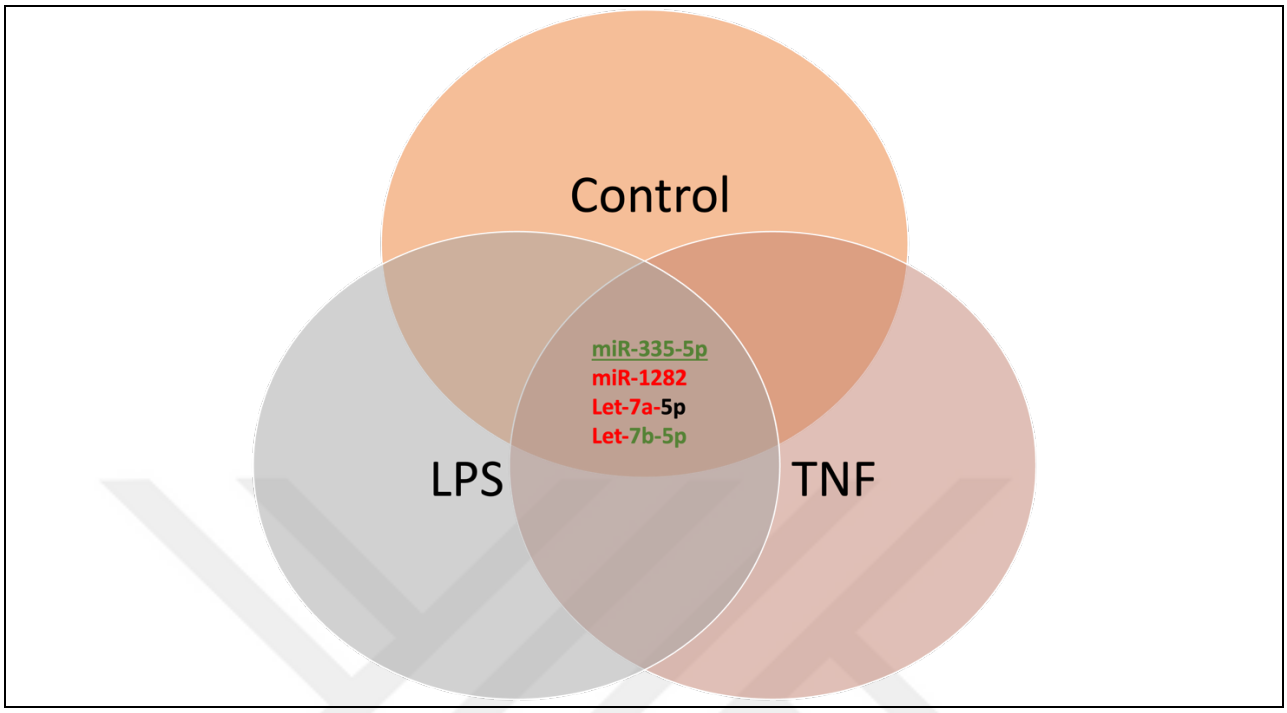


***Figure 30**: miRNA sequencing results of miRNA from exosomes produced by **Vascular Smooth Muscle Cells** under control, TNF and LPS treatment conditions. (miRNAs that have increased expression are represented by green color, miRNAs that have decreased expression are represented by red color, miRNAs that have not expression changes are represented by black color.)*

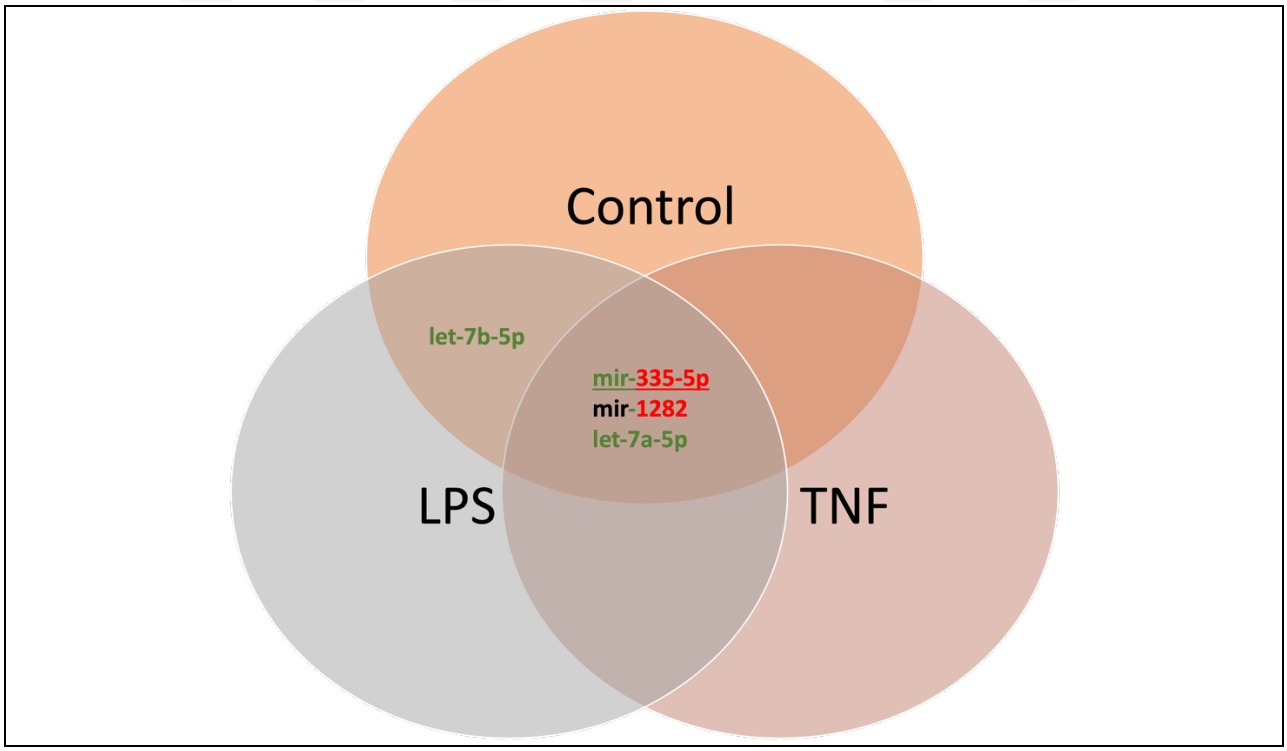


***Figure 31**: miRNA sequencing results of **Endothelial Cell**s Control, TNF and LPS treatments. (miRNAs that have increased expression are represented by green color, miRNAs that have decreased expression are represented by red color, miRNAs that have not expression changes are represented by black color.)*

3.9 Targets of miR-335-5p

miR-335-5p first 100 targets were taken from 2 different database; “Targetscan. org” and “miRDB.org”. According to their results, common targets were analysed by using “Bioinformatics.psb.ugent.be” tool. 33 common targets were obtained. (**Figure 32** and **Table 6**)

TargetScan and miRDB are tools that help us predict where our molecule of interest, hsa-miR-335-5p, might interact within the cell. They do this by looking at specific areas of the cell's genetic material. **TargetScan** ranks these possible interaction sites based on certain scores, while **miRDB** uses a type of computer learning called support vector machines (SVMs) to rate how confident we should be in each prediction. [262,263]

However, even though these tools are easy to use and offer some great insights, they also showed some inconsistencies and irrelevant results in studies. This could be because of limitations in how these tools make their predictions. For instance, they might over-focus on one part of the interaction process, not fully take into account how the action of our molecule can depend on its context, or overlook that its role can change depending on the tissue or the timing. These results emphasize that while these tools are helpful, we should use them along with other experimental data to get a more accurate and relevant picture of what's happening biologically. [262,263]

To minimize the impact of this limitation, we analyzed the first 100 targets from the two databases (TargetScan and miRDB) with the “Bioinformatics.psb.ugent.be” tool and found the common genes. (**Figure 32**)

Input files:

List names	number of elements	number of unique elements
Targetscan.org	100	100
miRDB.org	100	100
Overall number of unique elements		167

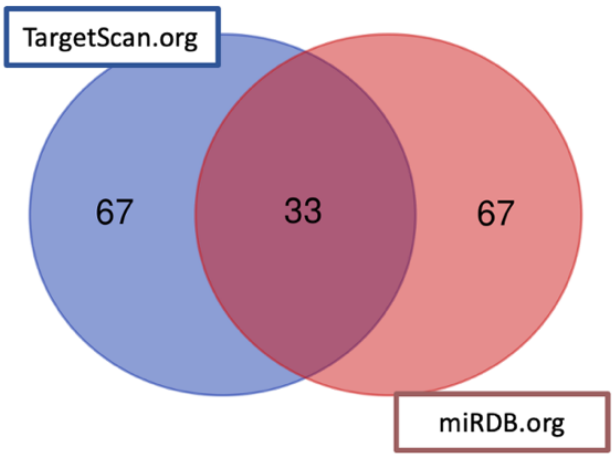


Figure 32: Common targets of miR-335-5p

NAA25	CALU	POU2F3
PRKAA2	CASP7	UBE2G1
SORCS1	HOXD8	FAM131B
FBXO28	NRXN1	NCKAP5
F13A1	RPRM	ARHGAP18
RASA1	GLYR1	SNIP1
DAAM1	KAT7	CCNF
GJA5	ARGLU1	CAMKK2
POU5F1	NXPH2	CNOT7
PSD3	SMARCA2	VAPA
KDM4C	ZMPSTE24	HAND1

Table 6: Common targets of miR-335-5p

Relation between 33 common targets of miR-335-5p were analysed by using “string-db.org” tool. (**Figure 33**)

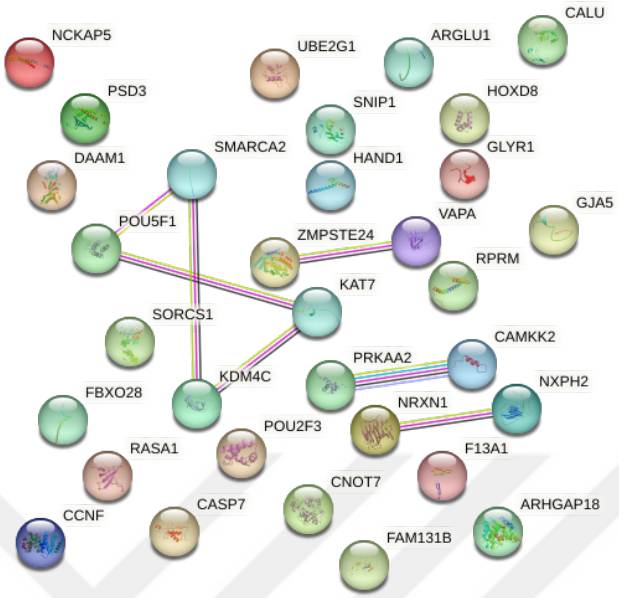


Figure 33: Relation between targets of miR-335-5p.

There could be a few reasons why we weren't able to find any correlation between the target genes identified using "TargetScan" and "miRDB" when we analyzed them with the "string-db.org" tool.

- **Limited dataset:** The string-db database may not have enough information on the genes you're investigating. It's possible that the interactions between these genes haven't been well-studied or documented yet. [264]
- **Low expression or activity in your specific context**: Even if these genes are potential targets of hsa-miR-335-5p, they may not be significantly expressed or active in the specific cellular context or condition we're interested in (e.g., VSMCs or ECs under atherosclerotic conditions).
- **Inherent tool limitations:** Both TargetScan and miRDB predict interactions based on computational algorithms, which have their own limitations and false positive rates. The predicted targets might not actually be biologically relevant or might not interact in the context of our specific study. [262,263]
- **Complex nature of miRNA-gene interactions:** miRNAs typically have multiple target genes and multiple miRNAs can target each gene. This complex network of interactions can sometimes make it difficult to discern clear correlations.
- **Absence of direct interactions**: String-db database mainly shows direct protein-protein interactions. However, the genes targeted by a miRNA don't necessarily need to interact with each other directly. Their protein products can be part of different pathways or processes that contribute to the same phenotype or cellular function. [264]

Given these possibilities, it's important to use additional experimental data and approaches to validate the potential targets of hsa-miR-335-5p and their role in atherosclerosis.

3.10 Common genes between 3 groups; "Targets of miR335", "Genes found in VSMC" and "Genes found in EC"

miR335 targets were found before (in the part of **3.8**). Additionally, we wanted to see the common genes that found either "targets of miR335" and "genes that were found in VSMC" or "targets of miR335" and "genes that were found in EC", or all "targets of miR335", "genes that were found in VSMC" and "genes that found in EC". Because of this reason, we found these groups of genes from different databases;

1st group: Genes as a target of miR335 were taken from "Targetscan.org" and "miRDB.org" databases.

2nd group: Genes that are found in VSMC were taken from "proteinatlas.org" database

3rd group: Genes that are found in EC were taken from "proteinatlas.org" database.

These three databases' common genes were analysed using "Bioinformatics.psb.ugent.be". (**Figure 34**) There are 9 common genes in the first and second groups. (**Table 7**) There are 3 common genes in the first and third groups. (**Table 8**) There are no common genes between 3 groups.

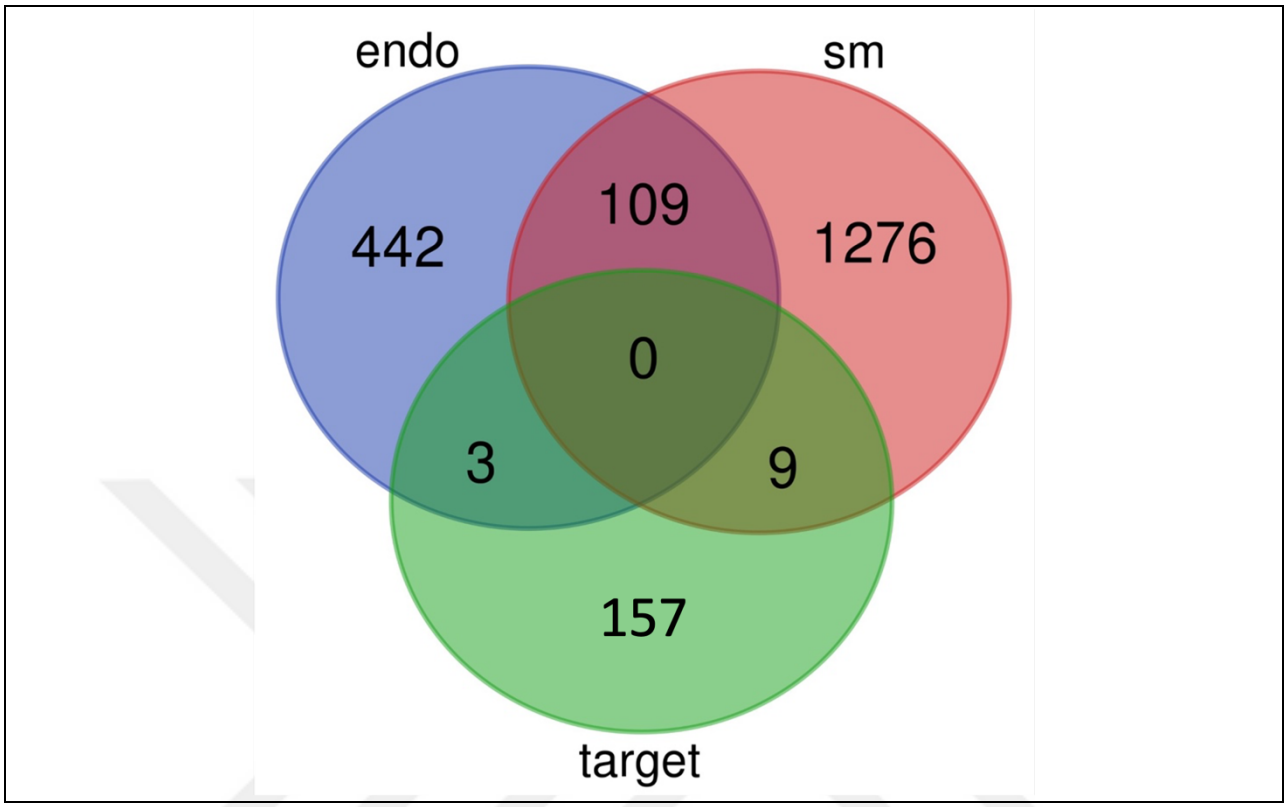


***Figure 34**: Number of common genes that are found in miR-335 targets, VSMC and EC. (endo=EC, sm=VSMC, target=miR-335)*

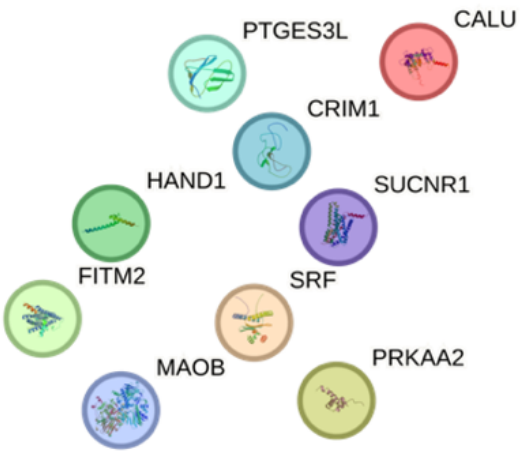
PTGES3L	CRIM1	PRKAA2
MAOB	HAND1	CALU
SUCNR1	SRF	FITM2

***Table 7**: Common genes that found in either "target of miRNA" (1st group) and "genes in VSMC" (2nd group).*

HOXD8	FLT1	PTPRB

***Table 8**: Common genes that found in either "target of miRNA" (1st group) and "genes in EC" (3rd group).*

Relation between common genes were analysed by using “string-db.org” tool. (**Figure 35, 36**)



Nodes:

Network nodes represent proteins	Node Color	Node Content
splice isoforms or post-translational modifications are collapsed, i.e. each node represents all the proteins produced by a single, protein-coding gene locus.	*colored nodes: query proteins and first shell of interactors* *white nodes: second shell of interactors*	*empty nodes: proteins of unknown 3D structure* *filled nodes: some 3D structure is known or predicted*

Your Input:

CALU	*Calumenin; Involved in regulation of vitamin K-dependent carboxylation of multiple N-terminal glutamate residues. Seems to inhibit gamma- carboxylase GGCX. Binds 7 calcium ions with a low affinity (By similarity); Belongs to the CREC family. (323 aa)*
SRF	*Serum response factor; SRF is a transcription factor that binds to the serum response element (SRE), a short sequence of dyad symmetry located 300 bp to the 5' of the site of transcription initiation of some genes (such as FOS). Together with MRTFA transcription coactivator, controls expression of genes regulating the cytoskeleton during development, morphogenesis and cell migration. The SRF-MRTFA complex activity responds to Rho GTPase-induced changes in cellular globular actin (G- actin) concentration, thereby coupling cytoskeletal gene expression to cytoskeletal dynamics. Required f [...] (508 aa)*
PRKAA2	*5'-AMP-activated protein kinase catalytic subunit alpha-2; Catalytic subunit of AMP-activated protein kinase (AMPK), an energy sensor protein kinase that plays a key role in regulating cellular energy metabolism. In response to reduction of intracellular ATP levels, AMPK activates energy-producing pathways and inhibits energy-consuming processes: inhibits protein, carbohydrate and lipid biosynthesis, as well as cell growth and proliferation. AMPK acts via direct phosphorylation of metabolic enzymes, and by longer-term effects via phosphorylation of transcription regulators. Also acts a [...] (552 aa)*
FITM2	*Fat storage-inducing transmembrane protein 2; Plays an important role in lipid droplet accumulation. Plays a role in the regulation of cell morphology and cytoskeletal organization; Belongs to the FIT family. (262 aa)*
HAND1	*Heart- and neural crest derivatives-expressed protein 1; Transcription factor that plays an essential role in both trophoblast giant cell differentiation and in cardiac morphogenesis (By similarity). Binds the DNA sequence 5'-NRTCTG-3' (non-canonical E-box) (By similarity). Acts as a transcriptional repressor of SOX15 (By similarity). In the adult, could be required for ongoing expression of cardiac-specific genes. (215 aa)*
PTGES3L	*Putative protein PTGES3L; Prostaglandin E synthase 3 like. (161 aa)*
CRIM1	*Processed cysteine-rich motor neuron 1 protein; May play a role in CNS development by interacting with growth factors implicated in motor neuron differentiation and survival. May play a role in capillary formation and maintenance during angiogenesis. Modulates BMP activity by affecting its processing and delivery to the cell surface. (1036 aa)*
MAOB	*Amine oxidase [flavin-containing] B; Catalyzes the oxidative deamination of biogenic and xenobiotic amines and has important functions in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues. MAOB preferentially degrades benzylamine and phenylethylamine; Belongs to the flavin monoamine oxidase family. (520 aa)*
SUCNR1	*Succinate receptor 1; Receptor for succinate. (334 aa)*

Your Current Organism:

Homo sapiens

NCBI taxonomy Id: 9606
Other names: H. sapiens, human, man

***Figure 35**: Relation of genes that is common in 1st group (targets of miR335) and 2nd group (genes found in VSMC)*

***Figure 36**: Relation of genes that is common in 1st group (targets of miR335) and 3rd group (genes found in EC)*

4. DISCUSSION:

Our experimental approach utilized an *in vitro* model that aimed to simulate the atherosclerotic environment by applying TNF and LPS treatments to co-cultures of vascular smooth muscle cells and endothelial cells. The treatment with these inflammatory stimuli was designed to mimic the inflammation and immune response processes typically observed in atherosclerosis.

The response of the cells to the treatment was assessed via immunofluorescence and qPCR experiments. The observed changes in these assays, including modifications in gene expression or protein localization, signify that the cells underwent some transformation or reaction, likely

mirroring their potential behavior under atherosclerotic conditions. These changes therefore validate the effectiveness and relevance of our *in vitro* model, demonstrating that it can effectively simulate aspects of the atherosclerotic environment and influence cell behavior in a way that is consistent with *in vivo* atherosclerosis.

The ability to mimic this environment *in vitro* provides a valuable tool for further study, as it offers an opportunity to examine the detailed mechanisms of atherosclerosis development and progression, as well as the roles of various cell types and inflammatory stimuli within this process. Such insights could guide the discovery of novel therapeutic targets or strategies. It should be acknowledged, however, that *in vitro* models, while useful, may not fully capture the complexity of *in vivo* systems, and further research using *in vivo* models or clinical samples would be needed to confirm and expand upon our findings.

Moreover, the observed changes in the cells post treatment, while indicative of some level of response, warrant further investigations to fully elucidate the nature of these changes. This will provide more concrete evidence about the specific pathways and mechanisms involved in the response of vascular smooth muscle cells and endothelial cells to a simulated atherosclerotic environment.

4.1 Cell-cell Communication Models for Atherosclerosis

4.1.1 Advantages and Disadvantages of Direct co-culture model

VSMC and EC were co-cultured by using the direct/transwell co-culture method. [73,265-268] The direct or transwell co-culture methods involve culturing multiple cell types in close proximity, allowing for intercellular communication and interaction. In the context of atherosclerosis research, this approach can be used to study interactions between endothelial cells, smooth muscle cells, and immune cells. There are some advantages for direct/transwell co-culture methods. First advantage is that co-culture methods enable the study of cell-cell communication and paracrine signaling, which are essential aspects of atherosclerosis. They allow for the investigation of the influence of one cell type on another, leading to a better understanding of disease mechanisms. We targeted to make the direct/transwell model more representative of cellular interactions in the blood vessel wall during atherosclerosis by co-culturing different cell types in our experiment. This can provide a more physiologically relevant platform for studying disease progression.

But there are some disadvantages of direct co-culture models. The first disadvantage is that the co-culture systems can be more technically challenging than monoculture models, requiring optimization of cell ratios, culture conditions, and barrier systems such as transwell inserts. Ensuring proper separation of cell types while allowing communication between them can be complex. Another disadvantage could be that the presence of multiple cell types and the resulting complex interactions can make it challenging to interpret the individual contributions of each cell

type to the observed effects. Disentangling the specific effects of different cell types can require additional experimental approaches, such as selective cell-type labeling or genetic manipulations.

4.1.2 Advantage and Disadvantages of the Indirect model

The indirect cell culture model, also known as monoculture, involves culturing a single cell type in isolation. In the context of atherosclerosis research, this model typically involves culturing endothelial cells or smooth muscle cells separately. [73] Indirect model allows researchers to study specific cell types in a controlled environment, focusing on their individual behavior and responses. It provides a simplified system for studying cellular processes related to atherosclerosis. But there are some disadvantages of the indirect model. Since the cells are cultured in isolation, the model lacks the intercellular communication and interaction that occurs *in vivo*. Atherosclerosis is a complex disease involving multiple cell types, such as endothelial cells, smooth muscle cells, and immune cells, which interact with each other in a dynamic manner. Another disadvantage of the indirect model is that this model may not fully capture the complex pathophysiology and disease progression of atherosclerosis. It fails to mimic the spatial organization and cellular crosstalk observed in the blood vessel wall, which plays a crucial role in the development of atherosclerotic plaques. But the main disadvantage of this model for our study is, when cells' conditioned media are switched, in addition to the exosomes they produce, other protein factors and signaling molecules produced are also transferred between cells.

4.1.3 VSMC treated with EC's isolated exosomes;

Exosomes are extracellular vesicles released by cells that play a significant role in cell-cell communication. Isolated exosome treatment methods involve exposing cells to exosomes derived from specific cell types involved in atherosclerosis, such as endothelial cells or immune cells. This approach can provide insights into the role of exosomes in modulating cellular functions and disease processes. Isolated exosome treatment provides a simplified system focusing on the effects of exosomes on recipient cells. [231-235] While it offers valuable information on intercellular communication, it may not fully capture the complexity of cellular interactions and spatial organization seen *in vivo*. And lastly, exosomes can exert indirect effects on recipient cells by influencing their gene expression, phenotype, or functional characteristics. Understanding the downstream effects and deciphering the specific contributions of exosomes can be challenging.

In summary, while the indirect cell culture model provides a simplified system to study individual cell types, the direct/transwell co-culture methods offer a more comprehensive approach for investigating cellular interactions and mimicking the complex environment of atherosclerosis. Isolated exosome treatment methods focus on intercellular communication via exosomes but offer a simplified model. The choice of model depends on the specific research goals and the trade-off between simplicity and physiological relevance. Since our study focused on the effect of exosomes formed as a result of cell-cell interaction on intercellular communication, we preferred trans well and isolated exosome treatment methods.

4.2 Exosome Isolation Method

The ultracentrifugation method is considered the gold standard for isolating exosomes from large volumes of cell-conditioned media due to several reasons. [269] While alternative methods such as ExoQuick or commercial isolation kits offer convenience and rapidity, ultracentrifugation remains widely used for research purposes, particularly when dealing with larger sample volumes.[270] There are some reasons to the preference for ultracentrifugation:

- Ultracentrifugation can effectively isolate exosomes from various biological fluids, including cell-conditioned media, blood plasma, and urine. This versatility allows researchers to work with diverse sample types and investigate exosomes from different sources.
- Ultracentrifugation is compatible with a wide range of exosome research applications, including proteomic and genomic analyses, as well as functional assays. This method provides intact exosomes that can be further characterized and utilized for downstream experiments.
- Ultracentrifugation has been extensively studied and standardized over the years, resulting in widely accepted protocols. This consistency and reproducibility enable better comparability of results across different research studies, facilitating the advancement of exosome research as a whole.
- Ultracentrifugation allows for the isolation of relatively pure exosome fractions, minimizing the co-isolation of contaminants such as proteins, lipids, and other non-exosomal vesicles. This purity is crucial for accurate characterization and functional analysis of exosomes.
- Ultracentrifugation, though requiring specialized equipment, is a cost-effective method in the long run. Once the equipment is available, the consumables required for ultracentrifugation are generally more affordable than other isolation methods. [270]

It is worth noting that while ultracentrifugation has its advantages, it also has limitations, such as its time-consuming nature and the need for specialized equipment and expertise. For smaller sample volumes or rapid isolation, alternative methods like ExoQuick or commercial kits can offer convenience. However, when it comes to isolating exosomes from large amounts of supernatant and prioritizing yield, purity, and versatility, ultracentrifugation remains the preferred method and is widely regarded as the gold standard in exosome isolation. [270]

4.3 Isolated Particles are “Exosomes”

The isolated particles were confirmed to be exosomes by two different methods; Western blot and Nano tracking analysis (NTA).

We performed to western blot to characterize the exosomes. It employed specific exosomal marker antibodies, CD63 and CD81. The results of the Western blot analysis clearly demonstrated the

presence of these exosomal markers in our samples. The Western blot bands corresponding to CD63 and CD81 proteins were observed at the expected molecular weights, indicating the successful detection of exosomal markers in the isolated samples.

CD63 and CD81 are widely recognized as reliable markers for exosomes, as they are known to be enriched in these extracellular vesicles. Overall, the Western blot analysis using CD63 and CD81 antibodies provided conclusive evidence of the presence of exosomes in our samples, affirming the successful isolation and characterization of these important extracellular vesicles.

The results of our nano-tracking analysis (NTA) revealed a significant finding regarding the size distribution of the analyzed samples. The obtained graph clearly demonstrates that the majority of the particles fall within the range of 30-200 nm, which aligns perfectly with the typical size range of exosomes. This observation strongly suggests that the analyzed samples predominantly consist of exosomes, as their size distribution closely matches the established characteristics of these extracellular vesicles. The graph showcases a prominent peak within the specified size range, indicating a high concentration of particles falling within the exosome size range. This consistent and concentrated distribution provides compelling evidence that the isolated particles are indeed exosomes, as their size matches the established dimensions for these small vesicles.

4.4 Phenotype changes in HAVSMC and EC in response to exosome treatment

Throughout this research, our initial goal was to establish an *in vitro* model of atherosclerosis. Therefore, we develop an *in vitro* model of atherosclerosis by co-culturing vascular smooth muscle cells and endothelial cells, with the addition of TNF and LPS as inducers of low-grade inflammation.

Tumor Necrosis Factor-alpha (TNFα) and Lipopolysaccharides (LPS) are frequently used in *in vitro* atherosclerosis models because they are known to promote inflammation, which is a key component of atherosclerosis development. [271]

TNFα is a potent pro-inflammatory cytokine produced by many types of cells including macrophages, lymphocytes, and endothelial cells. TNFα has been implicated in the development and progression of atherosclerosis due to its ability to promote the expression of adhesion molecules on endothelial cells, stimulate the production of other inflammatory cytokines, and enhance the activation and migration of immune cells, all of which contribute to the initiation and progression of the atherosclerotic process. [272]

LPS, or endotoxin, is a component of the outer membrane of Gram-negative bacteria. It is recognized by the immune system and can stimulate a strong immune response. In the context of atherosclerosis, LPS can promote inflammation by triggering the release of inflammatory cytokines and promoting the activation of endothelial cells. [273]

After the atherosclerotic model has been created *in vitro*, the smooth muscle cells and endothelial cells should each be analyzed using qPCR in order to check for their respective markers. This is an essential step that must be taken in order to prove that our technique is reliable for researching the miRNA markers that were isolated from the atherosclerotic model.

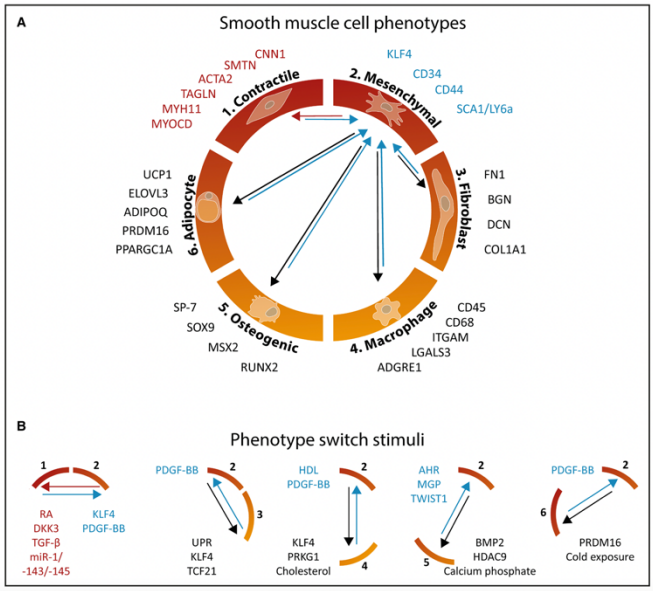


Figure 37 [274]

The markers ACTA2, SM22alpha, MHY11, KLF4, and OPN were used to see phenotypic changing of VSMC. ACTA2, SM22alpha and MHY11 are contractile-phenotypic markers of VSMC. KLF4 and OPN are synthetic-phenotypic marker of VMSC. Both ICAM and VCAM were applied in order to identify endothelial cells' changes.

The effect of endothelial exosomes induced by stimuli on smooth muscle cells have been partially described in the literature. These published articles discuss the transmission of exosomal content from endothelial cells to smooth muscles via exosomes and the potential for altering the phenotype of these muscle cells:

- Endothelial tet2 protein protects vascular smooth muscle cell from phenotype switch. Endothelial cell derived exosomes transfer tet2 protein to vascular smooth muscle cell. Li et all, 2020 [213], showed that by inhibiting TET2 and transferring from endothelial cell-derived exosomes to vascular smooth muscle cells, CD137 signaling activation increases neointimal development.
- Hergenreider et al., 2012[73], reported that to maintain the differentiation status of VSMCs and the atheroprotective benefits, KLF2 mediates miR-143/145 transfer from endothelial cells to VSMCs in EVs. KLF2-expressing endothelial cells' production of EVs prevented the development of atherosclerotic aortic lesions in ApoE-/- mice. According to the

findings, KLF2 mediates miR-143/145 transfer from endothelial cells to VSMCs in EVs in order to preserve VSMC differentiation status and atheroprotective benefits

- Bonn and Horrevoets et al., 2009[275], found that KLF2 binds to miR-143/145 and causes an expansion of the cluster that controls the VSMCs' phenotype. EVs generated by KLF2-stimulated HUVECs were enriched in miR-143/145, and in a HUVEC-VSMC co-culture, VSMCs showed a decrease in the expression of miR-143/145-targeted genes. The miRNA targets were reduced in VSMCs when miR-143/145-deficient ECs were cocultured with them.

Furthermore, existing knowledge indicates an expected phenotypic change in vascular smooth muscle (VSMCs), notably under the influence of inflammatory signals such as TNF/LPS. This phenotypic modulation is postulated to be linked with specific microRNAs (miRNAs), which are short non-coding RNA molecules involved in post-transcriptional regulation of gene expression. The miRNAs being studied in the context of VSMCs, include miR-155[217], miR-143/145 [219,225], and miR-221[226].

Despite the progress in this field, our research is set to provide a more comprehensive view, investigating unreported miRNAs through detailed sequencing methods. Our goal is to illuminate potential novel miRNAs that might be implicated in endothelial-to-smooth muscle signaling and contribute to the nuanced understanding of the progression of atherosclerosis.

In particular, during the genesis and evolution of arterial disease like atherosclerosis, smooth muscle cells (SMCs) show phenotypic plasticity, which facilitates fast response to shifts in environmental cues. They are able to undergo many phenotypic changes when exposed to atherosclerotic circumstances. (Figure 2) There are multiple genes involved in the switching process, and each gene is responsible for a different stage. When we are creating the model of atherosclerosis, we should evaluate the expression of numerous genes to determine whether there are any changes in the phenotype of the vascular smooth muscle cells and endothelial cells. Using quantitative polymerase chain reaction (qPCR), we examined the expression of the genes ACTA2, SM22alpha, MHY11, and osteopontin in vascular smooth muscle cells.

Markers of the contractile phenotype of smooth muscle cells include ACTA2, SM22apha, and MHY11. It is expected that their expression is reduced during the development of atherosclerosis. [23,276] Alpha smooth muscle actin, which is produced by the ACTA2 gene, is an isoform of the vascular smooth muscle actins that contributes to vascular motility and contraction and is normally expressed in vascular smooth muscle cells. [277] The dynamics and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells have been well documented. [277-279] SM22 alpha is a calponin-related protein uniquely expressed in mature smooth muscle. To define the changes in smooth muscle, we analyzed the expression of the SM22 alpha gene after the treatment of TNF and LPS in the co-culture model.

The MYH11 gene encodes for the protein smooth muscle myosin heavy chain 11. [280] It is a member of the myosin protein family involved in cell movement and material transport inside and between cells. We wanted to see if we could obtain the expression of known genes in our model. It is known that ACTA2, SM22alpha, and MHY11 expressions are decreasing in atherosclerotic conditions. The findings from our qPCR experiment support our atherosclerosis concept. According to our findings, the expressions of all of these contractile-phenotype smooth muscle cell markers are on the decline in the treatment of TNF and LPS.

Another gene in vascular smooth muscles is osteopontin (OPN), an osteogenic-like phenotype marker. It is expressed in SMC with synthetic phenotype. [281] In our project, we observed that OPN expression decreased in VSMCs treated with TNF and LPS.

The results of our qPCR experiment using the OPN primers also provide support for our atherosclerotic model.

In atherosclerotic conditions, endothelial cells have been reported to express ICAM and VCAM receptors. [282-284] These receptors are not present on healthy endothelial cells. According to our PCR results, VCAM1 and ICAM1 expressions were increased when EC are treated with TNF and LPS as expected based on the literature. [284]

When we have finished developing an *in vitro* model of atherosclerosis, we may go on to working on its exosomes and the miRNA indicators that can be retrieved from these exosomes. To see the changes that occurring into the cells in treatment conditions, we first directly separate RNAs from the cells, and then we utilize these RNAs in qPCR experiments by using certain markers.

However, in order to conduct investigations on exosomes, exosomes need to be extracted directly from the medium of the cells (supernatant). After they have been isolated, exosomes need to be defined using certain techniques, such as western blot and nano-tracking analysis. Following the characterization of exosomes, a miRNA isolation procedure using the "Qiagen miRNeasy Micro RNA Kit" was carried out. Using the Agilent Boianalyzer 2100 with RNA Pico 6000 chip, the amonts of the acquired miRNAs were quantified in terms of their concentration.

There are several published articles about miRNA markers which are playing a role in the mechanisms of atherosclerosis. miR-145 is one of these markers. They have shown that miR-145 is highly expressed in human atherosclerotic lesions, and they have also shown that its downregulation is connected with vascular smooth muscle cell dedifferentiation, which is an essential stage in the progression of atherosclerosis.[285] Other example of these markers is miR-133. microRNA-133 regulates the phenotypic switch of vascular smooth muscle cells, between the contractile phenotype and the synthetic phenotype in response to external stimuli, *in vitro*. [286] The expression of these miRNAs were analyzed by qPCR.

Small RNA sequencing is something that we want to do so that we may study additional potential marker possibilities. For this sequencing, we used “TAKARA SMARTer smRNA-Seq kit”.

4.5 miRNA Measurements

The measurement of miRNA concentration from the isolated exosomes was conducted using four different methods: Agilent 2100 Bioanalyzer with Agilent RNA Pico 6000 kit, Agilent Tapestation 4150 with Agilent High Sensitive RNA ScreenTape, Qubit 3.0 with RNA HS Assay kit and Nanodrop 2000C. Notably, each of these measurement tools yielded different results, which have important implications for the interpretation of our findings.

The Agilent 2100 Bioanalyzer, utilizing the Agilent RNA Pico 6000 kit, provided the first set of measurements for miRNA concentration. This method is the most commonly used method to measure miRNA yield. It should be considered that the Agilent 2100 Bioanalyzer measures the concentration based on electrophoretic mobility, relying on the migration pattern of the RNA molecules.

The second method employed was the **Agilent Tapestation 4150** with the Agilent High Sensitive RNA ScreenTape. This device is the most recently developed device among the devices used to quantify miRNAs. Among them and there is no study to check its efficiency for miRNA measurement. However, according to an Agilent application article testing the RNA ScreenTape assay of the Tapstation 2200, an older model of the Tapestation 4150, the instrument failed to provide satisfactory results in exosomal RNA measurements. The Tapestation system offers a different measurement approach compared to the Bioanalyzer, utilizing microfluidic gel electrophoresis to assess the size distribution and concentration of the RNA molecules. Therefore, the results obtained from the Tapestation analysis may differ from those of the Bioanalyzer due to variations in the measurement principles and algorithms.

The third method, the **Qubit 3.0**, along with the RNA HS Assay kit, was utilized to quantify the miRNA concentration. This method employs a fluorescence-based assay that measures the specific binding of a fluorescent dye to the RNA molecules present in the sample. It is important to note that Qubit-based assays provide a different measurement principle compared to electrophoresis-based methods like the Bioanalyzer and Tapestation. Qubit 3.0 RNA HS Assay kit is providing a detection range of 4-200 ng which is high for our sample concentration. This minimum range is the limitation to measuring our samples.

Lastly, **Nanodrop 2000c** system are spectrophotometric-based methods that estimate nucleic acid concentrations by measuring absorbance. Nanodrop 2000c is providing a detection range of 2-12000 ng which is high for our sample concentration. This minimum range is the limitation to measuring our samples. While these methods are quick and convenient, they can be influenced by the presence of contaminants or impurities, leading to potential inaccuracies in the obtained miRNA concentration values.

The observed differences in miRNA concentration measured by the four different methods can be attributed to several factors. The variations in measurement principles, sensitivity, and assay dynamics of each technique can lead to discrepancies in the results. Additionally, different sample handling procedures and reagent requirements among the methods may contribute to the observed variations. Overall, the variations in miRNA concentration observed among the Agilent Bioanalyzer, Agilent Tapestation, Qubit and Nanodrop measurements underscore the importance of selecting an appropriate method and interpreting the results cautiously. Understanding the nuances and differences in these measurement techniques will contribute to a more comprehensive analysis and interpretation of the miRNA concentration within the isolated exosomes.

Based on the article, they isolate exosomes from 85ml mast cell cultures containing 100-340 million cells by using ultracentrifuge. Then miRNA isolation was performed by using the miRNeasy mini kit (Qiagen). Their miRNA yield was 2µg, 13 ng/10^6 donor cells. [287]

Our miRNA yield was less than expected. (According to kit's handbook, ~15 ng RNA should be isolated from 1x10^6 cells.) We conclude that because of the low amount of miRNA, reads (that obtained after sequencing) were also so low. miR-335 had the highest read amount compared to the other miRNA.

When we compare our miRNA yield to other groups' experiment results, we can conclude that, low number of seeded cells could be the reason for lower miRNA yield.

4.6 miR-335-5p Expression in Atherosclerotic Conditions

In our sequencing results, changes of miR335-5p in expression patterns inside the exosomes were particularly marked, pointing towards the role of miR-335-5p in the response to inflammatory stimuli such as TNF and LPS.

Given the critical roles of VSMCs and ECs in vascular physiology and pathology, this discovery implicates miR-335-5p as a potential marker for atherosclerosis. [288] Considering that both TNF and LPS are implicated in the pathogenesis of atherosclerosis, the heightened expression of miR-335-5p in the treated groups may provide a mechanistic link to atherosclerotic progression. This suggestion is underlined by the potential of miRNAs in regulating gene expression post-transcriptionally, with alterations in their levels often leading to significant physiological changes.

To further clarify, my research signifies that miR-335-5p could be acting as a vital regulator in the atherosclerotic process, mediated through its impact on VSMCs and ECs under inflammatory conditions. This understanding potentially sets the stage for exploring miR-335-5p as a promising biomarker for atherosclerosis, and possibly, as a future therapeutic target.

mir-335 has previously been associated with EC, VSMC and inflammation.

- miR-335-5p expression is significantly decreased in human osteoarthritis chondrocytes, and overexpression using miR-335-5p downregulates the inflammatory mediators like IL-1B, IL-8, and TNFα. [289]

Effect on Endothelial Cells;

- In one report HUVECs that were exposed to H_2O_2, high glucose or TNFα showed upregulated expression of mir-335-5p.[288]
- According to a different study, endothelial cell overexpression of miR-335 led to an increase in the expression of many inflammatory and fatty acid transport genes. This suggests that miR-335 may contribute to endothelial cell lipid buildup and inflammation. It was shown that these effects were mediated by miR-335-induced NF-kB activation, which was subsequently controlled by the miR-335 target CHFR (checkpoint with forkhead and ring finger domains), a negative regulator of NFkB function. The deletion of Mir335-/- mice in the Apoe-/- mouse model was revealed to be protective against high-fat diet-induced atherosclerosis by *in vivo* study. [290]
- One study found that miR-335-5p targeted and bound to SIRT7 mRNA, which contributed to endothelial ROS-induced signal of senescence-associated secretory phenotype, leading to atherosclerosis. The study also found that SIRT7 overexpression rescued endothelial function from oxidative stress, and miR-335-5p forced expression attenuated the rescue effect. [288]

Effect on Smooth Muscle cells;

- PDGF-BB (Platelet-Derived Growth Factor-BB) can trigger the synthetic phenotype, proliferation and migration of VSMCs and the participation of these cells in vascular remodeling. PDGF-BB was used to establish pathological models. VSMCs were exposed to PDGF-BB , and the expression of miR-335-5p was significantly lower in the PDGF-BB treated cells than in the control cells. VSMC were transfected with miR-335-5p mimic to overexpress miR-335-5p. [291] Based on the provided information, it does not explicitly state that VSMCs become synthetic when overexpressed. However, it suggests that PDGF-BB can trigger the synthetic phenotype, proliferation, and migration of VSMCs when exposed to it. Additionally, the participation of these cells in vascular remodeling is implied.

In conclusion, miR-335 is an important regulator of atherosclerosis and inflammation in endothelial and vascular smooth muscle cells. When exposed to various environmental stimuli such high glucose levels, TNF, or H_2O_2, its expression can change, triggering an inflammatory response and fat accumulation. The activation of NF-kB and its regulation by CHFR, a miR-335 target, appear to be crucial pathways. It's interesting to note that the deletion of Mir335-/- seemed to offer some defense against high-fat diet-induced atherosclerosis, pointing to possible therapeutic implications. Furthermore, the development of the endothelium ROS-induced senescence-associated secretory phenotype that results in atherosclerosis may depend on miR-335-5p's

interaction with SIRT7 mRNA. It's also important to note that miR-335-5p may have a role in vascular remodeling because PDGF-BB-induced pathological circumstances in VSMCs result in lower expression of the gene. Further studies are needed to completely understand its diverse roles and therapeutic possibilities.

5. CONCLUSION

This thesis has paved the way toward identifying a novel atherogenic biomarker using *in vitro* vascular smooth muscle cell (VSMC) and endothelial cell (EC) culture models. Our investigations have led to the candidate miRNA marker, hsa-miR-335-5p, but further experimentation is required to establish its definitive role in atherosclerosis. We used a three culture models, including direct co-culture, indirect culture, and isolated exosome treatment models, to describe the exosomes and find exosomal miRNA signatures. These tests included NTA, Western blot, and RNA sequencing from isolated exosomes. The highlighted candidate, hsa-miR-335-5p, emerged as a potential biomarker. However, to affirm its role as an atherosclerotic biomarker, future experiments should explore its functional role in atherosclerosis, using animal models or patient samples, where feasible. This would involve tracking the expression and function of hsa-miR-335-5p in relation to atherosclerotic progression or regression. Furthermore, *in vitro* experiments using gene silencing or overexpression could provide insights into its role in atherogenic processes such as plaque formation, inflammation, and lipid accumulation. Another critical experiment would involve profiling a panel of atherosclerosis patient-derived exosomes to verify whether the elevated expression of hsa-miR-335-5p is a consistent feature. Overall, our work has initiated a promising direction, with hsa-miR-335-5p standing as a candidate worth deeper exploration in the search for reliable atherosclerotic biomarkers.

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