ABSTRACT

Cardiovascular disease is the leading cause of death in the United States. During the process of plaque development called atherosclerosis, oxidized low-density lipoproteins (oxLDL) penetrate the endothelial lining of the arterial wall. The damage to the endothelial wall induces a signaling pathway to trigger an inflammatory response. Monocytes then phagocytose oxLDL in an attempt to prevent damage to the endothelial wall and ultimately transform into foam cells that constitute plaque tissue. This study explores the prevention of arterial plaque buildup in atherosclerosis using miRNA let-7g. Through bioinformatics, lectin-type oxidized LDL receptor (LOX-1), a macrophage scavenger receptor protein that uptakes oxLDL, leading to foam cell formation, was identified as a potential target. After a thorough literature review, miRNA let-7g was found to be the most promising miRNA that inhibits LOX-1 expression. By preventing the expression of LOX-1, the macrophage will no longer respond to oxLDL signaling and ultimately inhibit plaque development. Our aim was to determine if LOX-1 expression in macrophages would increase in a dose dependent manner in response to increased oxLDL concentrations. LOX-1 expression in human macrophage primary cell cultures was measured using a flow cytometry assay. We found that oxLDL concentration was not correlated with macrophages' expression of LOX-1 receptor in a dose-dependent manner. This suggests that inflammatory signaling molecules are needed for LOX-1 upregulation and increased oxLDL uptake. It is expected that using let-7g in conjunction with an anti-inflammatory compound, such as rapamycin, will further inhibit oxLDL uptake by macrophages and result in a novel treatment for atherosclerosis.

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INHIBITING LOX-1 RECEPTOR IN MACROPHAGES IN ATHEROSCLEROSIS

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INTRODUCTION

Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death in the western world, contributing to approximately 23.5% of deaths each year in the United States alone (Center for Disease Control and Prevention, 2019). Cardiovascular disease is a general term used to describe various heart problems such as arrhythmias, defects, and conditions that involve the narrowing of blood vessels (Mayo Clinic, 2018). A primary contributor to cardiovascular disease is atherosclerosis. Atherosclerosis is a progressive, chronic disease caused by the formation and continuous buildup of plaque in the arteries (Libby et al., 2019). Arterial plaque initially forms when oxidized low-density lipoproteins (oxLDLs) clump together and burrow into the endothelial lining of an arterial wall, known as the intima (Lo & Plutzky, 2012). When oxLDLs damage the intima, an inflammatory response occurs. Macrophages carry a lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) receptor that potentially plays a role in the migration to damaged intima and the phagocytosis of the oxLDLs in an attempt to reverse the damage (Lo & Plutzky, 2012; Wang et al., 2015). The macrophages then die due to the continuous consumption of oxLDL and transform into foam cells. These dead foam cells remain in the arterial wall and start to form plaque. Without treatment, the uncontrolled buildup of plaque leads to the narrowing of arteries and constriction of blood flow to organs in the body. If an unstable plaque formation breaks off from the arterial wall, an acute, life-threatening ischemic attack such as stroke, myocardial infarction or pulmonary embolism can occur (National Heart, Lung, and Blood Institute, 2016). Each of these ischemic attacks affects a large portion of the United States population, making their

cumulative risk even greater. 795,000 Americans suffer from a stroke every year, 735,000 are affected by a heart attack, and an estimated 900,000 Americans suffer from a pulmonary embolism or deep vein thrombosis every year (Raskob et al., 2010). Finding a non-invasive, effective, and inexpensive treatment for atherosclerosis can drastically decrease the risk of these ischemic attacks.

miRNA

MicroRNAs (miRNAs) are a class of small, endogenous RNAs of 21–25 nucleotides in length. They play an important regulatory role in animals and plants by targeting specific mRNAs for degradation or translation repression. Additionally, microRNAs have a wide variety of functions and are involved in various critical biological processes in mammals (Wahid et al., 2010). Current research is the investigation of the roles and pharmaceutical capabilities of miRNAs as treatments for cancer and cardiovascular, viral, and neurological diseases. One of the benefits of utilizing miRNA as treatment is that it is naturally occurring, and thus there is the potential for miRNA replacement therapy, where synthetic miRNA are used to promote normal gene regulation. Another form of treatment could be the use of miRNA suppression to promote - rather than prevent - gene expression (Wahid et al., 2010).

Certain microRNAs have been proven to be involved in cardiac remodeling, the process in which the heart adapts to various stressors (Romaine et al., 2015). However, chronic cardiac remodeling is associated with a wide variety of CVD, such as myocardial infarctions or heart failure (Romaine et al, 2015).

Justification for Research

Atherosclerosis is a progressive, long-term disease caused by the buildup of plaque in the arteries. Over time, the plaque hardens and causes narrowing of the arteries, resulting in irreversible damage to the arteries and the heart due to the lack of proper blood flow throughout the body. It is crucial that efforts and resources focus on research to find a cure because atherosclerosis has the potential to impact anyone regardless of gender, race, and age.

Additionally, prolonged atherosclerotic symptoms can lead to cardiovascular disease (CVD) and complications, such as myocardial infarction (heart attack) and stroke. CVD has become a global epidemic, and the economic implications are monumental. Cardiovascular disease's indirect and direct costs sum up to roughly $555 billion in the U.S. alone, based on data collected in 2016, and this cost is projected to reach $1.1 trillion by 2035 (American Heart Association, 2017). These monumental numbers demonstrate the huge financial impact that CVD can have on our population.

Unfortunately, atherosclerosis cannot be entirely prevented or cured over an individual's lifetime. Besides preventative lifestyle measures, treatments for atherosclerosis are invasive and/or problematic in the long term (National Institutes of Health, 2016). Many of these invasive procedures also do not guarantee a cure and potentially just cause more pain than healing. Thus, this proves that there is a strong need for new, preventive treatment that can ultimately be minimally invasive for the patient and prevent future complications, surgeries, or medications.

Currently, a probable yet risky solution to treat severe atherosclerosis involves seeking professional medical procedures by means of surgery. One common method,

known as percutaneous coronary intervention (PCI), is a process that improves blood flow with one's heart by opening the various affected arteries using a 'mesh tube' that will keep an artery open. Another method, known as coronary artery bypass grafting (CABG) is a type of surgical procedure that uses one's existing arteries/veins from other parts of the body as a solution to 'bypass' the plaque filled arteries/veins (Toledo-Ibelles & Mas-Oliva, 2018). Though these procedures may help improve blood flow and decrease the risk of heart attack and stroke, they are in fact invasive and may have adverse side effects. Recovery time is necessary in order to follow through with such procedures, and there is always a risk of procedure failure. Grouped with high costs, this causes these invasive solutions to be risky and dangerous.

In terms of current non-invasive treatments, one more commonly used method to treat plaque formation in the use of blood thinners and anti-inflammatory drugs, such as aspirin. This more commonly used drug can help thin one's blood, thus allowing for temporary improvement in blood flow, seemingly improving one's situation with regards to symptoms. However, in reality, the use of such drugs is simply a means to delay progression and does not target the real problem at hand. Even with the use of anti-inflammatories, plaque formation is not decreased, and the drugs must be continuously administered. Prolonged use of these drugs is not only expensive but may also trigger adverse side effects while not solving the root of the issue.

Another field of non-invasive treatments includes the use of antioxidants to help alleviate the effects of atherosclerosis. However, the main issue with using them revolves around safety concerns. Many of the methods from previous studies are not standardized, and because comparing current research regarding clinical trials is laborious, there is

currently a lack of conclusions that point us in the direction of this potential solution.

Recent scientific advances have revealed the synthesis pathways and the regulatory mechanisms of miRNAs in animals and plants, and miRNA-based regulation is implicated in disease etiology and has been studied for treatment. Additionally, several preclinical and clinical trials have been initiated for miRNA-based therapeutics, as it has been increasingly considered as a viable treatment. Recent findings in miRNA studies may add new dimensions to small RNA biology and miRNA therapeutics.

LOX-1 is a receptor on the macrophage that initiates the interaction between the macrophage and oxidized LDLs (oxLDLs). When LOX-1 is expressed on the macrophage, it receives signals from oxLDL that has penetrated the artery and is drawn to it. Once at the site of oxLDL, the macrophage takes up the oxLDL to excess and dies. The dead macrophage then hardens as a foam cell and becomes plaque. Using the miRNA sequence let-7g has been shown to prevent the translation of *LOX-1* into the receptor (Ding et al., 2013). Without LOX-1, the macrophage does not receive signals to consume oxLDL. Without the signal, the macrophage never consumes oxLDL and plaque is never formed.

Research Questions and Hypotheses

The goal of this project was to interrupt the atherosclerotic pathway at the macrophage uptake of oxLDL, thereby preventing the differentiation of macrophages to foam cells. We used bioinformatics research and direct testing on a human macrophage primary cell line to answer the following questions:

(1) What receptors are involved in the uptake of oxLDL by macrophage cells that ultimately leads to the production of atherosclerotic plaque?

(2) What miRNAs interact with these receptors to inhibit the protein production of these receptors?

(3) Do the human macrophage primary cells express the LOX-1 protein?

(4) Is the production of LOX-1 inhibited in this cell line through treatment with microRNA let-7g?

We hypothesize that by introducing a miRNA mimetic of let-7g, we can prevent the expression of the LOX-1 protein that results in oxLDL uptake. Ultimately, this could pave the path for future research to determine whether this can prevent the formation of atherosclerotic plaque.

LITERATURE REVIEW

Atherosclerosis

Atherosclerosis is a long-term disease that can lead to stages of cardiovascular disease that are difficult to treat. Foam cell formation and plaque generation are progressive processes but can be mitigated with proper lifestyle choices. However, timely or costly preventive methods and genetic predispositions to atherosclerotic factors make it difficult to prevent atherosclerosis altogether. This review details the pathology, prevention methods, and current treatments available for atherosclerosis in order to better understand the basis of our study and the severity of the disease.

Pathology

When discussing the stages of atherosclerosis, it is essential to have a thorough understanding of the endothelial cells that line the artery walls. Endothelial cells maintain a functional vascular network via vascular homeostasis (Michiels, 2003). Additionally, endothelial cells are the primary responders to changes in the vascular system and play a central role in the mechanisms underlying the development of vascular disorders, including atherosclerosis (Michiels, 2003).

Cholesterol levels play a significant role in the development of atherosclerosis. High blood cholesterol is a significant risk factor for atherosclerosis (Nelson, 2013). Cholesterol travels through the body in small packs called lipoproteins, which are categorized into low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). Lipoproteins are made of fat and protein and help transport cholesterol through the blood. HDL clears from the body through the liver, which prevents the build-up of plaque in the arteries, while LDL carries cholesterol to the arteries, allowing cholesterol to collect in

the artery walls and contribute to atherosclerotic plaque formation (Linton et al., 2018). As LDL levels increase and HDL levels decrease, the risk of cardiovascular diseases rises because LDLs are the main component of arterial plaque (Hao & Friedman, 2014).

In atherosclerosis, LDLs in the blood first accumulate in the arterial intima, which is the innermost layer of the artery wall below the endothelium. Then, LDLs are oxidized by free radicals, which are oxidative agents that promote inflammation in the body and become oxidized LDL or oxLDL (Insull, 2009). The changes in the arterial wall due to LDL oxidation activate endothelial cells to coordinate the recruitment of immune cells such as lymphocytes, mast cells, and neutrophils to sites of oxLDL build-up. Endothelial cells also secrete MPC-1, a monocyte chemoattractant protein, which attracts monocytes to the area (Hao & Friedman, 2014). Immune cells then release more pro-inflammatory cytokines - small proteins that affect the behavior of cells around them - and recruit even more immune cells to the artery wall (Fatkhullina et al., 2016). Monocytes also secrete lipoprotein-binding proteoglycans, which furthers inflammation (Ilhan & Kalkanli, 2015).

As more immune cells are recruited to the site of oxLDL build-up, monocytes attached to the endothelium make their way into the arterial intima, where they proliferate and differentiate into macrophages (Lusis, 2000). Proteins called scavenger receptors (LOX-1, CD36, CD68, and so forth) are on the macrophage surface (Figure 1). The receptors recognize oxLDL and signal the macrophage to engulf the oxLDL to remove oxLDL from the bloodstream (Moore et al., 2013). However, following uptake, Moore et al. explain that there is little negative feedback, leading to macrophages becoming grossly engorged with oxLDL. The normal macrophage phenotype is altered, and they become

foam cells, compromising crucial immune functions. Over time, the foam cells die and spill their lipid-filled contents into the dead core of the atherosclerotic lesion (Lusis, 2000). Eventually, this dead lipid core can become 30% to 50% of the arterial wall's total volume (Insull, 2009) and leads to the formation of harmful atherosclerotic plaque. This process is depicted in Figure 2.

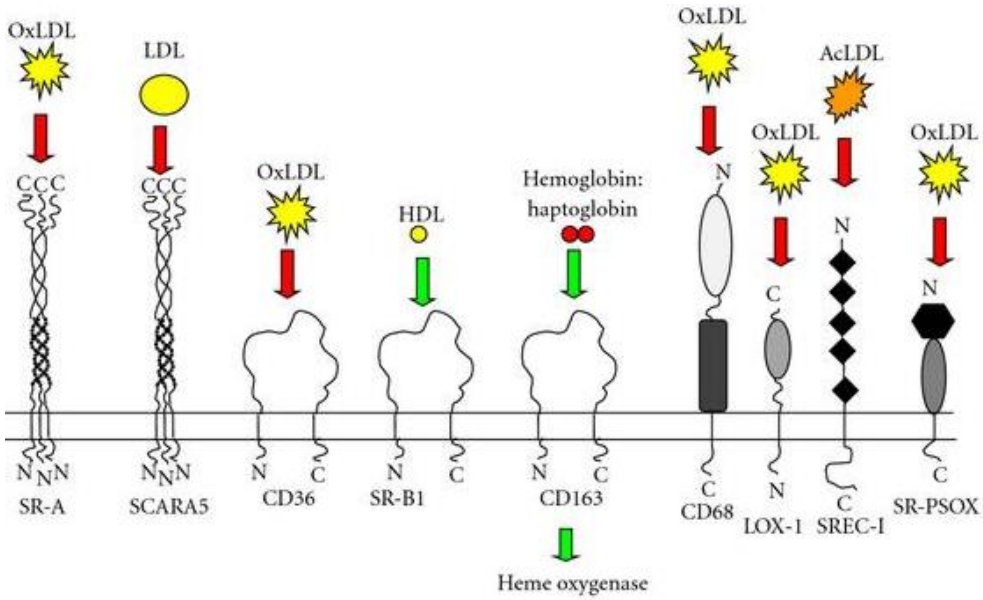

Figure 1. Macrophage scavenger receptors that respond to oxLDL (Stephen et al., 2010).

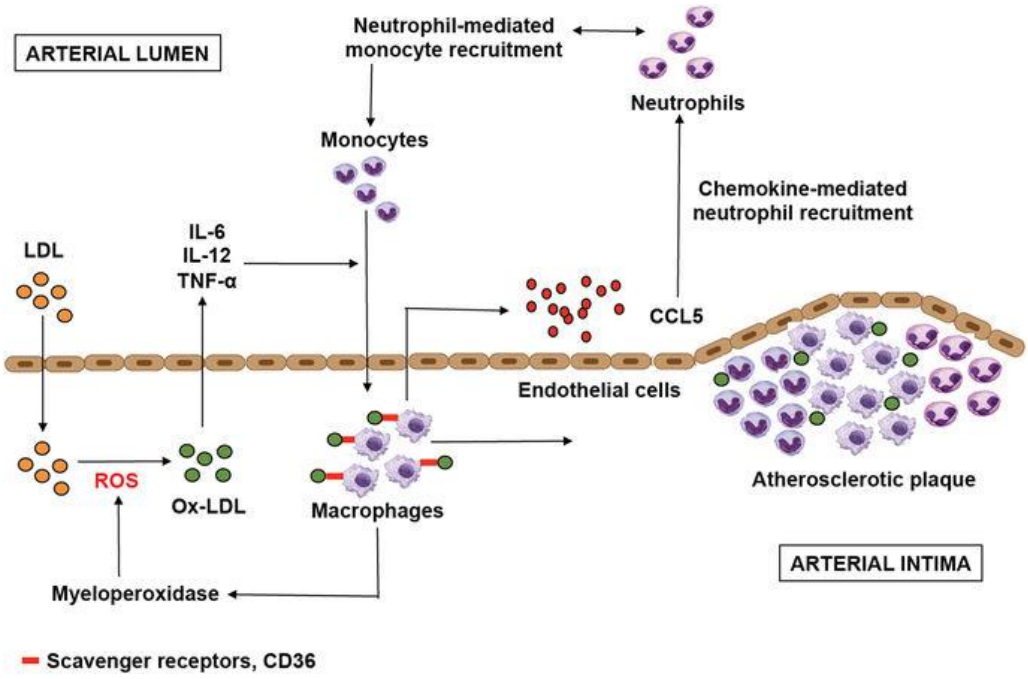

Figure 2. Inflammatory response pathway leading to the generation of atherosclerotic plaque buildup (Chhibber-Goel et al., 2016).

In atherosclerosis, the dominant type of lesion is the fibrous plaque lesion, which supports the dead lipid core that is already built up within the artery wall. Fibrous tissue, particularly collagen type I and type III, is added to form a cap over the lipid core. The fibrous cap is responsible for the strength of the structure due to its composition of calcium and elastin (Insull, 2009). The weak lipid core, however, is prone to deposition, and if the fibrous cap weakens or gets punctured, the entire plaque will rupture (Libby, 1995). Plaque is also reduced by cellular apoptosis, death, and elastin degradation with lipid deposition (Katsuda, 2003). When the plaque ruptures, it has great potential to cause a heart attack or stroke, leading to detrimental cardiovascular events.

Current Treatments

Current treatments to combat atherosclerosis are generally invasive or expensive. Oftentimes prescription drugs, medical procedures, and medical interventions are needed. These methods can be invasive or have harmful side-effects.

Surgical procedures are the most invasive. The most common methods include percutaneous coronary intervention (PCI), which opens blocked or narrowed arteries, and coronary artery bypass grafting (CABG), which bypasses narrowed coronary arteries (Mohr et al, 2013). However, Mohr et al. state that PCI can lead to complications such as blood vessel damage, kidney damage, as well as myocardial infarction, while CABG can potentially cause a stroke. Thus, less invasive atherosclerosis treatments are gaining popularity in both research and patient use. Current, less invasive treatments include anti-inflammatories, antioxidant therapies, and drug inhibitors that target specific proteins involved in atherogenesis.

Anti-inflammatories are compounds that reduce inflammation and oxidative stress at the sites of plaque formation. Studies have shown that arterial plaque has an increased number of inflammatory cells as well as inflammatory mediators (Charo & Taub, 2011). One popular anti-inflammatory treatment is the use of statins, which are lipid-lowering medications with an anti-inflammatory mechanism. Another promising anti-inflammatory is the drug Canakinumab, which targets a cytokine that mediates inflammatory response (Ridker 2017). However, its cost is high-approximately $73,000 per year in the United States, which is extremely expensive for the treatment of CVD (Sehested, 2019). Also, since it targets the body’s inflammatory response and immune system, there can be some adverse side effects in patients, such as immune infections and sepsis (Ridker 2017).

Ultimately, these drugs do not prevent or improve the development of atherosclerosis but only delay the progression.

Another approach is to target specific proteins that are critical in atherosclerosis. Recently, researchers at the NIH have identified a protein, Sirtuin 3 (SIRT3), as a way to resist or reverse the effects of obesity-related inflammation (Traba et al., 2015). SIRT3 ultimately blocks the receptor on macrophages and reduces the inflammation that contributes to atherosclerosis. Similarly, a second approach is to use the adhesion receptor protein CD146 to regulate macrophage foam cell formation (Luo et al., 2017). However, these avenues of research have encountered the same drawbacks as the anti-inflammatories described above, since there are other inflammatory pathways in which atherosclerosis can still progress through.

Antioxidants have also been researched to combat the oxidative stress that plays a role in atherosclerosis. Oxidative stress can be characterized as the imbalance between antioxidant levels and reactive oxygen species (ROS) in the body. An excess of ROS within the plasma and the arterial intima causes increased LDL oxidation (Adams, 1999). Free radicals also become hazardous when direct oxidation of critical cellular components like DNA and protein occurs (Yang, 2017). Antioxidant treatments protect against and eliminate ROS along with specific herbal derivatives, which can reduce inflammation. However, there is no substantial evidence to incorporate antioxidants or herbal remedies and treatments without having the risk of safety concerns (Tian et al., 2017).

Current advancements in treatment are geared towards noninvasive techniques. These noninvasive treatments are used as a preventative measure to slow the progression

of the disease or reverse it altogether. There is also a lower risk involved using noninvasive techniques, as there is usually a higher risk of injury or death with invasive surgical procedures.

Prevention Methods

Since CAD is the number one leading cause of death in the United States, preventative measures must be taken to delay the progression of this disease. Lifestyle changes are the cornerstone of atherosclerosis prevention. Simple changes to daily lifestyle habits can yield significant positive results. Eating right, regular physical activity, smoking cessation, and limiting stress are of the essential strategies for preventing CAD and managing risk factors.

Adopting a heart-healthy diet is one of the main ways for atherosclerosis prevention. Such a diet consists of fruits, vegetables, whole grains, fish, and low-fat dairy products. This also includes limiting sodium, added sugars, refined carbohydrates, and saturated fats. As mentioned above, limiting saturated fat intake is especially important because saturated fats contain small-dense LDL cholesterol, which is more susceptible to oxidation and thus atherosclerosis. Increased small-dense LDL means a decrease in HDL, often known as “good cholesterol,” in the blood. This results in high total cholesterol (TC): HDL ratio, which is a significant predictor of CAD. Diets consisting of increased added sugars and refined carbohydrates also increase the TC/HDL ratio resulting in the progression of atherosclerosis (DiNicolantonio et al., 2016).

Engaging in physical activity is another preventive method for atherosclerosis. Larson-Meyer et al. (2010) demonstrated that LDL, total cholesterol, and blood pressure were improved in those who engaged in regular physical activity and were placed on a

calorie-restricted diet in comparison to those who were only placed on the diet (Larson-Meyer et al., 2010). It is recommended that individuals engage in at least 30 minutes of exercise most days. Simple changes, such as taking the stairs or walking after a meal, can be done to increase daily activity.

Smoking cessation is also critical for the prevention of atherosclerosis. Cigarette smoke contains thousands of chemicals and has reactive oxygen species that upregulate inflammatory cytokines, cause endothelial dysfunction and contribute to oxidative stress, thus hindering proper control of plaque formation (Edirisinghe & Rahman, 2010). The cigarette smoke also can result in increased inflammation and foam cell formation leading to the rapid progression of atherosclerosis (Huang et al., 2016). Increased stress also can interfere with inflammatory responses leading to the progress of atherosclerosis. Stress influences the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. Increased release results in the release of cortisol, referred to as the body's stress hormone. Cortisol has been found to influence cardiovascular function negatively. It has been found that those with low-stress resilience has increased risk for the progression of cardiovascular disease (Robertson et al., 2017).

It is important to note that these are only a few prevention methods, and many more can be taken in order to delay atherosclerosis progression. If lifestyle changes are unsuccessful, medication and further treatments can be provided.

Scavenger Receptors and LOX-1

Scavenger receptors are membrane-bound receptors that bind to a variety of ligands (such as carbohydrates or cholesterol ester) but have been largely known for binding low-density lipoprotein (LDL). In particular, as a supergroup, the scavenger

receptors can bind different forms of LDL leading to subsequent responses within the body. The scavenger receptors can be categorized into several classes, ranging from A-J, depending on the specific domain of the receptor. In general, the receptor first receives a signal when a specific ligand binds its receptor. This triggers an intracellular signaling pathway, resulting in signal transduction and activation down the pathway. For scavenger receptors, this pathway tends to be the regulation of the host response, leading to events such as inflammation, cytokine signaling, and more depending on the specific receptor function and ligand. Overall, scavenger receptors have been found to be specifically linked to ROS generation, apoptosis, and angiogenesis. ROS generation and apoptosis are also linked in this manner. Scavenger receptors that promote ROS generation thus promote oxidative DNA damage, which results in apoptosis (Zani, 2015). These events all occur at a heightened rate during atherosclerosis, thus further strengthening the link between atherosclerotic conditions and scavenger receptors.

Examples of known and studied scavenger receptors are CD36 and SR-A1, which play roles in apoptosis, cell migration and adhesion, inflammation, and foam cell formation (Zani, 2015). Thus, the amplification of these oxLDL receptors can lead to very detrimental effects. However, as shown in Figure 3, a scavenger receptor can potentially be targeted in gene therapy in order to reduce the pro-inflammatory effects. Eliminating or reducing the expression of a pro-inflammatory receptor leads to a reduction in the subsequent signaling events that promote atherosclerosis. Knocking out SR-A1 or CD36 using RNA-interference has already shown promising results in reducing foam cell formation, and subsequently, plaque formation and other atherosclerotic

episodes (Mäkinen, 2010). This discovery is the basis of our study and potential treatment plan, as knocking out the receptor has demonstrated effective results.

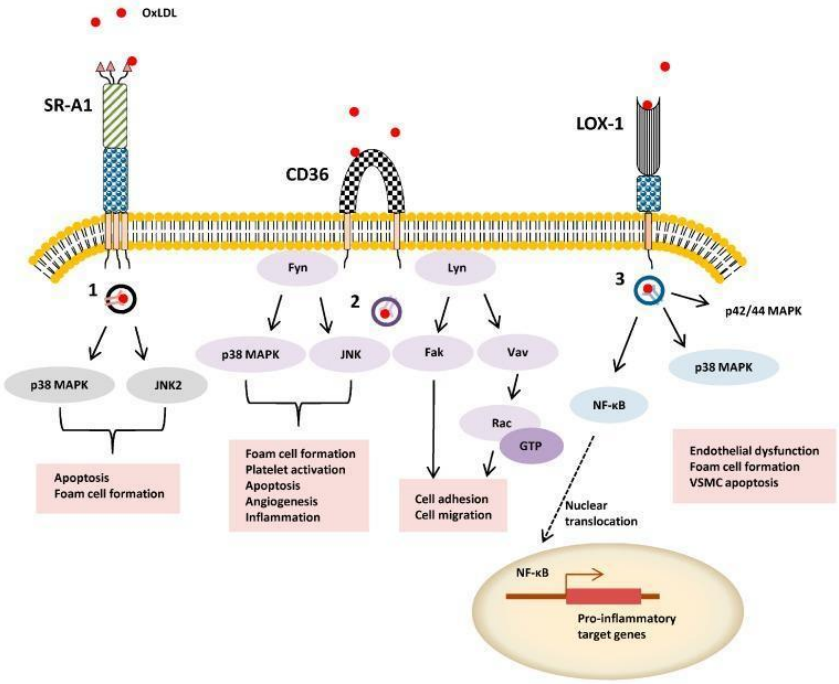

Figure 3: Intracellular signaling pathways of SR-A, CD36, and LOX-1 after oxLDL activation (Zani, 2015).

However, there has not yet been a development of human gene therapy treatment for preventing atherosclerosis. For this study in particular, one scavenger receptor is necessary and has been gaining attention as a potential target in cardiovascular disease treatment due to its enhanced expression during atherosclerosis. This receptor is the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1 receptor), which is one of the most crucial receptors of interest in the uptake of oxLDL. As a type II membrane receptor, its main function is the uptake and internalization of oxLDL, which, in turn, can lead to atherosclerotic pathways such as inflammation and oxidative stress. LOX-1 has been identified in vascular endothelial cells, macrophages, and smooth muscle cells

(Chen et al., 2001), but is of particular importance in the monocyte-derived macrophages due to the subsequent foam cell formation after oxLDL uptake. oxLDL binds with a high affinity to LOX-1, making it a key player in oxLDL internalization. This typically occurs through clathrin-mediated endocytosis or lipid raft-mediated uptake (Murphy, 2008).

LOX-1 expression is increased during atherosclerotic conditions, due to the increase in inflammation and reactive oxygen species (ROS) production (Thakkar, 2015). This leads to a reduction in macrophage migration since the macrophages are taking up the oxLDL (Wang, 2015). Again, this lack of macrophage migration ultimately leads to foam cell formation and plaque build-up due to the continuous consumption of oxLDL until the macrophage itself dies. Additionally, mutations within the LOX-1 gene also result in higher incidences of atherosclerosis (Mango, 2003). This speaks to its importance in the implication of atherosclerosis and cardiovascular disease. Thus, in order to prevent atherosclerotic conditions, LOX-1 expression could be targeted as a potential treatment plan. Knocking out or eliminating the expression of LOX-1 in its entirety has already been proven to significantly reduce atherosclerosis in LOX-1 knockout mice (Mehta, 2007). This is due to the reduction of oxLDL uptake and subsequent oxLDL clearance through the kidneys. Knocking out LOX-1 expression reduces the signaling pathways that are stimulated by the presence of oxLDL, which can lower foam cell formation and plaque generation.

miRNA

Biological Mechanisms

MicroRNAs, also referred to as miRNAs are single-stranded noncoding small RNAs that are typically 21-25 nucleotides long. Typically, miRNAs will downregulate

gene expression by binding to the 3′ UTR (untranslated region) in mature RNA, thereby suppressing protein production via cleavage or translation inhibition. It is estimated that 30% of protein-coding genes are regulated by miRNA (MacFarlane & Murphy, 2010).

While there is a general pathway for miRNA synthesis in animals, there are alternate pathways that exist and are proposed. The general biosynthesis mechanism also differs based on whether it is produced from intronic or intergenic regions of protein-coding genes (MacFarlane & Murphy, 2010). Animal intergenic miRNA biosynthesis begins with the transcription of the miRNA genes by RNA polymerase II or III, forming pri-miRNA (primary miRNA), which are large step-loop molecules (Wahid et al., 2010, p 1231-1243). This molecule is then processed by Drosha and DiGeorge syndrome critical region gene-8 (DGCR-8) to produce a pre-miRNA. The pre-miRNA is then transported to the cytoplasm with the help of exportin 5 (EXP5), which recognizes the short overhangs of the pre-miRNA. However, whether EXP5 is involved in miRNA biosynthesis in mammals is debated (MacFarlane & Murphy, 2010). In the cytoplasm, the RNase III enzyme Dicer cleaves the pre-miRNA hairpin, forming a miRNA/miRNA duplex composed of the mature miRNA strand along with the passenger strand, labeled with an asterisk and also initiates the formation of the protein complex, RISC (Wahid et al., 2010). RISC, whose exact assemblage mechanism is debated, then uses the mature miRNA as a template to recognize complementary sequences in mRNA (MacFarlane & Murphy, 2010).

The mechanism of downregulation that the miRNA/RISC protein complex employs depends on the specificity of the miRNA molecule. The two agreed-upon mechanisms that can be employed are either slicer-dependent or slicer-independent

regulation. If there is extensive base pairing between the miRNA and its target, which is in the 3’ UTR of an mRNA strand, the slicer-dependent mechanism will occur. In humans, argonaute-2 proteins in the RISC complex will cleave the mRNA, and mRNA degradation will occur. If the miRNA does not have extensive base pairing to the target mRNA, the silencer-independent mechanism is employed. In this form of gene suppression, translation is inhibited. There are various pathways that different miRNA use. One proposed process is the physical movement of the target mRNA away from translational machinery to cytoplasmic structures known as P-bodies. Research suggests that P-bodies are the site of reversible mRNA repression as well as mRNA degradation (MacFarlane & Murphy, 2010).

MicroRNAs have a wide variety of functions and are involved in various critical biological processes in mammals (Wahid et al., 2010). There is a wide variety of research that is currently investigating the roles as well as pharmaceutical capabilities of miRNAs as treatments for cancer and cardiovascular, viral, and neurological diseases. One of the benefits of utilizing miRNA as a form of therapy is that it is naturally occurring. Thus, there is the potential for miRNA replacement therapy, where synthetic miRNA is used to promote healthy gene regulation. Another form of treatment could be the use of miRNA suppression to encourage rather than prevent gene expression (Wahid et al., 2010).

Cardiovascular Disease Treatment

The regulatory functions of miRNA in cardiovascular processes have encouraged many researchers to focus on miRNA as a form of cardiovascular disease (CVD) treatment (Zhou et al., 2018). This can include both their use as a form of direct therapy as well as their employment as diagnostic biomarkers CVDs (Zhou et al., 2018).

MiRNAs are involved at various steps of the atherosclerosis pathway, such as endothelial cell dysfunction, inflammatory cell infiltration, lipid dysregulation, as well as smooth muscle cell differentiation (Romaine et al., 2015). Endothelial cell dysfunction, a starting process in atherosclerosis, is linked to miR-126-5p, which regulates the formation of endothelial cells.

The potential of various miRNAs as a form of biomarkers is summarized by Zhou (2018), who focuses mainly on circulating miRNAs. Circulating miRNAs have garnered lots of interest as potential biomarkers due to their durability, as they can travel in the bloodstream in naturally forming vesicles and thus are not degraded (Zhou et al., 2018).

miRNA let-7g

MiRNA let-7g is one of the best-characterized members of the Let-7 family of miRNAs and appears to play a uniquely primary role in the pathogenesis of vascular diseases (Mendell & Olson, 2012). Over the past five years, let-7g has been found as an important modulator in the development of atherosclerosis (Zampetaki & Mayr, 2012). Let-7g has been found to reduce plaque buildup and other effects of atherosclerosis in a variety of cells, including endothelial cells (Frangogiannis, 2014).

Let-7g reduces atherosclerotic progression by stabilizing atherosclerotic plaque. First, it negatively regulates LOX-1 through the intracellular Ca^{2+}-activated protein kinase C-oxLDL-LOX-1-let-7g pathway (Chen et al., 2011). Let-7g is an endogenous inhibitor of endothelial inflammation and protects endothelial cells by regulating TGF-β signaling and SIRT-1 signaling (Figure 4), which are pathways that promote atherosclerotic progression (Liao et al., 2014). This limits plaque inflammation to local areas and therefore improves vascular thrombosis. The increased let-7g expression also

results in less adhesion and migration of monocytes across the arterial endothelial wall, which is an important step in plaque build-up (Rom et al., 2015).

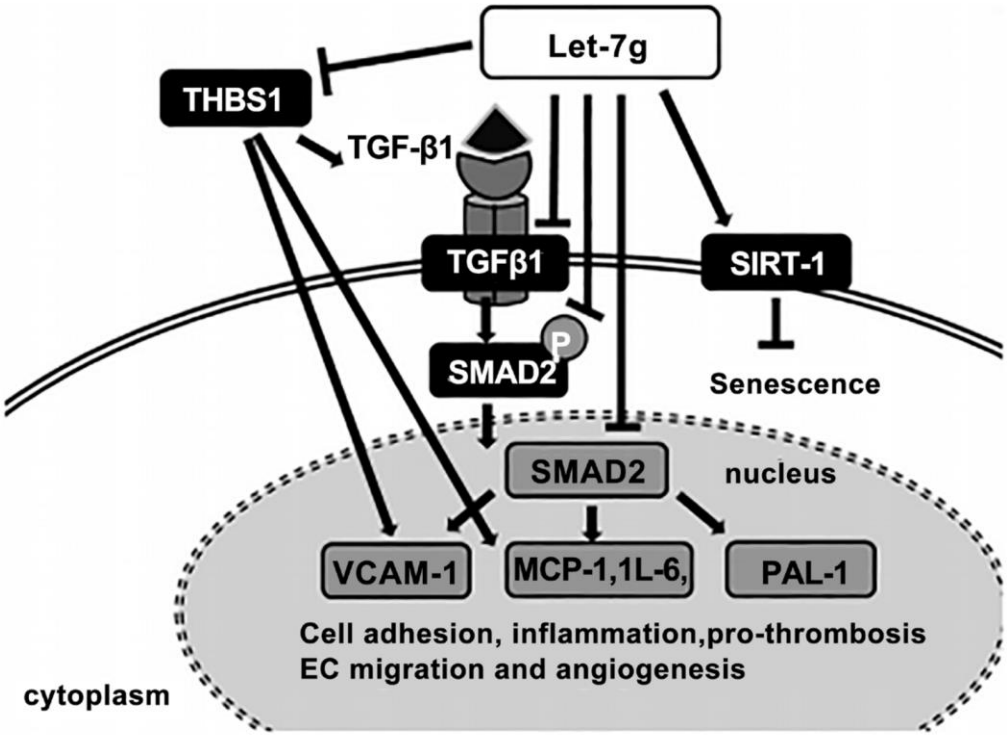

Figure 4. Diagram showing the effects of miRNA let-7g on endothelial cells. miRNA Let-7g modulates TGF-**β** and SIRT-1 signaling to prevent outcomes such as continued cell proliferation, inflammation, monocyte adhesion, and monocyte migration (Liao et al., 2014).

Another possible mechanism by which miRNA let-7g contributes to reducing atherosclerotic progression is by downregulating matrix metalloproteinases, or MMPs (Frangogiannis, 2014). MMPs belong to a family of proteases produced by inflammatory cells in atherosclerotic plaques to digest extracellular matrix, which helps vascular smooth muscle cells (VSMCs) migrate to the fibrous cap and cause plaque rupture

(Allahverdian et al., 2014). MiRNA let-7g may contribute to the reduction of atherosclerotic plaques by regulating MMPs. MiRNA let-7g has been shown to suppress the expression and activity of MMP-2 and MMP-9 in breast cancer (Qian et al., 2011). However, the role of MMPs in the atherosclerosis pathway is complicated; while some may weaken plaque caps, other MMPs are responsible for plaque stabilization. Some studies have shown that MMP-2, MMP-9, and MMP-14 promote VSMC migration and proliferation that increases fibrous cap thickness and maintains plaque stability (Liu et al., 2014). Furthermore, different MMPs have differential effects on plaque stabilization. More research on various MMPs' individual and synergistic effects on plaque stabilization should be studied.

Rapamycin

Biological Mechanism

Upon initial discovery, rapamycin, also known as sirolimus, was found to be produced by *Streptomyces hygroscopicus*, a soil bacterium in Easter Island. Rapamycin can be characterized by its antiproliferative properties as well as its ability to suppress the immune system (Lee & Kim et al., 2014). It exerts its immunosuppressive effects inhibiting the mammalian target of rapamycin (mTOR) signaling pathway which is critical for the regulation of cell growth and metabolism by controlling mRNA translation and ribosome synthesis (Guertin & Sabatini, 2017). There are currently two separate, yet functional, mTOR complexes.

Information from oxygen levels, growth factors, and nutrients such as amino acids are first integrated by mTOR complex 1 (mTORC1). Typically, the T cells in the immune system that produce cytokines (small proteins essential for immune function) activate the

PI3K/AKT pathway and mTOR. The activated mTORC1 can then phosphorylate ribosomal protein 6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) to initiate protein translation and subsequent protein synthesis to promote tumor growth and metastasis (Tong & Jiang, 2016). However, rapamycin disrupts this pathway by acting as a potent inhibitor of the TOK kinase. More specifically, rapamycin binds to the 12 kDa FK506-binding protein (FKBP12), forming the rapamycin-FK506 complex, which then blocks mTORC1 disrupting the progression of cell cycle and proliferation of T cells (Lee & Kim et al., 2014).

The mTOR complex 2 (mTORC2) is slightly different in that it is a rapamycin-insensitive protein complex. Here the mTROC2 integrates information from only growth factors and, in turn, promotes cytoskeleton organization in cells and cell survival via activation of protein kinase B, also known as Akt (Lee & Kim et al., 2014). However, with chronic exposure and administration of rapamycin, it has been found that the mTORC2 pathway can be inhibited as well. While the mechanism for this has not been found, it is proposed that inhibition of complex assembly is the reason why (Oh & Jacinto, 2011).

Due to its immunosuppression abilities, rapamycin has been beneficial for the treatment of many diseases such as cancer, neurodegenerative diseases, and diabetes (Zhang et al., 2014). However, the mTOR pathway promotes not only increased protein synthesis but also the proliferation of vascular smooth muscle cells and plaque formation in atherosclerosis (Cai et al., 2018). With the inhibitor properties of rapamycin on the mTOR pathway, rapamycin acts as a promising treatment for atherosclerosis.

Treatment of Cardiovascular Disease

As previously noted, rapamycin is readily used as an immunosuppressive drug, as it acts as an mTOR pathway inhibitor, which has been proven to be helpful for lowering the risk of rejection after organ transplants and for treating cancer patients (Dao et al., 2017). As a drug, it has a varying range of effects, from immunomodulating to anti-aging. However, it has been recently discovered that rapamycin has an impact on the cardiovascular system and its subsequent diseases as well. In particular, rapamycin has anti-atherosclerotic effects, in turn impacting the growth and progression of plaque due to its stimulation of autophagy pathways. The potential anti-atherosclerotic effects are wide-reaching, from promoting vasorelaxation, decreasing foam cell formation, to inhibiting the movement of monocytes. Rapamycin also tends to serve as a preventative treatment against the advanced stages of atherosclerosis. During advanced atherosclerotic stages, foam cell formation and plaque buildup are highly unstable, but rapamycin has the ability to stabilize plaques through its inhibition of apoptosis (Sun et al., 2018). However, it is unable to reduce the amount of lipid accumulation. Clinically, rapamycin can be applied to help treat atherosclerosis and the subsequent cardiovascular issues that follow, but this has proven to be both difficult and invasive.

Although a rapamycin/sirolimus-eluting stent has been used in clinical patients and found to be potentially more successful than traditional coronary stents (Morice et al., 2002), the procedure to both insert the stent during surgery and maintain its stability afterwards is difficult. There are several complications that can follow, such as the fracturing of the sirolimus-eluting stent (Ino et al., 2009) and hyperlipidemia, which may only further cause additional atherosclerotic issues as this results in an increase in lipids

(Kniepeiss et al., 2004). Since rapamycin affects endothelial cells as well, there is evidence of it potentially causing endothelial dysfunction, which can further aggravate atherosclerosis. There appears to be an optimal concentration of rapamycin for atherosclerotic treatment (dose dependency) that does not result in such side effects as endothelial cell damage and hyperlipidemia (Otsuka et al., 2015), but as of recently, that concentration is not well-known. Overall, rapamycin does have a therapeutic effect on atherosclerosis and may potentially be a strong treatment for it in the future. The mentioned studies have seen an application in animal trials and are now in practice with human patients. However, advancements are needed in order to minimize the dangers and invasiveness of the current clinical procedures utilizing rapamycin.

SUMMARY OF GOALS

Our study of microRNA let-7g and its effects on atherosclerosis was conducted by pursuing three goals:

I. Identify a receptor involved in the uptake of oxLDL as well as a miRNA sequence that inhibits its expression.

II. Confirm the expression of LOX-1 in human macrophage primary cell culture.

III. Measure the degree of inhibition of LOX-1 production in the macrophage cells after treatment with a miRNA mimic of let-7g.

We aimed to accomplish the first goal via a bioinformatic investigation of receptor proteins, for oxLDL, on the surface of human macrophage cells, as well as of corresponding, testable miRNA to inhibit such a protein. For the second goal, we hypothesized that human macrophage primary cell culture cells would express the LOX-1 protein. In addition to this general hypothesis, we expected the expression of the protein to increase in the presence of oxLDL. Concerning the third goal, we expected that, after treating the macrophage cells with the miRNA let-7g mimic, the cells would exhibit a decrease in the expression of the LOX-1 protein.

METHODOLOGY

Methodology Justification

After developing an understanding through the literature of macrophage cell culturing, LOX-1 receptor function, miRNA transfection, and other assays measuring the uptake of oxLDL, the following methods were performed.

Bioinformatics

The use of bioinformatics was important to better understand the genetic composition and ultimate protein structure of several oxLDL receptors on human macrophage cells. The resource genecards.org was helpful in determining the cell signaling pathways by which LOX-1 receptor takes up oxLDL, and its role in other pro-atherogenic events.

The first step to our approach includes the use of bioinformatics to find miRNA that targets proteins that uptake oxLDL, such as LOX-1, SCARB-1, and MSR1. Online bioinformatic analyses were performed to assess the predictable miRNA binding sites in order to validate the miRNA-mRNA interactions. miRNAs are small (18-24 nucleotides), single-stranded, noncoding RNAs. They regulate gene expression by binding to the 3'-untranslated region (UTR) of specific target mRNA (messenger RNA) sequences. Since any given miRNA can have several distinct miRNA-binding sites within its 3'-UTR, miRNAs have multiple levels of regulation over gene expression.

It is expected that increasing the expression of miRNA *in vitro* will repress protein production. Reliable genetic databases such as mirbase.org and genecards.org provided useful information about the genes for the LOX-1, SCARB1, and MSR1 proteins. By searching databases like miRBase, we accessed full annotations and

predicted gene targets of any miRNA sequence (Griffiths-Jones, 2006). Information gathered included the sequence of the gene, the 3' UTR, and the miRNAs which have a known effect on gene expression. Research databases like miRBase provided "integrated interfaces" for miRNA sequence data, annotation, and predicted gene targets (Griffiths-Jones, 2006).

Proteins that imbibe LOX-1 include proteins included scavenger receptors such as SCARB1, LOX-1, and 3 members of the class A type of scavenger receptors. Using genecards.org, the gene information for these proteins were found, and each web page was looked at individually. From these pages, the sequence of the 3' UTR, the sequence of the entire gene, and relevant miRNA were obtained and recorded. Secondly, the names of individual miRNA were searched for in mirbase.org. This website provided the sequence of the miRNA, any possible stem-loops, and relevant research articles on the specific miRNA. After all this information was gathered, it was deduced that LOX-1 would be the best protein to inhibit with a miRNA called let-7g. Overall, these websites helped us to deduce the miRNA that are associated with gene expression and protein production. Ultimately, the resulting miRNA were cross-referenced and searched for in other databases such as NCBI.

Macrophage Cell Culture

Human macrophage primary cell culture was purchased (Celprogen) and grown in Human Macrophage Cell Culture Complete Growth Media containing fetal bovine serum and antibiotics (Celprogen). The cell line was maintained in T25 flasks kept at 37°C, 5% CO_2. Media was replaced every other day and passed once every 7 days.

Analysis of LOX-1 Expression

Macrophages were plated with oxLDL tagged with fluorescence at varying concentrations (0ug, 5ug, 10ug, 20ug) to assess dose-dependent LOX-1 expression. Cells were run on a FACS Canto II flow cytometer (BD Biosciences, CA) and analyzed with FCS Express 6 (De Novo Software, Glendale, CA). Deris, doublets, and dead cells were excluded from analysis. Flow cytometry and CD45 flow cytometry was conducted to count live cells, cells expressing CD45, and cells expressing LOX-1 protein.

RESULTS

Flow cytometry analysis of macrophages to determine LOX-1 expression levels

We first assessed the effects of oxLDL on LOX-1 expression in human macrophages. The percentage of live macrophages expressing LOX-1 was calculated through flow cytometry (Figure 5). CD45-gated flow cytometry was also used to calculate the percentage of live CD45+ macrophages expressing LOX-1 (Figure 6).

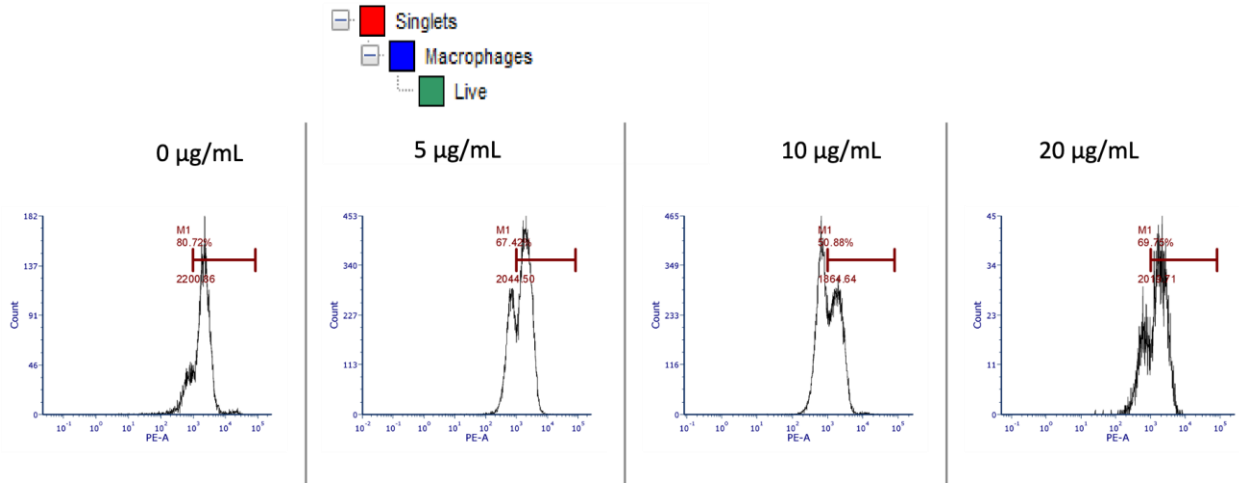

Figure 5. Live macrophage cell count and PE emission (nm) in various oxLDL concentrations. After incubating human macrophage cultures with various oxLDL

concentrations (0, 5, 10, and 20 ug/mL), percentage of LOX-1-expressing macrophages as calculated by running the cells on a FACS Canto II flow cytometer (BD Biosciences, CA) and analyzing them with FCS Express 6 (De Novo Software, Glendale, CA). The red markers represent the gates.

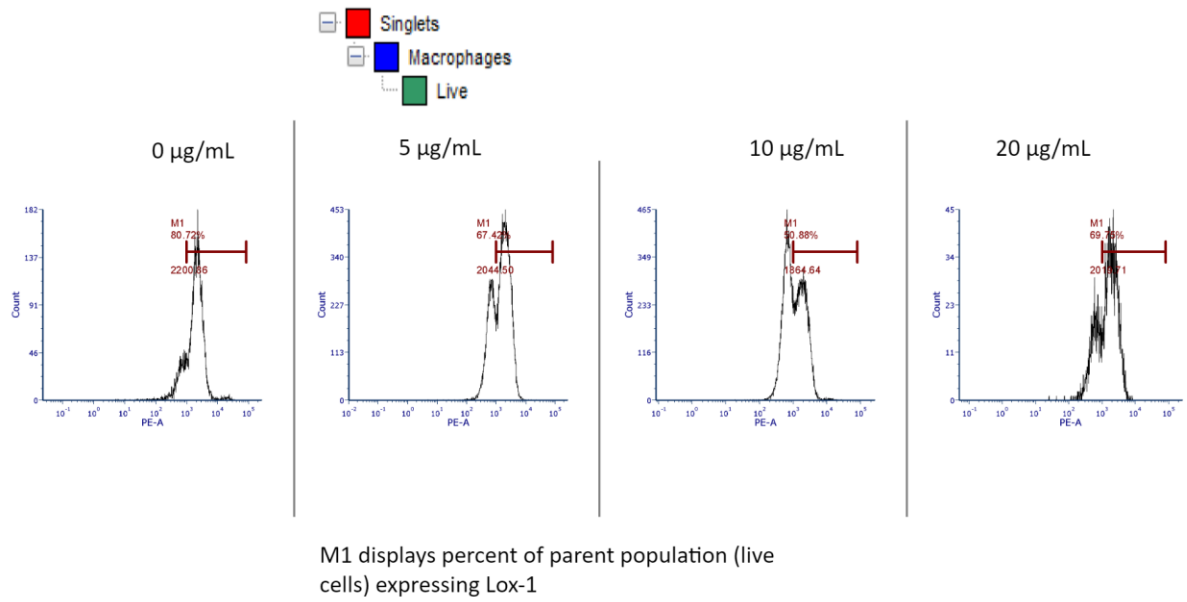

Figure 6. Live CD45+ macrophage cell count and PE emission (nm) in various oxLDL concentrations. After incubating human macrophage cultures with various oxLDL concentrations (0, 5, 10, and 20 ug/mL), percentages of LOX-1-expressing macrophages were calculated using FCS express software. The red markers represent the error bars. CD45 flow cytometry was performed.

Contrary to other literature, the percentage of LOX-1-expressing macrophages did not appear to have a positive correlation with oxLDL concentration (Figure 7). In fact, there appears to be almost no correlation between oxLDL concentration and LOX-1 expression, as indicated by the low R^2 value of 0.143 (Figure 7). Likewise, the percentage

of LOX-1-expressing CD45+ macrophages was not positively correlated with oxLDL concentration (Figure 8).

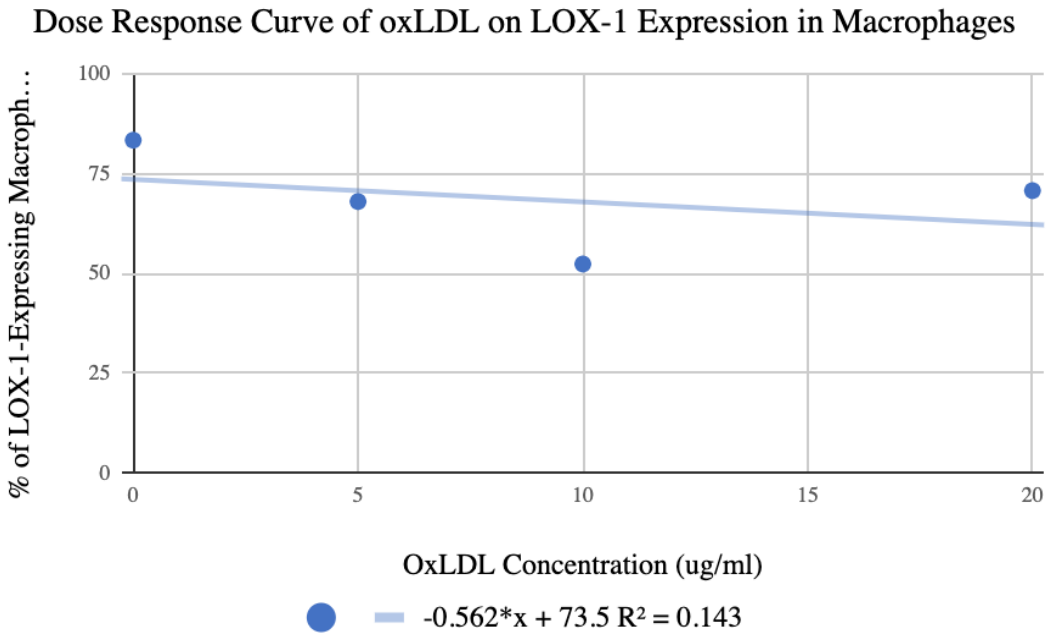

Figure 7. Dose response curve of oxLDL (ug/mL) on LOX-1 expression in macrophages. Percentage of all macrophages expressing LOX-1, including CD45+ macrophages, obtained from flow cytometry was plotted against oxLDL concentration. Linear line of best fit was calculated with equation $y = -0.562x + 73.5$, with an R^2 value of 0.143.

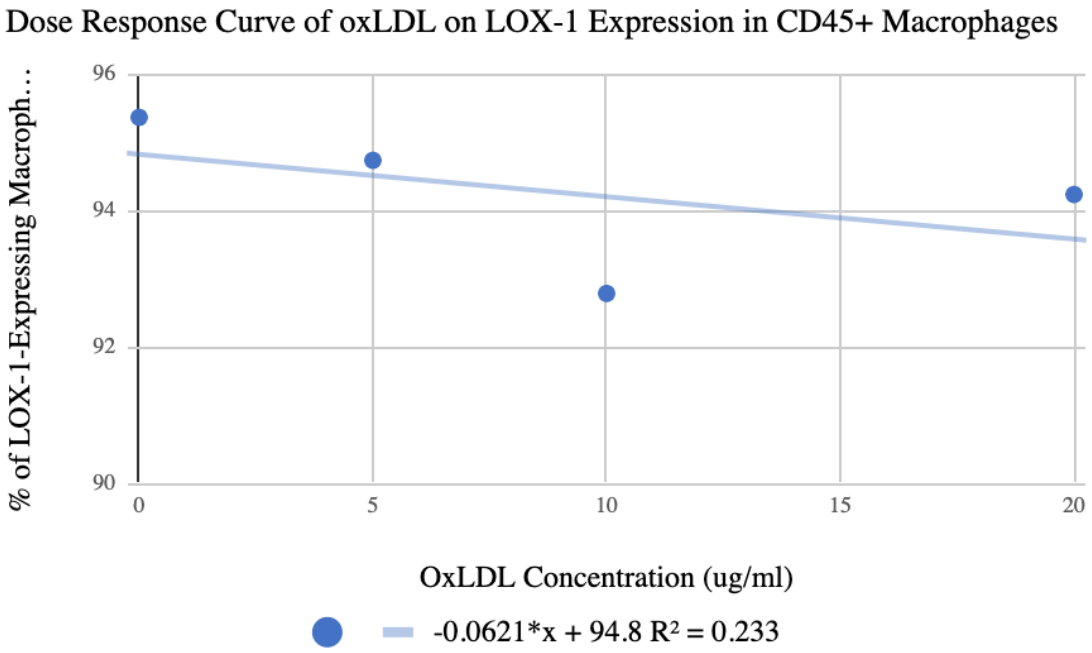

Figure 8. Dose response curve of oxLDL (ug/mL) on LOX-1 expression in CD45+ macrophages. Percentage of macrophages expressing LOX-1 obtained from CD45 flow cytometry was plotted against oxLDL concentration. Linear line of best fit was calculated with equation y = -0.0621x + 94.8, with an R^2 value of 0.233.

However, oxLDL appears to have the same consistent effect on macrophages, whether they are CD45+ or CD45-. There is a strong positive correlation between LOX-1 expression in CD45- and CD45+ after the same oxLDL treatments; the linear regression had an R^2 value of 0.905 (Figure 9).

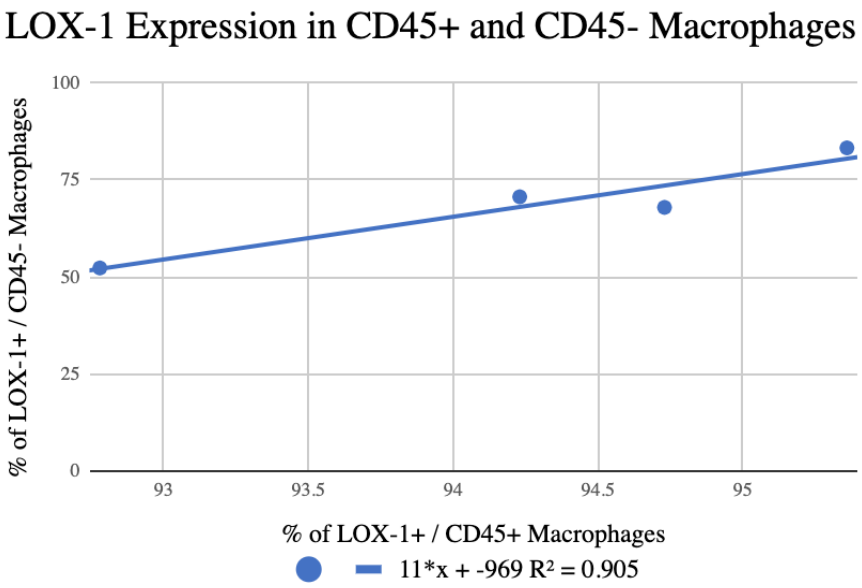

Figure 9. LOX-1 expression in CD45+ and CD45- macrophages at the same oxLDL concentrations. Corresponding percentages of LOX-1 expressing CD45- and CD45+ macrophages obtained from flow cytometry were plotted at these oxLDL concentrations: 0, 5, 10, and 20 ug/mL.

Flow cytometry analysis of poorly differentiated macrophages

Poorly differentiated macrophages expressed low levels of CD45. Among these cells, 98.15% of them were CD45- according to flow cytometry data (Figure 10). Of the CD45- cells, only 5.25% of them constitutively expressed LOX-1 (Figure 10). Only 1.85% of cells expressed CD45 (Figure 10). Of the CD45+ cells, 23% constitutively expressed LOX-1 (Figure 10).

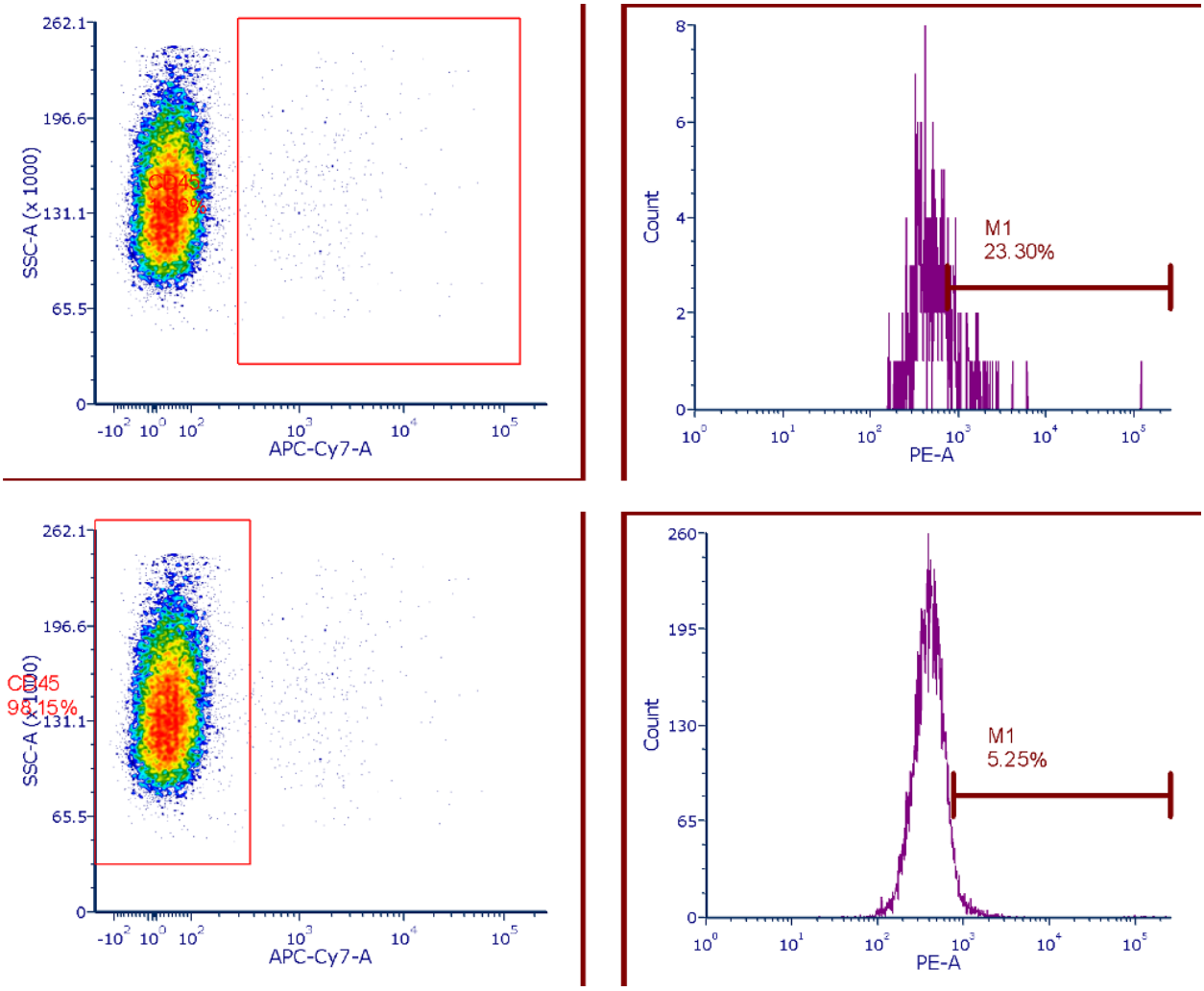

Figure 10. Flow cytometry analysis of poorly differentiated monocytes. Top right panel is emission intensity and corresponding macrophage counts of CD45+ cells (circled in red in the top left panel). Top left panel is emission intensity and corresponding macrophage counts of CD45- cells (circled in red in the bottom left panel). Purple markers indicate the percentage of macrophages that constitutively express LOX-1.

DISCUSSION

OxLDL does not independently increase macrophage expression of LOX-1 receptor

OxLDL does not appear to stimulate LOX-1 receptor production on its own in pure macrophage culture. Contrary to previous literature, there appeared to be no positive correlation between oxLDL addition and LOX-1 expression in any macrophage culture of this study. In fact, there was no correlation between oxLDL concentration and percentage of macrophages expressing LOX-1.

Several reasons could account for this. First of all, previous literature found that LOX-1 expression greatly increases in the presence of oxLDL in addition to inflammatory molecules such as TNF-α (Kume et al., 2000). Atherosclerosis is an inflammatory process, so an inflammatory ligand may be needed to stimulate LOX-1 production despite the presence of oxLDL.

Secondly, there appeared to be a steady decrease in LOX-1 expression from 0 to 10 ug/uL oxLDL; perhaps the percentage of macrophages expressing LOX-1 at 20 ug/uL was due to human error (Figures 7 & 8). Conversely, the increase in LOX-1 expression from 10 to 20 ug/uL could in fact be due to oxLDL concentrations greater than a certain threshold value. Previous literature has found that the binding of oxLDL to LOX-1 is enough to activate the NF-kB inflammatory pathway, which further upregulates the expression of LOX-1 (Kattoor, Goel, & Mehta, 2019). If oxLDL alone is enough to upregulate LOX-1, this could cause the increase in LOX-1 expression from 10 to 20 ug/uL. Macrophage expression of LOX-1 should be analyzed in oxLDL concentrations of greater than 20 ug/uL.

The results indicate that more testing is needed with increased replicates and a wider range of oxLDL concentrations in order to properly confirm or disprove findings.

LOX-1 expression is dependent on monocyte differentiation

Macrophage cells arise from monocytes, which differentiate into macrophage cells after various signaling events. Immature, poorly differentiated macrophage cells express very low levels of LOX-1 (Figure 10). Of the immature CD45+ macrophage cells, 23% constitutively expressed Lox-1 (Figure 10), compared to 95% when fully differentiated (Figures 6 & 8). Further, among the immature CD45- macrophage cells, only 5.25% of them expressed Lox-1 (Figure 10).

These data suggest that LOX-1 expression is dependent on monocyte differentiation, and that this should be noted in future experiments. Additionally, this confirms previous literature that LOX-1 is expressed in differentiated macrophages but suggests that even a low percentage of undifferentiated monocytes express LOX-1 (Kume et al., 2000).

Future Methodology

Since human differentiated macrophages in this study did not express LOX-1 in a dose dependent manner when oxLDL is present, it is important to understand how LOX-1 expression can be manipulated. Further research is needed to obtain optimal oxLDL concentrations at which LOX-1 can be studied. In the context of this project, the next step would be to use let-7g miRNA mimic to downregulate LOX-1 expression. It is expected that LOX-1 expression will decrease and oxLDL uptake will decrease in a dose-dependent manner. After examining the role of miRNA let-7g in inhibiting the LOX-1 receptor expressed in the macrophages, this miRNA treatment will be compared to an

existing drug with known anti-atherosclerotic effects. The drug chosen for comparison was rapamycin (Sirolimus), which has been found to reduce the increase in LOX-1 mRNA and protein levels when oxLDL is present. It does this by reducing mTOR (mechanistic target of rapamycin) phosphorylation, inhibiting transcription factor NF-κB activation, and suppressing LOX-1 expression. In order to do this, the macrophages will be transfected with a miRNA let-7g mimic, and oxidized LDL uptake into the cells will be measured. It is expected that LOX-1 expression and oxLDL uptake will be reduced. After this experiment, the results of transfection with let-7g and rapamycin will be compared to assess which is best at reducing oxLDL uptake. This could have implications for drug development in the context of heart disease treatment.

In order to complete this experiment, the macrophages will need to be treated according to a specific set of conditions, and their uptake of oxidized LDL will need to be measured using flow cytometry. In the first part of the experiment, a specific amount of the previously cultured human macrophages will be added in triplicate to the 24-well plate. They will then be treated using the following conditions:

- Row one, three wells: Positive control group, macrophages will be treated only with Myricetin. Myricetin has been reported to be an inhibitor of oxLDL uptake through CD36 transcriptional reduction in macrophages.
- Row two, three wells: Macrophages will only be treated with rapamycin.
- Row three, three wells: Macrophages will only be treated/transfected with miRNA let-7g mimic.
- Row four, three wells: Macrophages will be treated with both rapamycin and miRNA.

- Row five, three wells: Negative control group two, macrophages will not be treated with anything.

Following this treatment period, fluorescent oxLDL called oxLDL-DyLight 488 will be added to each of the wells in the plate. The macrophages will then be incubated for 4 to 24 hours before the flow cytometry protocol begins to observe the final uptake of oxidized LDL in the macrophages.

Anticipated Results

It is expected that under treatment condition row one, oxLDL uptake will be reduced because Myricetin is a known inhibitor of oxLDL receptors such as CD-36. The purpose of finding these results is to establish a baseline that we can compare further results to. Additionally, the treatment scenario in row five will establish a baseline amount of oxLDL that these cells take up. It is anticipated that under treatment condition row two, there will be decreased oxLDL uptake. This level will be compared to Myricetin to understand its relative efficacy as a possible drug for atherosclerosis prevention. The third treatment condition will allow us to compare oxLDL uptake after LOX-1 inhibition with miRNA let-7g. We expect to see a decrease in oxLDL uptake after the miRNA let-7g treatment. This would support previous research that suggested that miRNA let-7g can inhibit expression of LOX-1 protein by targeting its 3' untranslated region, therefore reducing the amount of oxLDL taken up by the cell. If there is a higher level of oxLDL uptake in the treatment with let-7g in comparison to the treatment with Myricetin, we would be able to confirm that miRNA let-7g is a more effective treatment for inhibiting the LOX-1 receptor than rapamycin. After establishing how effective let-7g and rapamycin are individually at decreasing LOX-1 expression and oxLDL uptake, the

macrophage cell line will be treated with them both. It is expected that when they are used in combination, the lowest amount of LOX-1 dependent uptake of oxLDL will be observed. After concluding the optimal concentrations of miRNA let-7g and rapamycin that diminish ox-LDL uptake, these drugs could be used as an injection to prevent further development of atherosclerotic lesions in heart disease patients.

Future Directions

MiRNA has proven successful in many *in vitro* studies, but its behavior *in vivo* is a cause for concern. An array of miRNA have been studied with success in targeting and inhibiting mRNA expression in vitro. Within the body, miRNA degenerates rapidly, has poor cellular uptake, and clears rapidly following administration in the body. These characteristics make miRNA an ineffective treatment in vivo when used on its own. Developing a compatible delivery method is essential to the efficacy of miRNA.

Liposomes & Drug Delivery

Developing a compatible delivery method is essential to the efficacy of miRNA as a drug treatment. Nanoparticles composed of lipids, polymers, and metals have all been studied with varying levels of stability and efficacy (Ozpolat, 2013). Of these nanoparticles, a lipid-based nanosome, otherwise known as a liposome, has proven to improve stability and increase bioavailability. Liposomes are one of the most common delivery methods *in vitro*.

Liposomes are optimal for treatment pertaining to atherosclerosis. The stability of liposomes has been studied in animal models and there is a direct relationship between cholesterol content and liposomal stability. This is optimal for treatment of the atherosclerotic pathway because of the relationship between hyperlipidemia and

atherosclerosis (Lobatto et al, 2012). The composition of the liposome contributes to the characteristics of the nanoparticle. A liposome of equimolar amounts of cholesterol and phosphatidylcholine showed the highest levels of miRNA transfection into the liposome. Meanwhile, cholesterol-free liposomes have been found to have decreased stability (Kirby et al., 1980). Cholesterol is integral to a stable liposome and, fortunately will then cooperate well with the let-7g miRNA transfection. A particular study manipulated liposomes encapsulating miR-34a and let-7g to treat a lung tumor in mice. The treatment found "significantly decreased lung tumor burden" to approximately half of the mice treated with miRNA (Alshehri et al., 2018). The liposome delivery system has high efficacy and is commonplace in miRNA studies.

Polymer Vesicles

Polymer vesicles, also referred to as polymersomes, are a viable and versatile drug delivery method due to their adjustable membrane contents, targeting abilities, and capability of containing a variety of molecules (Zhao et al., 2017). Unlike liposomes, polymersomes are made of "macromolecular amphiphiles architectures", a distinctive composition that allows them to have superior "colloidal stability" and protection of any drug contents (Zhao et al., 2017). In this section, we will take a look at asymmetrical polymersomes, which are polymeric capsules with asymmetrical membranes that allow them to have a superior endocytosis rate.

Due to their more stable membranes compared to that of liposomes, polymersomes may serve as a superior alternative for drug delivery. Additionally, polymersomes have the ability to contain hydrophobic, hydrophilic and amphiphilic compounds in their membranes. This is a notable advantage because future directions

may lead to other contents other than microRNA or Rapamycin being contained for delivery.

In this case, the use of the previously described ‘asymmetric polymersomes’ is advantageous due to the fact that they will allow for an increased endocytosis rate and efficient drug loading capacity, both which allow for the polymersome to protect drug properties and allow for more control in drug release (Zhao et al., 2017). Furthermore, in the case for protein delivery, asymmetric polymer vesicles are capable of encapsulating larger amounts of proteins efficiently than normal polymer vesicles, and the membrane of these vesicles will also prevent protein degradation. As such, these vesicles could be potentially used in the future as a means of drug delivery. The versatile and adjustable properties, along with greater protection for vesicle contents highlight the visibility that polymersomes showcase in a potential future application.

REFERENCES

Adams, A. K., Wermuth, E. O., & Mcbride, P. E. (1999). Antioxidant vitamins and the prevention of coronary heart disease. *American Family Physician,* 60(3), 895.

Alshehri, A., Grabowska, A., & Stolnik, S. (2018). Pathways of cellular internalisation of liposomes delivered siRNA and effects on siRNA engagement with target mRNA and silencing in cancer cells. *Scientific Reports,* 8(1), 3748. https://doi.org/10.1038/s41598-018-22166-3

Allahverdian, S., Chehroudi M, A.C., McManus, B.M. et al. (2014) Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation, 129,* 1551-1559. https://doi:10.1161/CIRCULATIONAHA.113.005015

American Heart Association (2017). Cardiovascular disease: A costly burden for America - Projections through 2035. *American Heart Association*. https://healthmetrics.heart.org/wp-content/uploads/2017/10/Cardiovascular-Disease-A-Costly-Burden.pdf

Cai, Z., He, Y., & Chen, Y. (2018). Role of mammalian target of rapamycin in atherosclerosis. *Current Molecular Medicine, 18.* https://doi:10.2174/1566524018666180926163917

Center for Disease Control and Prevention. (2019, December 2). Heart disease facts. https://www.cdc.gov/heartdisease/facts.htm

Charo, I. F., & Taub, R. (2011). Anti-inflammatory therapeutics for the treatment of atherosclerosis. *Nature Reviews Drug Discovery*, *10*(5), 365–376. https://doi: 10.1038/nrd3444

Chen, K.C., Hsieh, I.C., Hsi, E. et al. (2011) Negative feedback regulation between microRNA let-7g and the oxLDL Receptor LOX-1. *Journal of Cell Science, 124*, 4115-4124. https://doi:10.1242/jcs.092767

Chen, M., Masaki, T., & Sawamura, T. (2002). LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: Implications in endothelial dysfunction and atherosclerosis. *Pharmacology & Therapeutics, 95*(1), 89-100.

Chen, M., Narumiya, S., Masaki, T., & Sawamura, T. (2001). Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding. *Biochemical Journal*, 355(Pt 2), 289–296. https://doi:10.1042/0264-6021:3550289

Chhibber-Goel, J., Singhal, V., Bhowmik, D., Vivek, R., Parakh, N., Bhargava, B., & Sharma, A. (2016). Linkages between oral commensal bacteria and atherosclerotic plaques in coronary artery disease patients. *NPJ Biofilms and Microbiome*s, *2*(7). https://doi.org/10.1038/s41522-016-0009-7

Dao, V., Liu, Y., Pandeswara, S., Svatek, R., Gelfond, J. A., Liu, A., Hurez, V., & Curiel, T. J. (2016). Immune-stimulatory effects of rapamycin are mediated by stimulation of antitumor γδ T Cells. *Cancer Research, 76*(20), 5970–5982. https://doi:10.1158/0008-5472.CAN-16-0091

Ding, Z., Wang, X., Schnackenberg, L., Khaidakov, M., Liu, S., Singla, S., … Mehta, J. L. (2013). Regulation of autophagy and apoptosis in response to ox-LDL in vascular smooth muscle cells, and the modulatory effects of the microRNA hsa-let-7g. *International Journal of Cardiology*, *168*(2), 1378–1385. https://doi: 10.1016/j.ijcard.2012.12.045

Dinicolantonio, J. J., Lucan, S. C., & O'Keefe, J. H. (2016). The evidence for saturated fat and for sugar related to coronary heart disease. *Progress in Cardiovascular Diseases, 58*(5), 464–472. https://doi: 10.1016/j.pcad.2015.11.006

Edirisinghe, I., & Rahman, I. (2010). Cigarette smoke-mediated oxidative stress, shear stress, and endothelial dysfunction: role of VEGFR2. *Annals of the New York Academy of Sciences, 1203*(1), 66–72. https://doi:10.1111/j.1749-6632.2010.05601.x

Fatkhullina, A. R., Peshkova, I. O., & Koltsova, E. K. (2016). The role of cytokines in the development of atherosclerosis. *Biochemistry (Moscow), 81*(11), 1358–1370. https://doi: 10.1134/s0006297916110134

Frangogiannis, N.G. (2014) MicroRNAs and endothelial function: Many challenges and early hopes for clinical applications. *Journal of the American College of Cardiology, 63,* 1695-1696. https://doi:10.1016/j.jacc.2013.10.056

Guertin, D. A., & Sabatini, D. M. (2007). Defining the role of mTOR in cancer. *Cancer Cell, 12*(1), 9–22. https://doi:10.1016/j.ccr.2007.05.008

Hao, W., & Friedman, A. (2014). The LDL-HDL profile determines the risk of atherosclerosis: A mathematical model. *PLOS ONE, 9*(3), e90497. https://doi:10.1371/journal.pone.0090497

Harrington, R. A. (2017). Targeting inflammation in coronary artery disease. *New England Journal of Medicine, 377*(12): 1197-1198. https://doi:10.1056/NEJMe1709904

Huang, B., Svensson, P., Ärnlöv, J., Sundström, J., Lind, L., & Ingelsson, E. (2016). Effects of cigarette smoking on cardiovascular-related protein profiles in two community-based cohort studies. *Atherosclerosis, 254*, 52-58.

Ilhan, F., & Kalkanli, S. T. (2015). Atherosclerosis and the role of immune cells. World *Journal of Clinical Cases, 3(*4), 345–352. https://doi.org/10.12998/wjcc.v3.i4.345

Ino, Y., Toyoda, Y., Tanaka, A., Ishii, S., Kusuyama, Y., Kubo, T., Takarada, S., Kitabata, H., Tanimoto, T., Mizukoshi, M., Imanishi, T., Akasaka, T. (2009). Predictors and prognosis of stent fracture after sirolimus-eluting stent implantation. *Circulation Journal*., *73*(11), 2036-2041. https://doi:10.1253/circj.cj-09-0343

Insull, W. (2009). The pathology of atherosclerosis: Plaque development and plaque responses to medical treatment. *American Journal of Medicine, 122*(1 Suppl), S3–S14. https://doi:10.1016/j.amjmed.2008.10.013

Katsuda, S., & Kaji, T. (2003). Atherosclerosis and extracellular matrix. *Journal of Atherosclerosis and Thrombosis, 10*(5), 267–274. https://doi:10.5551/jat.10.267

Kattoor, A. J., Goel, A., & Mehta, J. L. (2019). LOX-1: Regulation, signaling and its role in atherosclerosis. *Antioxidants (Basel, Switzerland)*, *8*(7), 218. https://doi:10.3390/antiox8070218

Kirby, C., Clarke, J., & Gregoriadis, G. (1980) Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *The biochemical journal*, *186*(2), 591–598. https://doi:10.1042/bj1860591

Kniepeiss, D., Iberer, F., Schaffellner, S., Jakoby, E., Duller, D., Tscheliessnigg, K. (2004). Dyslipidemia during sirolimus therapy in patients after liver

transplantation. *Clinical Transplantation*, *18*(6), 642-646. https://doi:10.1111/j.1399-0012.2004.00253.x

Kume, N., Moriwaki, H., Kataoka, H., Minami, M., Murase, T., Sawamura, T., Masaki, T., Kita, T. (2000). Inducible expression of LOX-1, a novel receptor for oxidized LDL, in macrophages and vascular smooth muscle cells. *Annals of the New York Academy of Sciences, 902,* 323-327. https://doi:10.1111/j.1749-6632.2000.tb06332.x

Larson-Meyer, D.E., & Willis, K.S. (2010). Vitamin D and athletes. *Current Sports Medicine Reports, 9*(4), 220–226. https://doi:10.1249/JSR.0b013e3181e7dd45

Li, J., Kim, S. G., & Blenis, J. (2014). Rapamycin: One drug, many effects. *Cell Metabolism*, *19*(3), 373–379. https://doi: 10.1016/j.cmet.2014.01.001

Liao, Y.C., Wang, Y.S., Guo, Y.C., Lin, W.L., Chang, M.H, Juo, S.H. (2014). Let-7g improves multiple endothelial functions through targeting transforming growth factor- Beta and SIRT-1 signaling. *Journal of the American College of Cardiology*, *63*, 1685-1694. https://doi:10.1016/j.jacc.2013.09.069

Libby, P. (1995). Molecular bases of the acute coronary syndromes. *Circulation, 91*(11), 2844–2850. https://doi: 10.1161/01.CIR.91.11.2844

Libby, P., Buring, J.E., Badimon, L., Hansson, G.K., Deanfield, J., Bittencourt, M.S., Tokgözoğlu, L., Lewis, E.F. (2019) Atherosclerosis. *Nature Review Disease Primers* 5, 56. https://doi:10.1038/s41572-019-0106-z

Linton, M.F., Yancey, P.G., Davies, S.S., Jerome, W.G.J., Linton, E.F., & Vickers, K.C. (2018). The role of lipids and lipoproteins in atherosclerosis. In De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, Koch C,

Korbonits M, McLachlan R, New M, Purnell J, Rebar R, Singer F, Vinik A (Eds.), *Endotext*. South Dartmouth (MA): MDText.com, Inc.

Liu, X.Q., Mao, Y., Wang, B. et al. (2014) Specific matrix metalloproteinases play different roles in intraplaque angiogenesis and plaque instability in rabbits. *PLoS ONE, 9,* e107851. https://doi:10.1371/journal.pone.0107851

Lo, J., & Plutzky, J. (2012). The biology of atherosclerosis: general paradigms and distinct pathogenic mechanisms among HIV-infected patients. *Journal of Infectious Diseases, 205,* S368–S374. https://doi:10.1093/infdis/jis201

Lobatto, M. E., Calcagno, C., Metselaar, J. M., Storm, G., Stroes, E. S., Fayad, Z. A., & Mulder, W. J. (2012). Imaging the efficacy of anti-inflammatory liposomes in a rabbit model of atherosclerosis by non-invasive imaging. *Methods in Enzymology, 508,* 211–228. https://doi.org/10.1016/B978-0-12-391860-4.00011-2

Lusis, A. J. (2000). Atherosclerosis. *Nature, 407*(1), 233-242. https://doi: 10.1038/35025203

Luo, Y., Duan, H., Qian, Y., Feng, L., … & Yan, X. (2017). Macrophagic CD146 promotes foam cell formation and retention during atherosclerosis. *Cell Research, 27*(3), 352-372. https://doi:10.1038/cr.2017.8

Macfarlane, L.-A., & Murphy, P. R. (2010). MicroRNA: Biogenesis, function and role in cancer. *Current Genomics, 11*(7), 537–561. https://doi: 10.2174/138920210793175895

Makinen, P. I., Lappalainen, J. P., Heinonen, S. E., Leppanen, P., Lahteenvuo, M. T., Aarnio, J. V., Yla-Herttuala, S. (2010). Silencing of either SR-A or CD36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of

these receptors. *Cardiovascular Research, 88*(3), 530–538. doi: 10.1093/cvr/cvq235

Mango, R. (2003). Association of single nucleotide polymorphisms in the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction. *Journal of Medical Genetics, 40*(12), 933–936. doi: 10.1136/jmg.40.12.933

Mayo Clinic. (2018). Heart disease: Symptoms and causes. https://www.mayoclinic.org/diseases-conditions/heart-disease/symptoms-causes/syc-20353118

Mehta, J. L., Sanada, N., Hu, C. P., Chen, J., Dandapat, A., Sugawara, F., … Sawamura, T. (2007). Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circulation Research*, *100*(11), 1634–1642. doi: 10.1161/circresaha.107.149724

Mendell, J.T. and Olson, E.N. (2012). MicroRNAs in stress signaling and human disease. *Cell*, *148*, 1172-1187. https://doi:10.1016/j.cell.2012.02.005

Michiels, C. (2003). Endothelial cell functions. *Journal of Cellular Physiology*, *196*(3), 430–443. https://doi.org/10.1002/jcp.10333

Mohr, F. W., Morice, M. C., Kappetein, A. P., Feldman, T. E., Ståhle, E., Colombo, A., ... & Serruys, P. W. (2013). Coronary artery bypass graft surgery versus percutaneous coronary intervention in patients with three-vessel disease and left main coronary disease: 5-year follow-up of the randomised, clinical SYNTAX trial. *Lancet, 381*(9867), 629-38. https://doi: 10.1016/S0140-6736(13)60141-5

Moore, K. J., Sheedy, F. J., & Fisher, E. A. (2013). Macrophages in atherosclerosis: A dynamic balance. *Nature Reviews Immunology, 13*(10), 709–721. https://doi:10.1038/nri3520

Morice, M.C., Serruys, P.W., Sousa, J.E. et al. (2002). A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *New England Journal of Medicine, 346*(23), 1773-80. https://doi:10.1056/NEJMoa012843

Murphy, J. E., Vohra, R. S., Dunn, S., Holloway, Z. G., Monaco, A. P., Homer-Vanniasinkam, S., … Ponnambalam, S. (2008). Oxidised LDL internalisation by the LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2. *Journal of Cell Science*, *121*(13), 2136–2147. doi: 10.1242/jcs.020917

Nelson, R. H. (2013). Hyperlipidemia as a risk factor for cardiovascular disease. *Primary Care: Clinics in Office Practice, 40*(1), 195–211. doi: 10.1016/j.pop.2012.11.003

Oh, W. J., & Jacinto, E. (2011). mTOR complex 2 signaling and functions. *Cell Cycle*, *10*(14), 2305–2316. https://doi: 10.4161/cc.10.14.16586

Otsuka, F., Byrne, R. A., Yahagi, K., Mori, H., Ladich, E., Fowler, D. R., Kutys, R., Xhepa, E., Kastrati, A., Virmani, R., Joner, M. (2015). Neoatherosclerosis: Overview of histopathologic findings and implications for intravascular imaging assessment. *European Society of Cardiology*. 36, 2147–2159. doi:10.1093/eurheartj/ehv205

Ozpolat, B., Sood, A. K., & Lopez-Berestein, G. (2014). Liposomal siRNA nanocarriers for cancer therapy. *Advanced Drug Delivery Reviews, 66,* 110-116.

Raskob, G. E., Silverstein, R., Bratzler, D. W., Heit, J. A., & White, R. H. (2010, March 20). Surveillance for deep vein thrombosis and pulmonary embolism: Recommendations from a national workshop. https://doi.org/10.1016/j.amepre.2010.01.010

Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C.,... & Glynn, R. J. (2017). Anti-inflammatory therapy with canakinumab for atherosclerotic disease. *New England Journal of Medicine, 377*(12), 1119-1131. https://doi:10.1056/NEJMoa1707914

Robertson, J., Schiöler, L., Torén, K., Söderberg, M., Löve, J., Waern, M., … Åberg, M. (2017). Mental disorders and stress resilience in adolescence and long-term risk of early heart failure among Swedish men. *International Journal of Cardiology*, *243*, 326–331. https://doi: 10.1016/j.ijcard.2017.05.043

Rom, S., Dykstra, H., Zuluaga-Ramirez, V., Reichenbach, N. L., & Persidsky, Y. (2015). miR-98 and let-7g* Protect the blood-brain barrier under neuroinflammatory conditions. *Journal of Cerebral Blood Flow & Metabolism*, *35*(12), 1957–1965. https://doi: 10.1038/jcbfm.2015.154

Romaine, S. P. R., Tomaszewski, M., Condorelli, G., & Samani, N. J. (2015). MicroRNAs in cardiovascular disease: an introduction for clinicians. *Heart*, *101*(12), 921–928. https://doi: 10.1136/heartjnl-2013-305402

Qian, P., Zuo, Z., Wu, Z., Meng, X., Li, G., Wu, Z., Zhang ,W., Tan, S., Pandey, V., Yao, Y., Wang, P., Zhao, L., Wang, J., Wu, Q., Song, E., Lobie, P.E., Yin, Z., Zhu, T. (2011). Pivotal role of reduced let-7g expression in breast cancer invasion and

metastasis. *Cancer Research, 71*, 6463-6474. https://doi:10.1158/0008-5472.CAN-11-1322

Sehested, T. S. G. (2019). Cost-effectiveness of canakinumab for prevention of recurrent cardiovascular events. https://doi:10.1001/jamacardio.2018.4566

Stephen, S. L., Freestone, K., Dunn, S., Twigg, M. W., Homer-Vanniasinkam, S., Walker, J. H., Wheatcroft, S. B., & Ponnambalam, S. (2010). Scavenger receptors and their potential as therapeutic targets in the treatment of cardiovascular disease. *International Journal of Hypertension, 2010*, 646929. https://doi.org/10.4061/2010/646929

Sun, R., Fan, Y., Liang, X. et al. (2018). Rapamycin and FTY720 alleviate atherosclerosis by cross talk of macrophage polarization and autophagy. *BioMed Research International*, 2018, 2314-6133. https://doi:10.1155/2018/1010248

Thakkar, S., Wang, X., Khaidakov, M., Dai, Y., Gokulan, K., Mehta, J. L., & Varughese, K. I. (2015). Structure-based design targeted at LOX-1, a receptor for oxidized low-density lipoprotein. *Scientific Reports, 5*(1). https://doi: 10.1038/srep16740

Tian, J., Liu, Y., Liu, Y., Chen, K., & Lyu, S. (2017). Cellular and molecular mechanisms of diabetic atherosclerosis: Herbal medicines as a potential therapeutic approach. *Oxidative Medicine and Cellular Longevity, 2017*(9080869). https://doi:10.1155/2017/9080869

Toledo-Ibelles, P., & Mas-Oliva, J. (2018). Antioxidants in the fight against atherosclerosis: Is this a dead end? *Current Atherosclerosis Reports, 20*(7), 36. https://doi:10.1007/s11883-018-0737-7

Tong, M., & Jiang, Y. (2015). FK506-Binding proteins and their diverse functions. *Current Molecular Pharmacology*, *9*(1), 48–65. https://doi: 10.2174/1874467208666150519113541

Traba, J., Kwarteng-Siaw, M., Okoli, T. C., Li, J., Huffstutler, R. D., Bray, A., Waclawiw, M. A., Han, K., Pelletier, M., Sauve, A. A., Siegel, R. M., & Sack, M. N. (2015). Fasting and refeeding differentially regulate NLRP3 inflammasome activation in human subjects. *Journal of Clinical Investigation, 125*(12), 4592–4600. https://doi.org/10.1172/JCI83260

Wang, X., Ding, Z., Lin, J., Guo, Z., Mehta, J.L. (2015). LOX-1 in macrophage migration in response to ox-LDL and the involvement of calpains. *Biochemical and Biophysical Research Communications*, 467(1), 135-9. https://doi: 10.1016/j.bbrc.2015.09.100.

Wahid, F., Shehzad, A., Khan, T., & Kim, Y. Y. (2010). MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1803*(11), 1231–1243. https://doi:10.1016/j.bbamcr.2010.06.013

Yang, X., Li, Y., Li, Y., Ren, X., Zhang, X., Hu, D., ... & Shang, H. (2017). Oxidative stress-mediated atherosclerosis: Mechanisms and therapies. *Frontiers Physiology*, *8*(1). https://doi:10.3389/fphys.2017.00600

Zampetaki, A. and Mayr, M. (2012) MicroRNAs in vascular and metabolic disease. *Circulation Research, 110,* 508-522. https://doi:10.1161/CIRCRESAHA.111.247445

Zani, I. A., Stephen, S. L., Mughal, N. A., Russell, D., Homer-Vanniasinkam, S., Wheatcroft, S.B., Ponnambalam, S. (2015). Scavenger receptor structure and function in health and disease. *Cells, 4*(2), 178–201. https://doi:10.3390/cells4020178

Zhang, Y., Bokov, A., Gelfond, J., Soto, V., Ikeno, Y., Hubbard, G., … Fischer, K. (2013). Rapamycin extends life and health in C57BL/6 mice. *Journals of Gerontology: Series A*, *69A*(2), 119–130. https://doi: 10.1093/gerona/glt056

Zhao, Y., Li, X., Zhao, X., Yang, Y., Li, H., Zhou, X., & Yuan, W. (2017). Asymmetrical polymer vesicles for drug delivery and other applications. *Frontiers in Pharmacology*, *8*, 374. https://doi.org/10.3389/fphar.2017.00374