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Exploring the Efficacy of miR-33 Antagonism in Promoting Regression of Intracranial Atherosclerosis in an Nonhuman Primate Model

Peter Hecker
University of Kentucky, peter.hecker@uky.edu
Author ORCID Identifier:
https://orcid.org/0000-0002-3371-472
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Peter Hecker, Student

Ryan E. Temel, Major Professor

Howard Glauert, Director of Graduate Studies

EXPLORING THE EFFICACY OF miR-33 ANTAGONISM IN PROMOTING REGRESSION OF INTRACRANIAL ATHEROSCLEROSIS IN A NONHUMAN PRIMATE MODEL

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Peter I. Hecker
Lexington, Kentucky
Director: Dr. Ryan E. Temel, Professor of Physiology
Lexington, Kentucky
2023

https://orcid.org/0000-0002-3371-472

ABSTRACT OF DISSERTATION

EXPLORING THE EFFICACY OF miR-33 ANTAGONISM IN PROMOTING REGRESSION OF INTRACRANIAL ATHEROSCLEROSIS IN A NONHUMAN PRIMATE MODEL

Atherosclerosis, characterized by lipid accumulation and arterial inflammation, is a major contributor to global morbidity and mortality. Despite significant progress in understanding atherosclerosis in extracranial arteries, the study of intracranial atherosclerosis (ICAS) has been relatively neglected, despite its crucial role in stroke and vascular cognitive impairment. Challenges related to ICAS, including its location within the cranium and limited availability of suitable animal models, have hindered research progress in this area. Although nonhuman primates (NHPs) are commonly used for studying extracranial atherosclerosis, a comprehensive understanding of ICAS pathophysiology in these animals is lacking. By subjecting NHPs to a high-fat/cholesterol diet, we successfully induced measurable ICAS, providing a unique opportunity to investigate underlying mechanisms and potential therapeutic strategies for ICAS regression. This study presents a robust NHP model of ICAS development and explores the potential of miR-33 antagonism for promoting atherosclerosis regression. Mouse studies have shown that inhibiting miR-33a can stabilize or regress atherosclerosis in extracranial arteries, but their translatability is limited. To address this, we employed an NHP model that closely mimics human miR-33a and miR-33b expression and atherosclerosis development. Our investigation aims to assess the effectiveness of miR-33 antagonism in promoting ICAS regression in 61 NHPs, using histological characterization and digital pathology techniques to evaluate ICAS morphology and composition. Surprisingly, our results showed no histological evidence supporting the efficacy of miR-33 antagonism in improving ICAS regression measures. This study significantly contributes to our understanding of ICAS and its potential treatment strategies by establishing a reliable animal model for ICAS development. However, further investigation is required to determine the role of miR-33 antagonism in atherosclerosis regression. These findings have important implications for future research and the development of therapeutic strategies to improve brain health and function while reducing the burden of ICAS on stroke and vascular cognitive impairment.

KEYWORDS: Intracranial Atherosclerosis, Vascular Dementia, microRNA, miR-33, Nonhuman Primates, Stroke

Peter I. Hecker

(Name of Student)

06/19/2023

Date

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By
Peter I. Hecker

Ryan E. Temel
Director of Dissertation

Howard Glauert
Director of Graduate Studies

06/19/23
Date

DEDICATION

This work is dedicated with love and gratitude to my dad and Boone - the standing guideposts in my life's journey. Though physically absent, their profound influence on my character and achievements remains undiminished.

From my dad, I gleaned the invaluable lessons of responding to adversity with patience and understanding, fostering kindness, and choosing empathy over judgment. These lessons have been the cornerstone of my growth as a soldier, a leader, an academic, and a man.

Boone, you left to soon man, you taught me that true friendships can be forged in the most unexpected places. During one of the lowest and hardest challenges of my life, you were the unwavering pillar that kept me going. From you, I learned the power of consistency in a relationship and the essential role of trust, which requires effort from both parties.

This accomplishment belongs to all of us.

The memories we built together will forever inspire me as I venture into the unknown chapters ahead.

This dissertation signifies not an end, but the commencement of the journey you both set me upon.

I love you guys for you have shaped me in the most profound of ways and I am eternally blessed to have had you by my side.

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To be a great leader, one must first learn to be led. Rarely does an individual develop into a leader without first observing and experiencing effective leadership. A leader's role involves making difficult decisions while providing the necessary resources for their team to succeed, all while preparing the next generation to carry the torch. Leadership, in this cyclical way, requires compassion, a deep understanding of the essence of leadership, and the strength of humility to foster collaboration among team members. It is great leaders that inspire and motivate future leaders.

I am deeply grateful to my exceptional academic mentors who have guided me with grace and unwavering support throughout this journey. Among them, I owe my deepest gratitude to Dr. Ryan Temel. Your unwavering support for my unorthodox ideas and provision of resources and space for their growth into expert opinions have been invaluable. Your boundless curiosity and compassion have inspired me to embrace humility in my pursuit of knowledge and to understand the significance of empathy in guiding future students. Even during challenging times when I fell short of expectations, you persisted, lifted me up, challenged me, and guided me to completion. Beyond being a mentor, you have become a friend with whom I can always share a well-deserved beer with.

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and heartfelt appreciation that I acknowledge every one of you for your contributions to my success.

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1.1 Atherosclerotic Vascular Disease

Atherosclerosis is the primary vasculopathy in cardiovascular and cerebrovascular disease.[1] Globally, atherosclerosis is the number one contributor to ischemic and/or occlusive cardiovascular and cerebrovascular events, accounting for approximately 85% of all heart attacks and strokes[1] and 17.8 million deaths annually.[2] In the United States, atherosclerosis remains one of the leading causes of disability, with approximately 121 million adults (nearly 37% of the population) predicted to be at risk for developing atherosclerosis,[3] 18 million (8%) to have been diagnosed with atherosclerotic vascular disease (AVD), and over 6 million of those with AVD are at risk for a clinical event.[4] Moreover, AVD, is responsible for one out of every four deaths in the United States, with coronary artery disease (CAD) and stroke being the most common manifestations.[2]

The development and progression of atherosclerosis is strongly influenced by modifiable risk factors, including unhealthy lifestyle habits, such as poor diet and lack of physical activity that contribute to comorbidities like obesity, hyperglycemia, insulin resistance, hypertension, dyslipidemia, and hypercholesterolemia.[3] Additionally, related to these risk factors, the age-adjusted prevalence of AVD in the United States has been steadily increasing, underscoring the need for improved risk prevention and management strategies to curb the escalating burden of cardiovascular and cerebrovascular events.[3]

Efforts to mitigate the public health crisis of atherosclerosis on global and national levels have primarily focused on targeting the major modifiable risk factors and/or comorbidities through lifestyle interventions, public health campaigns, and pharmacological therapies aimed at optimizing metabolic functions associated with the

pathogenesis of AVD.[3] Addressing the atherosclerotic process and its contribution to cardiovascular and cerebrovascular events is crucial for reducing the morbidity and mortality associated with these conditions and improving the overall health of populations worldwide.

This literature review aims to provide a comprehensive overview of atherosclerosis, a complex and multifactorial disease that affects both the cardiovascular and cerebrovascular systems. Specifically, the review will discuss the contributing factors, pathogenesis, treatment strategies, and spatial consequences of atherosclerosis in both extracranial and intracranial arteries. While the review will cover all aspects related to atherosclerosis, special attention will be given to the impact of intracranial atherosclerosis on stroke and vascular dementia, emphasizing the need for more research in this area. Additionally, the review will address the lack of experimental animal models of intracranial atherosclerosis and the potential for nonhuman primates as a unique translational model. By examining these topics, the review aims to deepen our understanding of atherosclerosis as a whole and the importance for the continual development of effective prevention and management strategies that can address residual risks associated with AVD and reduce the morbidity and mortality associated with this disease.

1.2 Atherosclerotic Vascular Disease: The Risk Factors

Atherosclerotic vascular disease (AVD) is a progressive condition characterized by inflammation and lipid accumulation in the arterial wall, leading to the development of atherosclerotic plaques. If left unaddressed, these plaques worsen vascular dysfunction, disrupt blood flow, and can ultimately result in severe cardiovascular and cerebrovascular

events, such as coronary artery disease (CAD) and ischemic stroke. Several modifiable metabolic risk factors are associated with the development and progression of AVD, including obesity, hyperglycemia, hypertension, and dyslipidemia. By managing these metabolic risk factors through a healthy diet, regular exercise, and/or therapeutic drug interventions, it is possible to significantly reduce the risks of AVD and its associated complications, such as CAD and stroke.

1.2.1 Diet and Physical Activity

Poor dietary intake was associated with 6.58 million cardiovascular related deaths and 11.3 million global deaths in 2021.[5] Eating a healthy diet is a key factor in maintaining optimal metabolic function. It's not just about calories; it's also about the quality of nutrients going into the body. In Western societies, animal proteins, fruits, vegetables, fats, oils and processed carbohydrates are readily available and integral to the cultural diet. However, food commercialization in these Westernized societies has promoted a "Western" diet characterized by consumption of nutrient-poor, calorie-rich foods such as processed and refined sugars, unhealthy fats, and low intake of reduced caloric nutrient-dense foods like fruits, vegetables, and whole grains. When consumed in excess, these nutrient-poor, calorie-rich foods can lead to metabolic dysfunctions, including weight gain, hyperglycemia, insulin resistance, hypertension, and/or dyslipidemia, considerably increasing the risk for AVD. Unmanaged metabolic dysfunctions contribute to the development and progression of vasculopathies, such as atherosclerotic plaques, by promoting inflammation, oxidative stress, and endothelial dysfunction within the arterial wall.

The Framingham Heart Study has long since set up the connection between diets high in saturated and trans fats and the development, morbidity, and mortality of cardiovascular disease.[6] Subsequent studies comparing Mediterranean diets, which are low in saturated fats and high in monounsaturated fats, to Western diets have corroborated these findings. In countries where Western diets are prevalent, morbidity and mortality rates have been positively associated with the average percentage of dietary energy intake from saturated fats. Conversely, in countries where the Mediterranean diet is more common, morbidity and mortality have been inversely associated with a protective ratio of increased dietary monounsaturated fats to decreased saturated fats.[7]

However, recent research suggests that the protective effects of the Mediterranean diet may not be solely attributed to the consumption of monounsaturated fats but also the increased intake of whole grain carbohydrates, fruits, vegetables, and fiber. Additional studies on the Western diet have linked elevated risk for metabolic dysfunction and AVD related events to the consumption of red and processed meats, fried foods, and refined sugars in place of whole foods with healthy unprocessed proteins, fresh fruits, and fresh vegetables.[8] Despite this evidence, the modern Western diet continues to include these calorically dense, nutrient-poor proatherogenic foods in place of AVD risk lowering, nutrient dense whole foods.

The average caloric intake and macronutrient distribution of today's Western diet can vary depending on the country and specific population studied. For Instance, in the United States the average daily caloric intake is estimated to be around 2,500-2,700 kcal for men and 1,800-2,000 kcal for women. The macronutrient distribution of the Western diet typically consists of a high proportion of carbohydrates (45-65%), with a significant

portion coming from refined grains and added sugars; a moderate to high amount of fat (30-40%), with a high intake of saturated and dietary cholesterol coming from meat and dairy; and relatively low protein intake (10-35%), often sourced from animal products.[9] The combination of an unhealthy diet and a sedentary lifestyle exacerbates the risk of developing metabolic dysfunction, including insulin resistance, glucose intolerance, and dyslipidemia, which are all significant contributors to the development and progression of AVD. Studies have consistently shown that adopting a healthier diet and increasing physical activity can improve metabolic parameters and reduce AVD risk.[10]

Physical inactivity has been identified as an independent risk factor for AVD. Reduced caloric/energy expenditure from lack of daily physical movement and sedentary behavior leads to increased adiposity, insulin resistance, and systemic inflammation. The Nurses' Health Study and the Health Professionals Follow-up Studies have demonstrated that individuals with low levels of physical activity are at a higher risk of developing metabolic dysfunction and CAD/AVD compared to those who are more regularly active.[10–12]

Conversely, regular daily physical activity has been shown to reduce AVD risk through various mechanisms such as improved lipid profiles, enhanced endothelial function, and reduced blood pressure.[14–17] Additionally, physical activity has been associated with the attenuation of inflammation, as evidenced by lower C-reactive protein levels in physically active individuals.[13] Engagement in regular aerobic exercise can foster a lipoprotein and lipid profile that reduces AVD risk through increased plasma high density lipoprotein cholesterol (HDL-C) levels and HDL function and reduced plasma low density lipoprotein cholesterol (LDL-C), triglycerides, and total cholesterol levels.[14–16]

Considering the complex interplay between diet, physical activity, and metabolic function, it is essential to understand the individual metabolic factors associated with AVD. These factors include obesity, hyperglycemia, insulin resistance, hypertension, and dyslipidemia.

1.2.2 Obesity

Obesity is a well-established risk factor for AVD, as it promotes a pro-inflammatory state, insulin resistance, and dyslipidemia.[17]. The prevalence of obesity has dramatically increased in Western societies, particularly in the United States. According to the World Health Organization, in 2016, more than 1.9 billion adults worldwide were overweight, and of these, over 650 million were obese.[19] In the US, the age-adjusted prevalence of obesity among adults has risen from 30.5% in 1999-2000 to 42.4% in 2017-2018.[18] This increase in obesity rate reflects the growing trend of metabolic dysfunction in these populations and highlights the urgent need to address this public health crisis.

Obesity often serves as an outward reflection of metabolic dysfunctions that promote and exacerbate vasculopathy, such as AVD, through several inflammatory mechanisms.[19] Weight gain and abdominal fat expansion are early signs and key contributors to the development of hyperglycemia, hypertension, and dyslipidemia/hypercholesterolemia.[20] These metabolic abnormalities, in turn, further promote AVD progression and elevate the risk of cardiovascular events. As such, addressing obesity is crucial in mitigating the impacts of associated metabolic complications on vascular health.

Obesity is associated with chronic low-grade inflammation that contributes to further metabolic dysfunction. Obesity has been shown to trigger a shift in the immune response within adipose tissue and other organs, such as the liver, from a predominantly anti-inflammatory to a pro-inflammatory state.[21] In adipose tissue, this shift is characterized by an increase in the number and ratio of pro-inflammatory macrophages to anti-inflammatory macrophages, which leads to the production of cytokines that contribute to further metabolic dysfunction.[22] Similarly, in the liver, obesity may result in activation of inflammatory pathways, macrophage infiltration, and the production of inflammatory chemokines and cytokines, which can contribute to the development of nonalcoholic fatty liver disease (NAFLD).[19]

Obesity has also been linked to insulin resistance through multiple mechanisms, including the pro-inflammatory state induced by adipose tissue inflammation and the secretion of adipokines that interfere with insulin signaling.[19,22] This intimate connection between obesity and insulin resistance contributes to atherosclerotic plaque development by promoting a chronic inflammatory state and exacerbating hyperglycemic conditions, which further impair metabolic function and increase AVD risk. Additionally, obesity may contribute to the development of hypertension by increasing the activation of the renin-angiotensin-aldosterone system, promoting sodium retention, and impairing blood vessel function through endothelial dysfunction and arterial stiffness.[23]

Obesity is also associated with dyslipidemia characterized by increased Very Low Density Lipoprotein Cholesterol (VLDL-C) and reduced plasma HDL-C levels. In individuals with obesity, there is an increased influx of free fatty acids (FFA) to the liver due to higher adipose tissue lipolysis. The liver then processes these FFA into triglycerides,

which are later incorporated into VLDL particles and secreted into the bloodstream. This results in higher circulating levels of VLDL-C.[24] Additionally, obesity can impair the function of enzymes involved in HDL-C metabolism, such as lecithin-cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP), leading to reduced HDL-C levels and impaired reverse cholesterol transport.[25] These combined effects of obesity on inflammation, insulin resistance, hyperglycemia, hypertension, and dyslipidemia significantly amplify the risk and progression of AVD.

1.2.3 Hyperglycemia and Insulin Resistance

While obesity provides an overt sign of metabolic dysfunction, hyperglycemia often stays hidden, silently contributing to vasculopathy, such as AVD, until the clinical presentation of insulin resistance and diabetes mellitus becomes apparent. Hyperglycemia, characterized by fasting plasma glucose levels above 100 mg/dL, is a significant risk factor for diabetes mellitus (DM) and for the development of AVD.[27] In 2019, the global prevalence of diabetes was estimated at 9.3%, affecting 463 million people, with diabetes mellitus type 2 (DM2) accounting for 90 to 95 percent of all diabetes diagnoses. Notably, one in two people living with diabetes remain undiagnosed.[26] In the presence of diabetes, the risk of cardiovascular disease and mortality rates increase significantly. Studies have found that adults with diabetes face a 75% higher risk of mortality, with cardiovascular disease accounting for a sizable part of the excess mortality. Moreover, adults with diabetes face a 2-4 times higher risk of developing cardiovascular and cerebrovascular AVD-related complications, which escalates with deteriorating glycemic control.[27]

Persistent hyperglycemia worsens endothelial cell (EC) dysfunction, promoting the development and progression of atherosclerotic vascular disease. For instance,

hyperglycemic conditions enhance oxidative stress via reactive oxygen species (ROS) formation that activates protein kinases, leading to the upregulation of proinflammatory cytokines that increase vascular permeability at the endothelial layer.[28,29] Furthermore, advanced glycation end products (AGEs), which form due to hyperglycemia, also increase ROS activity, disrupting the structure and function of the extracellular matrix and ultimately impairing endothelial protective functions.[30] It is also worth noting that hyperglycemia hinders endothelial nitric oxide synthase (eNOS) activity, leading to decreased nitric oxide (NO) production and a subsequent loss of vascular tone,[32] which may contribute to proatherogenic non-laminar blood flow and reduced shear stress.[31] In addition to proinflammatory mechanisms related to ECs, persistent hyperglycemia can elevate triglycerides and increase VLDL particles in circulation while reducing plasma HDL-C levels, promoting a proatherogenic lipid profile that increases AVD risk.[32]

Common causes of hyperglycemia include an unhealthy diet high in sugar and a sedentary lifestyle with reduced physical activity, which can lead to insulin resistance, a key factor in the development of diabetes.[33] Insulin resistance occurs when the body's cells do not respond adequately to insulin, impairing glucose uptake and leading to elevated blood glucose levels. This dysfunctional glucose metabolism is intricately linked to the development of diabetes mellitus, which has two primary types: type 1 (DM1) and type 2 (DM2). DM1 is an autoimmune disorder where the immune system destroys insulin-producing pancreatic beta cells, while DM2 is primarily considered a metabolic condition that is more prevalent than DM1 and develops from a combination of insulin resistance and impaired insulin secretion.[34]

Insulin resistance in DM2 promotes dyslipidemia characterized by elevated triglycerides and lipid exchange processes between apoB and apoA-containing lipoproteins, resulting in the formation of smaller, more proatherogenic LDL particles and HDL particles with reduced functionality. Smaller LDL particles are said to be more susceptible to oxidation and intimal retention, while a decrease in large, buoyant HDL particles impairs their protective properties against atherosclerosis development and progression.[32] Additionally, in DM2, platelet activation and aggregation are heightened due to increased oxidative stress, elevated levels of inflammatory markers, and endothelial dysfunction, which potentially contribute to thrombi formation within complex and vulnerable atherosclerotic plaques, thus increasing the risk for ischemic or occlusive events.[35] Furthermore, hyperglycemia and insulin resistance in DM2 have been associated with kidney disease and poorly controlled hypertension,[36,37], which both contribute to a further increased risk for AVD-related events across the cardiovascular and cerebrovascular systems.

Hyperglycemia, insulin resistance, and DM2 are critical factors contributing to the development of AVD. The molecular mechanisms underlying these conditions, such as oxidative stress, inflammation, endothelial dysfunction, dyslipidemia, and platelet activation, play significant roles in the development and progression of complex and vulnerable atherosclerotic plaques, increasing the risk for ischemic or occlusive events that contribute to global morbidity and mortality rates.

1.2.4 Hypertension

Hypertension (HTN), a silent and pervasive disease, remains a significant global health concern. Defined as a persistent elevation in systolic blood pressure (SBP > 130

mmHg) and/or diastolic blood pressure (DBP > 80 mmHg) over time, HTN is typically diagnosed after a series of blood pressure (BP) measures taken during rest and activity.[38] Modifiable lifestyle factors, particularly poor diet and physical inactivity are key contributors to the onset and progression of hypertension.[38]

The global prevalence of HTN is staggering, with approximately 1.13 billion people affected worldwide in 2015,[39] while in 2017 an estimated 63% of adults 45-75 years living in the United States are reported to have HTN.[40] Moreover, in the United States alone, hypertension is present in approximately 49% of adults with cardiovascular disease and 62% of adults with cerebrovascular disease.[41] The risk of atherosclerotic cardiovascular and cerebrovascular events in individuals with HTN is significant, and this risk is further exacerbated when both HTN and hypercholesterolemia are present.[42] Indeed, individuals with both HTN and hypercholesterolemia have a 1.5 to 2 times higher likelihood of developing atherosclerotic related coronary artery disease (CAD) and stroke compared to those with either condition alone.[43] Like obesity, hyperglycemia, and insulin resistance, HTN exacerbates the development and progression of atherosclerotic lesion by inducing endothelial dysfunction.

Changes in normal blood flow and fluid dynamics across endothelial cells can activate various bio-mechanosensitive signaling pathways and promote endothelial dysfunction that increases the susceptibility to a variety of vasculopathies such as atherosclerosis.[43] Blood flows normally in a smooth and uniform manner, when it travels a straight arterial path and can be characterized by concentric, laminar layers. However, when the arterial path deviates from a straight course, this orderly laminar blood flow can be disrupted, giving rise to turbulent or disrupted flow dynamics.[44] Deviations from

laminar flow dynamics frequently occur at arterial branching points, such as those in the coronary arteries, or in regions where arteries become convoluted, like those among the circle of Willis. Moreover, arterial areas of disrupted blood flow dynamics are most often associated with areas of initial atherosclerotic lesion development and areas where complex and vulnerable lesions are most pervasive.[44]

Persistent HTN-induced disruptions in laminar flow initiating several proinflammatory mechanisms, further exacerbating EC dysfunction and creating a more proatherogenic milieu within the vascular wall, characterized by increased leukocyte adhesion, intimal lipid accumulation, and further arterial wall inflammation.[43] An essential factor in mitigating this process is shear stress, the frictional force exerted by blood flow on the endothelial cell surface. Under normal laminar flow ECs experience unidirectional shear stress which induces endothelial nitric oxide synthase (eNOS) expression promoting an anti-inflammatory phenotype;[45] however, reduced shear stress due to altered fluid dynamics, such as those influenced by HTN, promotes a more proinflammatory phenotype resulting in vasculopathy that promotes the development and progression of atherosclerosis.[46,47]

Reduced laminar shear stress has been shown to mediate endothelial dysfunction through the regulation of key genes involved in inflammation and thrombosis that contribute to the progression of complex and vulnerable atherosclerotic plaques.[46] For example, under normal laminar sheer stress the induction of transcription factor Kruppel-like factor 2 (KLF2) leads to increased expression of several genes involved in the anti-inflammatory, anti-thrombotic, and vasodilatory pathways, and decreased expression of genes involved in vasoconstriction, thrombosis, and inflammation. KLF2 activation also

leads to increased endothelial nitric oxide synthase (eNOS) expression and activity, resulting in increased nitric oxide (NO) production and subsequent vasodilation. Furthermore, KLF2 has been shown to inhibit platelet activation and adhesion, thus reducing thrombotic activity.[47]

In contrast, disturbed flow patterns, such as those observed in bifurcations, vascular remodeling (AVD/stenosis), and HTN may result in decreased KLF2 expression and activation, leading to a pro-atherogenic endothelial phenotype characterized by increased expression of pro-inflammatory, pro-thrombotic, and pro-oxidant genes, as well as decreased eNOS expression and activity, leading to reduced NO production and impaired vasodilation.[47] Diminished NO production can then lead to increased thrombotic activity and vasoconstriction, which are important contributors to complex and vulnerable atherosclerotic plaques, and subsequent ischemic and/or occlusive events.[48]

In addition to its effects on eNOS expression, reduced KLF2 expression has been associated with increased levels of reactive oxygen species (ROS) in endothelial cells, which can promote oxidative stress and contribute to endothelial dysfunction in atherosclerosis. Moreover, decreased KLF2 expression has been shown to promote the expression of pro-inflammatory molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1),[49] which can promote the recruitment of monocytes and leukocytes to the arterial wall where they may enhance the uptake of oxidized LDL by endothelial cells, leading to the formation of foam cells and the development of atherosclerotic plaques.[50]

The metabolic disorders of obesity and diabetes, as well as hypertension, all contribute to the initiation and exacerbation of endothelial dysfunction and inflammation

through molecular proinflammatory and bio-mechanosensitive signaling pathways, ultimately setting the stage for atherosclerosis. However, it is the retention of cholesterol and low-density lipoproteins (LDL) because of genetic and/or metabolic hypercholesterolemia and dyslipidemia that drive the development and progression of AVD.

1.2.5 Dyslipidemia and Hypercholesterolemia

Although hypertension, hyperglycemia, insulin resistance, and obesity are significant risk factors for developing atherosclerotic vascular disease (AVD), dyslipidemia and hypercholesterolemia have been well established as central players in this intricate disease process[52,53]. The prevalence of hypercholesterolemia has evolved into a pressing global public health issue, with an estimated 39% of adults worldwide living with elevated plasma cholesterol levels, resulting in approximately 2.6 million deaths annually.[51] Globally, hypercholesterolemia, particularly elevated low-density lipoprotein cholesterol (LDL-C), has been identified as the primary risk factor for developing AVD and related ischemic and/or occlusive coronary and cerebral events.[52]

In the United States, the prevalence of hypercholesterolemia has followed an upward trend over recent decades. Over 100 million American adults exhibit elevated plasma total cholesterol (TC) levels, with more than 36 million adults presenting high plasma LDL-C levels exceeding 130 mg/dL.[3] The escalating prevalence of hypercholesterolemia in the United States and across the globe highlights the pressing need for public concern.

Dyslipidemia and hypercholesterolemia are two interconnected terms describing an individual's lipid and lipoprotein profile. Dyslipidemia refers to an abnormal lipid profile, characterized by elevated levels of lipids such as cholesterol, free fatty acids (FFAs), and triglycerides (TAG), and/or abnormal lipoprotein distribution or function. Hypercholesterolemia, conversely, specifically refers to elevated levels of total cholesterol (TC) and LDL-C in the bloodstream and plasma.

This section aims to enhance the foundational understanding of how dysfunctional lipid and lipoprotein metabolism sets the stage for the development and progression of atherosclerotic lesions. We will first discuss cholesterol, delving into its metabolism, transport, and role in the pathogenesis of atherosclerotic lesions. Comprehending the fate of cholesterol will enable us to understand the various other lipids and lipoproteins involved, which are also intimately associated with the pathogenesis of AVD.

1.2.5.1 Lipids and Lipoproteins

The discovery of cholesterol in the body dates to the 18th century when François Poulletier de la Salle first discovered the waxy substance in gallstones.[53] As researchers ventured into the 20th century, they began investigating cholesterol's role in atherosclerosis, with Anitschkow's groundbreaking 1913 experiment demonstrating the development of atherosclerosis in cholesterol-fed rabbits.[54] Later, in the mid-20th century, Gofman's pioneering work using ultracentrifugation techniques led to the identification of lipoprotein classes and established their association with cardiovascular disease (CVD).[55]

Cholesterol esters and other non-polar lipids are packaged and transported in lipoproteins, which are complex particles featuring a hydrophobic lipid core surrounded by a hydrophilic membrane composed of phospholipids, free cholesterol, and apolipoproteins (Apo). These

apolipoproteins serve as structural proteins, receptor ligands, immune system modulators, lipase activators/inhibitors, and lipid transporters in the bloodstream. Various lipoprotein classes can be distinguished by their size, density, associated apolipoproteins, and the lipids they carry. For example, chylomicrons are linked with apoB-48 and transport dietary triglycerides and cholesterol from the intestine to peripheral tissues and the liver. In contrast, very-low-density lipoproteins (VLDL) are associated with apoB-100 and primarily shuttle TAG from the liver to different tissues throughout the body. Low-density lipoprotein particles also associate with apoB-100 and deliver cholesterol to cells, while HDL particles are linked with apoAI and II and remove cholesterol from cells, transporting it back to the liver for elimination.[56]

Dietary fatty acids and cholesterol are converted to TAG and cholesterol esters (CE) in the liver where they are packaged with apoB-100 (and apoC-1, apoC-II, apoCIII, and apoE) into VLDL particles (VLDL-P) before being transported to various tissues. In tissue lipoprotein lipase (LPL) lipolyzes TAG within the VLDL-P releasing free fatty acids (FFAs), and as TAG is depleted the VLDL-P reduces in size to form a smaller remnant IDL particle (IDL-P). The end-product of VLDL metabolism is a smaller CE rich TAG poor apoB-100 containing LDL particle (LDL-P) that is then circulated in the blood to target tissues where it binds plasma membrane receptors and is endocytosed by LDL receptor-mediated endocytosis.[57]

Conversely, the nascent HDL particle (HDL-P) is synthesized in the liver and intestine, with its main structural protein ApoA-I as a key component in its biogenesis. ApoA-I receives cholesterol and phospholipids from enterocytes and hepatocytes through the transporter ATP-binding cassette transporter 1 (ABCA1) forming a discoidal HDL particle.

The HDL-P circulates in the blood and lymph receiving free cholesterol (FC) and phospholipids from tissues and other lipoproteins. Neutral lipids are added to the core of the HDL-P through the action of lecithin cholesterol acyltransferase (LCAT), which converts free cholesterol to cholesteryl ester, and cholesteryl ester transfer protein (CETP), which exchanges HDL cholesteryl ester for VLDL triglyceride. The HDL-P then facilitates reverse cholesterol transport (RCT) by delivering its cholesterol to the liver where uptake is mediated by scavenger receptor class B type I (SR-BI). After cholesterol clearance, the now smaller HDL-P is released by SR-BI into circulation.[58]

Although correlated, it is crucial to differentiate between LDL-P, which refers to the LDL particle itself, and LDL-C, which specifies the cholesterol it contains. LDL particle profiles, which reflect size and carrying capacity, can vary significantly among individuals, with smaller and denser LDL particles considered more proatherogenic.[59] Similarly, HDL-C measures the cholesterol in the plasma carried by HDL-P, and HDL-P profiles can also differ significantly, with diverse subspecies possessing varying functional capacities and potential to mitigate atherosclerotic risk.[60] This implies that evaluating plasma LDL-C and HDL-C levels alone, without considering the lipoprotein particles themselves, may underestimate the proatherogenic or pro-resolving functional capacity of LDL-C and HDL-C in the development or regression of AVD, respectively.

While the importance of examining the lipoprotein profile in its entirety is well-supported, limitations remain in defining diagnostic criteria and approaches for routine measurements of lipoprotein particle function to improve risk prediction in conjunction with standard cholesterol measures. Nonetheless, measures of LDL-C and HDL-C levels continue to be used as proxies for assessing the risk of atherosclerotic vascular disease (AVD). Elevated

LDL-C levels above the recommended optimal guidelines (>129 mg/dL) and reduced HDL-C levels below the optimal guidelines (<40 mg/dL) have been consistently associated with an increased risk for AVD.[3,61] Therefore, while improvements in diagnostic criteria and approaches are needed to enhance the accuracy of risk prediction, the current standard measures of LDL-C and HDL-C levels should not be ignored. Indeed, early detection and treatment to reduce plasma LDL-C and raise HDL-C remain the first line defense to reducing risk for AVD related ischemic and/or occlusive events.

1.2.5.2 LDL

Studies on inherited autosomal-dominant (ADH) hypercholesterolemia have provided valuable insights into the significance of LDL receptor (LDLR) activity on regulating LDL-C in the body and the pathogenicity of apoB containing lipoproteins. The discovery of the LDLR and its role in cholesterol metabolism was a significant scientific breakthrough that began in the early 1970s when researchers began investigating the genetic causes of familial hypercholesterolemia (FH), a condition characterized by abnormally high plasma LDL-C levels. In the seminal study published in 1974 Goldstein and Brown used cultured human fibroblasts to identify the LDLR as the key receptor responsible for controlling the uptake of LDL into cells. Comparing cells from a normal subject to those from a patient with homozygous FH, they demonstrated that cells from the patient with FH lacked a LDLR receptor and were unable to accept LDL cholesterol. In a follow-study in 1976,[62] Goldstein and Brown exposed human fibroblasts to radiolabeled LDL to demonstrate that receptor-mediated cellular uptake of LDL-C was both saturable and specific.

The expression of LDLR gene is regulated at multiple levels, including transcriptional and post-transcriptional mechanisms. The level of intracellular cholesterol is one of the main factors that regulate LDLR gene expression. When cellular cholesterol levels are low, the transcription factor sterol regulatory element-binding protein 2 (SREBP-2) is activated, which then binds to the LDLR promoter region and activates its transcription, leading to increased LDLR protein expression on the cell surface. This allows for increased uptake of LDL-C from the blood replenishing cellular cholesterol stores. Conversely, when cellular cholesterol levels are high, SREBP-2 activation is suppressed, leading to a decrease in the transcription of the LDLR gene and a reduction in LDLR protein expression on the cell surface, which in turn reduces the uptake of LDL-C from the blood.[63]

The regulation of LDLR expression by SREBP-2 is a critical mechanism for controlling cholesterol metabolism in the body and maintaining cholesterol homeostasis. Goldstein and Brown's studies on the LDLR receptor were essential in establishing the link between LDL receptor activity and defects among individuals with FH, laying the foundation for subsequent genetic studies that identified thousands of mutations in the LDL receptor gene as the primary cause of the disorder and the most common cause of ADH.[64] However, it was population-based studies on individuals with ADH, specifically FH, which provided significant insights into the causal role of elevated LDL-C as the primary risk factor in the development of AVD and subsequently ischemic/occlusive events. These studies also demonstrated that reducing LDL-C was associated with reduced AVD mortality.

FH is one of the most common genetic causes of premature coronary artery disease (CAD) due to a persistent lifelong elevation in plasma LDL-C levels. In the United States Heterozygous FH is thought to be present in 1/250 individuals, whereas homozygous is

extremely rare affecting 1/160000 to 1/250000 individuals.[65] However, the true burden of FH is largely unknown, as it is often underdiagnosed, confounded by the vast number of individuals at risk for CAD due to more common modifiable risk factors. Nevertheless, heterozygous FH individuals exhibit plasma total cholesterol (TC) levels of 310-580 mg/dL and plasma LDL-C levels of 155-325mg/dL, which often leads to adults developing CAD before the age of sixty. In contrast, homozygous FH individuals have total cholesterol levels of 460-1160 mg/dL and plasma LDL-C levels of 400-1000mg/dL, resulting in early CAD development and, if left untreated, death before the age of 20.[66] Early diagnosis and treatment with cholesterol-lowering medications can attenuate atherosclerosis development and prevent CAD in those with FH.

A rare gain-of-function (GOF) mutation in the protein proprotein convertase subtilisin/kexin type 9 (PCSK9) has also been characterized as ADH mutation, which further highlights the crucial role of LDLR activity in LDL-C metabolism and the significant association of LDL-C with the development and mortality of atherosclerotic vascular disease (AVD). GOF PCSK9 mutations, which occur in approximately 12.5% of individuals with ADH, increase the degradation of LDL receptors, reducing the availability of LDLR for LDL clearance in the liver. This leads to a systemic increase in LDL-C and consequently heightens the risk for AVD.[67] Conversely, loss-of-function (LOF) mutations in PCSK9 have a contrasting effect on plasma LDL-C levels. These mutations cause inefficient degradation of LDL receptors, resulting in a higher number of receptors clearing LDL in the liver and a systemic reduction of LDL-C. Notably, even moderate lifelong reductions in LDL cholesterol levels due to PCSK9 LOF mutations can lead to a substantial decrease in the incidence of coronary events, regardless of non-lipid-related cardiovascular

risk factors. In fact, the Atherosclerosis Risk in Communities (ARIC) study found an 88% reduction in the risk of developing ischemic heart disease among individuals with inborn PCSK9 LOF mutations.[68] ADH mutations such as FH and PCSK9 that affect LDLR functional capacity have revealed critical insights into cholesterol metabolism and risks associated with elevated LDL-C.

Apart from uncovering the functional capacity of LDLR, the identification of ADH related mutations in apoB gene has been crucial in enhancing our understanding of the pathogenicity of apoB containing LDL-P in AVD. A landmark study by Vega and Grundy (1986)[69] discovered a mutation in the apoB gene, which reduced the binding affinity of LDL-P to their corresponding receptors leading to persistent elevations in LDL-C similar to that observed in FH.[70] The significance of this research should not be understated, as these findings laid the groundwork for later studies that characterized and established apoB containing lipoproteins, such as LDL-P as key pathogenic contributors to the development of AVD.[72] The outcomes of this research collectively highlighted that solely relying on LDL-C measures might overlook other pathogenic factors intrinsic to the lipoprotein particles themselves.[59,71]

Following Vega's discovery, a myriad of subsequent genetic exploration identified numerous distinct apoB mutations that contribute to elevated plasma LDL-C concentrations and an increased risk for AVD.[72] Collectively, these studies have not only expanded our understanding of the genetic basis of the LDLR, apoB-containing lipoproteins, and LDL-C metabolism but also encouraged the development and improvement of diagnostic methods and tools.[56] This progress has enabled the establishment of targeted therapeutic interventions for the reduction of LDL-C in ADH and

more common forms of hypercholesterolemia, thereby substantially reducing the impact of elevated LDL-C on cardiovascular and cerebrovascular health.[73] Despite the effectiveness of therapeutics in lowering plasma LDL-C and TC levels, a residual risk persists, particularly in individuals with atherosclerotic plaques that have developed over an extended period. Consequently, addressing the residual risk that remains from LDL-C lowering alone is a significant challenge in the field, leading many to explore alternative approaches of targeting HDL function and metabolism as a potential route for therapeutic drugs that would address this residual risk.

1.2.5.3 HDL

In opposition to genetic factors that elevate LDL-C, decreased HDL-C levels also exhibit a robust genetic component, with heritability estimates typically ranging between 40-60%.[74,75] Historically, a considerable proportion (>30%) of coronary artery disease (CAD) cases associated with AVD reveal low plasma HDL-C levels (<40mg/dL) in affected individuals.[76] Predominantly, mutations underlying monogenic HDL deficiency and gene variations impacting HDL-C levels in the population are present in genes encoding proteins vital for HDL formation, maturation, and catabolism, such as apolipoprotein A-I (apoA-I), ATP binding cassette transporter A1 (ABCA1), cholesterol ester transfer protein (CETP), and lecithin: cholesterol acyltransferase (LCAT). One study discovered that approximately 16% of individuals with HDL-C levels in the bottom 5% possessed rare mutations in these genes, compared to 2% in those with HDL cholesterol levels in the top 5%.[75]

Low HDL-C (<40mg/dL) has long been regarded as a biomarker for AVD.[76] Epidemiological studies consistently demonstrate an inverse relationship between HDL-C levels and the risk of AVD.[77] This association appeared to be independent of other

established risk factors, such as LDL-C and TAG.[78,79] This observation gave rise to the "HDL-hypothesis," proposing that high HDL-C is athero-protective, and thus, increasing HDL-C would reduce the risk of AVD and related events. However, more recent findings indicate that HDL-P measures, which pertain to the number, structure, and function of HDL-P in circulation, may be a superior predictor of cardiovascular risk than standard HDL-C measures alone.[60,80,81]

Historically, the HDL particle is known for its capacity to remove excess cholesterol from peripheral tissues and return it to the liver, a process termed reverse cholesterol transport (RCT).[82] In addition to RCT, the HDL-P has demonstrated anti-inflammatory and antioxidant effects that may alleviate the inflammatory burden associated with atherosclerosis.[83] Furthermore, HDL-P has been shown to exhibit vasodilatory effects that may enhance endothelial function, smooth muscle tone, and fluid dynamics, thus reducing the risk of atherosclerotic plaque rupture.[84,85] Studies also revealed that HDL-P can protect against thrombosis, another critical factor in the development of atherosclerotic vascular disease. This antithrombotic effect of HDL is mediated by its ability to inhibit platelet activation and aggregation and to promote fibrinolysis.[86]

The significance of HDL structure, encompassing size, shape, and lipid and protein composition, considerably influences its athero-protective properties.[87] For example, smaller, denser HDL particles have been associated with a greater athero-protective capacity compared to larger, less dense particles.[80,88] Moreover, the apolipoproteins contributing to HDL structure also play a critical role in HDL-P's functional capacity.[89]

Apolipoprotein A-I (apoA-I), the major protein component of HDL, interacts with cellular receptors to promote cholesterol efflux from peripheral tissues.[82] Mutations that impair

cholesterol efflux, such as those observed in ABCA1 gene mutations, result in an accumulation of cellular cholesterol and a reduction in HDL-C levels.[75] Moreover, when ABCA1-mediated cholesterol efflux is impaired, nascent HDL-P are less stable and more susceptible to renal clearance, which further reduces circulating HDL-P. Due to the diminished formation and stability of HDL-P, the overall capacity of RCT is reduced.[90] This leads to decreased cholesterol removal from peripheral tissues and macrophages, which can contribute to the formation of cholesterol-laden proinflammatory foam cells and the progression of atherosclerotic plaques. Furthermore, suppressed levels of mature HDL-P can impact HDL's antioxidant and anti-inflammatory functional capacity, essential for reducing the oxidation of LDL-P and inflammation within artery walls.[83] Indeed, the ABCA1 gene plays a crucial role in maintaining both HDL-C levels and HDL-P athero-protective properties.

However, other proteins, such as LCAT and CETP, also play important roles in HDL metabolism. For instance, mutations in CETP illustrate the importance of considering HDL-P measures over HDL-C. When a loss-of-function mutation occurs in the CETP gene, it results in decreased cholesterol ester (CE) transfer between apoB-containing lipoproteins (LDL, VLDL) and apoA-containing HDL-P, leading to increased HDL-C levels.[91] However, several clinical trials using CETP inhibitors have shown that this increase in HDL may not reduce the risk of AVD.[92] Indeed, many studies have demonstrated that CETP deficiency or inhibition is associated with larger, CE-enriched HDL-P, a profile linked with reduced capacity for RCT.[82] Moreover, these large lipid-rich HDL-P have also been shown to exhibit impaired antioxidant capacity and anti-inflammatory functions.[59]

Conversely, mutations in the LCAT gene are associated with reduced HDL-C and dysfunctional maturation of nascent HDL-P. The enzyme LCAT catalyzes the esterification of free cholesterol (FC) to CE within HDL-P, thus contributing to the maturation and remodeling of HDL-P.[93] Therefore, mutations leading to reduced LCAT activity or nonfunctional LCAT protein result in impaired cholesterol esterification, accumulation of FC in HDL-P, and reduced HDL-C levels.[94] Furthermore, the inability to esterify FC within HDL-P hinders the maturation of nascent HDL particles into their mature spherical state, a process that reduces the overall capacity of HDL RCT and may lead to an increased risk for development and/or progression of AVD.[94]

The importance of HDL-P structure and function over HDL-C levels in cardiovascular disease is further highlighted by recent clinical trials targeting HDL-C levels. Despite promising preclinical data, these trials have largely failed to demonstrate a reduction in cardiovascular events with HDL-C raising therapies.[95] These studies reflect the limitations of HDL-C as a biomarker for assessing AVD risk. The multifunctionality of HDL-P, encompassing reverse cholesterol transport, anti-inflammatory, and antioxidant properties, highlights the complex nature of their protective role against atherosclerosis. These insights on HDL biology underscore the complexity of HDL-P and signify the importance of addressing specific HDL-P functions as potential therapeutic targets that may potentially reduce the residual AVD risks left behind from traditional cholesterol-lowering therapies.

With a basic foundational understanding of the lifestyle, genetic, and metabolic risk factors that lead up to the development and/or progression of AVD, we will now move onto the pathophysiology of the atherosclerotic lesion and the clinical implications of atherosclerosis in extracranial and intracranial arteries.

1.3 The Atherosclerotic Lesion

1.3.1 The Artery

Arteries, blood vessels responsible for transporting oxygen-rich blood from the heart to the body, possess a unique anatomical structure enabling them to endure various biomechanical forces associated with hemodynamic flow.[96] The innermost layer of an artery comprises a single layer of endothelial cells (EC) that line the intimal space. This endothelial layer functions as a barrier between blood, the intima, and the remaining arterial wall, regulating the exchange of nutrients and waste products from circulation and within the arterial wall.[97] The media layer of the artery is separated from the intimal layer by the internal elastic lamina (IEL) and consists of vascular smooth muscle cells (VSMC) that can contract and relax, providing the necessary biomechanical tone to regulate blood flow and maintain blood pressure.[98] The adventitia constitutes the outermost layer that surrounds the artery and is composed of connective tissues containing blood vessels, nerves, and immune cells.[99]

The arterial anatomy's cellular, structural, and biomechanical components are crucial in maintaining optimal vascular health and resistance against vasculopathies such as atherosclerosis. The endothelial layer serves as the first line of defense against proatherogenic risk factors, including diet, lack of physical activity, obesity, hyperglycemia, insulin resistance, hypertension, and hypercholesterolemia. Damage to the endothelial layer due to these factors may lead to lipid and immune cell accumulation within the intimal space, contributing to the initial development of atherosclerotic lesions.[97] Similarly, the vascular smooth muscle cells in the media can contribute to the formation of atherosclerotic plaques by migrating into and proliferating within the intima, where they

may undergo dedifferentiation into a proinflammatory phenotype.[100] Furthermore, inflammation in the adventitia can exacerbate the progression of atherosclerotic lesions.[99] Understanding the anatomy of arteries is essential for comprehending the pathogenesis of atherosclerosis that will be discussed.

1.3.2 The Lesion

1.3.2.1 Initiation

The pathophysiology of the atherosclerotic lesion entails intricate interactions between various cell types: ECs, VSMCs, macrophages (0M), and lymphocytes (LYM). These cellular interactions drive the pathogenesis of endothelial dysfunction, inflammation, oxidative stress, matrix degradation, and vascular remodeling, which contribute to the overall development and progression of atherosclerotic lesions and atherosclerotic vascular disease (AVD). The initial stages of plaque formation involve endothelial injury and dysfunction, leading to lipid accumulation, foam cell formation, and the migration and proliferation of VSMCs within the intima.[101] Subsequently, vulnerable atherosclerotic plaques develop, characterized by a complex lipid-rich acellular necrotic core, mineralization, destabilization of the extracellular matrix (ECM) resulting in fibrous cap thinning, and eventual plaque destabilization and rupture, causing acute thrombosis and ischemic or occlusive events in the cardiovascular and cerebrovascular arteries.[102]

Under normal circumstances, the subendothelial layer, or intimal space, serves as a protective buffer between the endothelial and medial layers. This subendothelial layer may undergo adaptive remodeling in either an eccentric or diffuse manner, referred to as eccentric intimal thickening (EIT) or diffuse intimal thickening (DIT), respectively. EIT

and DIT are considered nonatherosclerotic lesions and arise as a response to various stimuli such as aging, shear stress, or injury. Histologically, DIT is characterized by an expanded area within the intima, atypical accumulation of VSMCs, and an absence of lipids and inflammatory cells. Nevertheless, EIT and DIT may signify an initial stage of the arterial wall's response to injury associated with endothelial dysfunction and inflammation, potentially progressing into the early stages of atherosclerotic plaque development.[103]

Fatty streak formation is considered the earliest phase of atherosclerosis with some observations of its development before birth, suggesting a potential fetal programming scenario of a pro-atherosclerotic phenotype induced by maternal genes related to cholesterol metabolism.[104] The study of Napoli, C., et al. identified intimal thickening, lipid accumulation, the presence of resident 0M, apoB retention, and oxLDL in aortic cross-sections from fetuses and premature newborns. Early fatty streak development indicators were most prevalent in fetuses from hypercholesterolemic mothers.[104] Despite the limited number of follow-up studies on fetuses and premature newborns, autopsy examinations on infants who succumbed to natural causes have also revealed instances of fatty streak formation, referred to as "infantile atherosclerosis".[105] These findings provide some evidence of atherosclerosis in predevelopment stages of life independent of exposure to traditional risk factors related to environment and lifestyle.

The development of early fatty streaks involves a complex interplay of various cellular and molecular processes, including LDL retention and oxidation (oxLDL), endothelial dysfunction, proinflammatory cascades, leukocyte adhesion, lipid retention, matrix proteoglycan release, and further intimal thickening.[106,107] The exact mechanism which LDL-P enters the intima preceding LDL-P retention is not fully understood and remains a

topic of active research. Nevertheless, LDL retention within the arterial intima is thought to be a critical initiating event in the development of early fatty streaks.[106]

Negatively charged ECM molecules within the intima, such as proteoglycans, can retain LDL particles by binding the positively charged amino acid residues on apoB-100 containing lipoproteins.[107] Extracellular lipid accumulation within the intima begins as LDL is retained as either intact or partially degraded LDL-Ps, initiating an inflammatory response resulting in endothelial dysfunction.[106,108] Furthermore, LDL retention increases the susceptibility of LDL-P to undergo modifications such as oxidation, forming oxLDL-P, even before the presence of inflammatory cells like macrophages.[104] OxLDL-P exacerbates inflammation and endothelial dysfunction, accelerating the development of fatty streaks and the progression of atherosclerotic lesions.[109]

1.3.2.2 Progression

Pathological intimal thickening (PIT) commences as lipids and proinflammatory cells accumulate within the intimal space. This proinflammatory milieu creates an environment for endothelial dysfunction that induces the expression and activation of adhesion molecules (e.g., vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), selectins), procoagulant molecules (e.g., tissue factor), and chemokines (e.g., interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1)). These molecules collectively induce a proinflammatory endothelial phenotype, promoting the adhesion of circulating leukocytes to the endothelium and facilitating their migration into the subendothelial space (intima).[97]

Upon entering the intima, monocytes differentiate into macrophages (0M), a process driven by local growth factors and cytokines such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). As extracellular lipids accumulate in the intima, LDL-P and oxLDL bind scavenger receptors on the surface of macrophages, such as scavenger receptor class A members (SRA) and cluster of differentiation 36 (CD36), a class B scavenger receptor. This initiates macrophage phagocytosis and lipid uptake, culminating in foam cell formation.[110] The growing proinflammatory milieu of cytokines and chemokines perpetuates the recruitment of additional leukocytes and further modification of LDL-Ps, thereby maintaining a chronic inflammatory state. This persistent proinflammatory environment promotes the migration and proliferation of VSMC into the subendothelial space where they begin remodeling and thickening the intima, which is best described as pathological intimal thickening (PIT).[111]

During the initial stages of PIT, VSMCs, which are usually present in the medial layer of the arterial wall, migrate to the intima under the influence of growth factors such as platelet-derived growth factor (PDGF).[112] In the intima, VSMCs can dedifferentiate switching from a contractile to a synthetic phenotype, characterized by increased proliferation and synthesis of ECM components, such as collagen, elastin, and proteoglycans.[113] Proteoglycan-rich ECM contribute to feed forward mechanisms that further drive the progression of atherosclerosis by enhancing LDL retention, inflammation, and the formation of a lipid-rich necrotic core.

For instance, proteoglycans in the newly formed ECM can bind to LDL, increasing its susceptibility for modification.[113–115] Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is up regulated in both macrophage and VSMCs in response to

modified LDL and proinflammatory cytokines within the intima such as TNF-a, interleukin 1 (IL-1) and interferon gamma (IFNy).[113–115] Modified oxLDL binds to LOX-1 on the surface of VSMC inducing the release of growth factors that perpetuate inflammatory signaling and the proliferation of VSMC within the intima.[115] Moreover, under the influence of proinflammatory mediators the transcription factor (TF) kruppel-like factor 4 (KLF4) is upregulated in VSMC promoting phenotype switching towards a more macrophage-like cell capable of phagocytosis lending to the growing pool of lipid laden foam cells.[116]

Under the American Heart Association (AHA) classification system the preceding pathology described as PIT is considered a Type III lesion. This system categorizes atherosclerotic lesions into types (I-VI) based on the histological appearance of the lesion. It emphasizes the various components of the plaques, such as lipid content, macrophage infiltration, and fibrous tissue. This classification system focuses primarily on the progression of atherosclerosis from early to advanced stages.[103] More recently a "modified" AHA classification based on morphological description has been proposed. This system is more focused on the specific morphological features and thrombotic potential of atherosclerotic plaques. It incorporates additional categories, such as thin fibrous cap atheroma (TFCA), plaque rupture, erosion, calcified nodule, and fibrocalcific plaque, to better describe the complexity and heterogeneity of plaques[102,117]

There are advantages and disadvantages for each system that should be considered when assessing atherosclerotic lesions, as I will be doing in the following text. For instance, the AHA system provides a clear and straightforward way for describing and understanding the natural sequence of the progression of atherosclerotic lesions.[103] However, the AHA

system lacks granularity required to describe the morphology and composition of complex and advanced atherosclerotic plaques that may limit its usefulness for guiding research and clinical based decision making. Alternatively, the modified AHA system provides feature rich descriptions of compositional nuances within atherosclerotic plaques that underline their potential for rupture and thrombosis. While potentially aiding in the development and implementation of targeted interventions,[117] the modified AHA classification system can require a greater degree of expertise and resources that might not be widely available, especially for large scale applications where several histological strategies of preparing, imaging, and analyzing are required. For this discussion, I will avoid jumping between systems and will only be providing the most common morphological and compositional observations that are related to the pathology being discussed.

Histopathological composition of PIT can be characterized by diffuse extracellular lipid accumulation and lipid laden foam cells beneath a proteoglycan-rich matrix observed with both HE and Movat chemical staining.[107] Substantial amounts of vascular smooth muscles cells within the intima are readily observed with antibodies against alpha actin (a-actin) or smooth muscle myosin heavy chain.[118] Diffuse numbers of inflammatory cells such as macrophage may be observed throughout the intima when stained with antibodies against cluster of differentiation 68 (CD68) and 163 (CD163).[119,120] However, VSMC that have undergone phenotype can lose the expression of a-actin and smooth muscle myosin heavy chain and gain expression CD68 and/or CD 163.[119,120] This can complicate the identification of “true” macrophage cells within the atherosclerotic plaque. Therefore, caution is needed when characterizing macrophage expression/involvement and may require additional strategies for validation. Nevertheless, pathological intimal thickening

is characterized by the presence of diffuse extracellular lipids, foam cells, proinflammatory cells and VSMC within a proteoglycan-rich matrix, and the absence of necrosis or necrotic core.

1.3.2.3 Features of the Advanced Atherosclerotic Lesion

As vascular remodeling progresses, the lesion can gradually encroach upon the lumen of the blood vessel, resulting in the formation of a stenotic lesion that reduces the total lumen area and restricts the flow of blood.[117] Lesion remodeling may either occur in a concentric fashion around the entirety of the vascular wall or in an eccentric fashion forming a "shoulder lesion" on just one side of the vessel. Concentric lesions are often seen in PIT and hyperplasia post stent procedures and can result in stenosis. While atherosclerotic lesions do form concentrically, eccentric formation is most often observed among advanced lesions and is considered be more vulnerable to rupture.[121,122]

Independent of stenosis, continual intimal remodeling and inflammation progresses PIT into a fibroatheroma (FA) with a well-defined necrotic core, exhibiting the first signs of a true atherosclerotic plaque.[122] With continued plaque progression foam cells and macrophage-like VSMCs may undergo cell death beneath the fibrous ECM formed during PIT. This cell death may occur through either apoptosis or necroptosis due to excessive intracellular lipid accumulation and the proinflammatory milieu within the intima.[122] Apoptosis is a programmed cell death, a controlled mechanism that removes damaged or unnecessary cells without inciting inflammation. Conversely, necroptosis is an uncontrolled cell death resulting from extreme cellular distress or injury. Necrotic cells undergo swelling, membrane rupture, and the release of intracellular contents subsequently enhancing the inflammatory response within the intima.[123]

Apoptotic cells display specific "eat-me" signals on their surface, such as phosphatidylserine (PS) that are recognized by various receptors on phagocytic macrophages that leads to subsequent binding to apoptotic cells and the initiation of phagocytosis. Following phagocytosis, the apoptotic cell is internalized within phagolysosomes where they are metabolized by lysosomal enzymes. The end products of apoptotic cells are then either recycled within the cell or effluxed by the macrophage.[123]

Compared to apoptotic cells, recognition and clearance of necrotic cells is impaired. During necroptosis cells do not exhibit the same controlled “eat-me” signals that are on the surfaces of apoptotic cells making their recognition and uptake by macrophages inefficient. Instead, necrotic cells release damage-associated molecular patterns (DAMPs) which initiate a localized inflammatory response recruiting inflammatory cells to the site of necrosis.[123] Furthermore, the release of DAMPs such as, high mobility group box 1 (HMGB1), heat shock proteins (HSPs), and S100 proteins further exacerbating the proinflammatory environment and impairing efferocytosis of apoptotic cells.[124]

Efferocytosis is the process by which apoptotic and necrotic cells are recognized, engulfed, and cleared by macrophages, a process crucial to the homeostasis and prevention of necrotic core development. Localized cellular debris begins to accumulate within the fibrous ECM when apoptotic and necrotic cells are not efficiently recognized and metabolized by macrophages. The extracellular contents released by unprocessed apoptotic and necrotic cells begin the formation of the necrotic core beneath an ECM fibrous cap forming a fibroatheroma (FA).[125]

The most noticeable difference observed with HE staining between PIT and FAs is the presence of a localized acellular, lipid-laden necrotic core resting underneath a collagen-

rich fibrous cap. The necrotic core can consists of extracellular lipid pools, cholesterol clefts, cholesterol crystals, and cellular debris as products of impaired efferocytosis.[122] Compared to PIT, FA may have reduced expression of IHC markers for VSMC, such as a-actin, and increased expression of proinflammatory markers, such as CD-68, especially around the necrotic core and within the fibrous cap.[122] Furthermore, compared to the proteoglycan-rich ECM observed in PIT, the FA will have higher amounts of fibrous tissue, mainly collagen, that is most readily observed using Movat, Masons Trichrome, or Picrosirius Red Fast Green (PSRF) chemical staining.[101]

The fibrous collagen-rich matrix plays a vital role in plaque stabilization and protection by providing resistance to mechanical stress and rupture of the necrotic core, and synthetic VSMCs are crucial to this defensive process.[126] Both 0M and VSMC produce metalloproteinases (MMPs) such as MMP-2 and MMP-9 that are involved in the degradation of the collagen and elastin and play a significant role in the remodeling of the fibrous cap. The proteolytic activity of MMPs is inhibited through a negative feedback loop provided by the release of tissue inhibitors of metalloproteinases (TIMPs) from synthetic VSMCs, that binds the active sites on MMPs, inhibits their enzymatic activity, and helps maintain ECM homeostasis within the fibrous cap.[126,127]

However, continual LDL retention, lipid accumulation, inflammation and oxidative stress within the intima enhances the activity of MMPs while reducing the expression of TIMPs disrupting the negative feedback loop and enhancing the degradation of ECM overlaying the necrotic core.[127] Moreover, proinflammatory mediators continue to enhance VSMC phenotype switching away from collagen producing synthetic VSMC towards more proinflammatory macrophage-like VSMCs.[128] Therefore, a reduction of synthetic VSMCs

and elevation in proteolytic MMP activity leads to reduced collagen deposition and increased collagen breakdown, a process that results in the thinning and destabilization of the fibrous cap, predisposing the necrotic core to rupture and a thrombic event.[129]

Alternatively to laying down a collagen-rich matrix, synthetic VSMCs may also produce bone matrix proteins promoting intraplaque calcification.[130] However, in the milieu of cytokines (e.g., TNF-a, IL-1B) and growth factors such as osteoprotegenerin, VSMCs may also differentiate into osteoblast-like cells that can contribute to calcification inside the plaque.[131] Moreover, microcalcification can begin to develop as apoptotic and necrotic cells release matrix vesicles containing calcium and phosphate that precipitate around the necrotic core.[132] The process of calcification can be stabilizing but more often infers destabilization and increased risk for plaque rupture.[133]

Further destabilizing processes in the plaque can continue with persistent exposure to a proinflammatory environment of cytokines (e.g., TNF-α, IL-1β), along with growth factors (e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)), that may stimulate angiogenesis or neovascularization within the plaque.[133] Alternatively, neovascularization may also occur following severe inward remodeling as a consequence of stenosis. With reduced blood flow, oxygen supply to the plaque's deeper layers becomes limited, leading to hypoxia. In turn, hypoxia can provoke the cellular release of hypoxia-inducible factor-1 (HIF-1) from resident lesion cells that may then upregulate the expression of angiogenic growth factors such as VEGF and stimulate neovascularization within the plaque.[134]

Consequently, newly formed blood vessels deep within the intima promote both growth and expansion of the lesion through the supply of nutrients and oxygen to the plaque.[135]

Neovascularization facilitates the infiltration of inflammatory cells deep into the plaque feeding forward the proinflammatory processes.[133] Furthermore, neovascularization creates immature blood vessels susceptible to leakage especially when exposed to proinflammatory stimulus, such as cytokines within the lesion. The rupture of these neovessels has been shown to lead to intraplaque hemorrhage exacerbating inflammation, impaired efferocytosis, necrotic core expansion and degradation of the fibrous cap.[136]

There are a variety of ways intraplaque calcium may present histologically and each presentation has different implications for plaque stability. For instance, small darkly stained (HE) calcium, or microcalcifications, deposit within the fibrous cap reducing the plasticity of the ECM and contributing to a localized increase in biomechanical stress increasing the chance for rupture.[137] Conversely, deep within the plaque proximal to the internal elastic lamina (IEL), large, well-organized, continuous layers of calcium maybe observed as sheet-like calcification deposits. These sheet-like calcium deposits are most often observed in aged fibroatheromas lacking large necrotic cores, and here the calcium provides mechanical support and stabilization to the lesion.[137] However, histological observations may also reveal small irregular and protruding (eruptive) nodular calcifications that interrupt the surrounding tissue architecture lending to destabilization and chance for rupture, especially when found in or around a thinning fibrous cap.[138]

Observing the extent of lesion calcification in situ within the intima can be challenging. Calcium deposits such as sheets and nodules can disrupt the surrounding architecture of the lesion and this can lead to tissue destruction through sheering or tearing while processing cross-sections on a standard microtome, as the hard calcium maybe displaced taking tissue with it. Moreover, while histological processing techniques such as

decalcification may improve routine sectioning, they may also result in stripping calcium from the lesion leaving only remnants of positive calcium staining around the borders of where it was previously deposited making difficult to quantify or classify the full extent of calcification within the lesion.

Nevertheless, fibrocalcific plaques are a hallmark presentation of advanced and complex atherosclerotic lesions. The typical fibrocalcific plaques are collagen-rich lesions with significant luminal stenosis. In the absence of inflammatory cells, the interplay between ECM collagen and calcium becomes significant, potentially working together to contribute to the formation and characteristics of fibrocalcific plaques. This collaborative mechanism may also play a role in stabilizing fibrocalcific atheroma lesions. However, under continual pro-atherosclerotic conditions the ECM may slowly become degraded thinning the fibrous cap over the necrotic core leading to destabilization of the lesion. Furthermore, in thin cap fibroatheromas (TCFA) calcium loses its contribution to stability and is believed to promote further destabilization, increased susceptibility to intraplaque hemorrhage and/or rupture[137,138]

Most often factors preceding atherosclerotic plaque rupture and thrombosis include inflammation, macrophage infiltration and activation, MMP release with ECM degradation, calcification, and intraplaque hemorrhage. Indeed, histological cross sections of advanced atherosclerotic lesions often reveal evidence of a complex interplay of cellular and molecular mechanisms, including lipid accumulation, inflammation, cell migration and proliferation, ECM synthesis and degradation, and plaque calcification. Histopathological observations behind these cellular and molecular mechanisms leading up to plaque instability and rupture have been crucial to our understanding of atherosclerotic lesion

progression. Observations from histological studies suggest that with each additional layer of complexity that develops within the atherosclerotic lesions there comes an increasing risk for plaque rupture, thrombosis, and an ischemic event that may precipitate a heart attack or stroke.

1.4 Extracranial Atherosclerosis

Building upon our analysis of atherosclerotic plaque formation, progression, and subsequent complications, we now turn our attention to the repercussions of atherosclerosis across extracranial and intracranial vascular regions. While all atherosclerosis stems from a common etiology, its consequences differ depending on the location of the plaque and the probability for a tissue damaging ischemic event. Atherosclerotic vascular disease mutually links cardiovascular and cerebrovascular health. This section will briefly explore Extracranial Atherosclerosis (ECAS) and its ramifications for heart health before penetrating deeper into Intracranial Atherosclerosis (ICAS) and its implications for brain health.

Coronary artery disease (CAD), synonymous with ischemic heart disease, emerges as extracranial atherosclerotic plaque progressively accumulates and compromises the lumen of coronary arteries, thereby curtailing blood supply to the heart. This chain of events precipitates angina, potentially escalating to a heart attack, myocardial infarction.[139] With approximately 19.1 million global deaths in 2020, CAD stands as the foremost cause of mortality worldwide. Moreover, an estimated 244.1 million individuals reportedly suffered from some form of CAD in the same year, with the highest prevalence rates emerging in low-income countries in Asia, Eastern Europe, North Africa, and the Middle East.[3] In the

United States, per the Centers for Disease Control and Prevention (CDC), one in twenty American adults over the age of twenty are living with CAD, which translates to roughly 5% of the population. Furthermore, the CDC estimates that approximately 805,000 Americans experience a heart attack annually and that CAD claimed nearly 695,000 lives in 2021.

1.5 Intracranial Atherosclerosis

1.5.1 Stroke

Conversely, a stroke is a cerebrovascular event that disrupts the brain's blood supply, leading to neuronal injury and possibly permanent disability or death. Strokes can be of two types, ischemic or hemorrhagic. The latter, characterized by the rupture of intracerebral arteries and subsequent blood seepage into brain tissue, accounts for 10-20% of global strokes and 8-15% of strokes in the United States.[140] In contrast, globally ischemic strokes constitute 62% of all stroke cases and 87% of stroke cases in the United States.[141,142]

A primary cause of ischemic stroke is intracranial atherosclerotic plaque development, resulting in invasive stenosis or plaque rupture with occlusive thrombi/emboli within a cerebral artery.[143] The incidence of ischemic stroke varies according to age, gender, race/ethnicity, and geographic region. The American Heart Association (AHA) estimated in 2022 that about 6.7 million Americans aged 20 or older have experienced either an initial or recurrent stroke.[3] In the U.S., the anticipated fatality rates for seniors range from 5 to 15% for those aged 18 to 49, with a significant rise every subsequent decade, peaking at around 30 to 40% for those aged 80 and older.[3] The lifetime risk (LTR) of ischemic stroke is higher in females, although the average age of onset is later (75 years) compared to males

(68 years).[144] Among ethnic groups, African Americans, Hispanic Americans, and Asian Americans exhibited a higher incidence of ischemic stroke relative to non-Hispanic white Americans.[3]

The incidence ischemic stroke continues to rise in young adults; however, there has been a reduction in ischemic stroke related mortality among the elderly.[3] This decrease in elderly mortality may be attributed to advancements in treating atherosclerotic risk factors, timely stroke identification, and emergency/surgical interventions addressing transient ischemic attacks, which often precede a full-blown ischemic stroke.

Despite advances to reduce stroke related mortality, the survival of a stroke greatly increases the odds for a follow-up or reoccurring stroke. Research suggests that individuals who have survived a stroke face an increasing yearly risk of recurrence, which poses a significant threat, particularly among the aging population. Within the initial 90 days post-stroke, the likelihood of a second stroke can be as high as 13% surpassing the general population's stroke risk by 15 times.[145,146] Prevention of reoccurring strokes requires quick clinical intervention to reduce proatherogenic vascular risk factors such as hyperglycemia, hypertension, and hypercholesterolemia.[147] The clinical approach to rapidly reduce risk factors associated with stroke reoccurrence does save lives. However, independent of increased lifespan, the clinical approach appears to fall short in addressing the consequences of residual neuropathology developed from past stroke events, and how those consequences may lead to functional deficits and cognitive impairments in the aging brain.

1.5.2 ICAS and the Blood Brain Barrier

Intracranial atherosclerotic stenosis (ICAS) significantly contributes to the development of neuropathology associated with ischemic stroke which in turn may lead to Vascular Cognitive Impairment (VCI). Healthy brain tissue and cellular function hinge on a continual supply of oxygen-rich blood, tight regulation of nutrient exchange and waste disposal, and homeostatic hemodynamics throughout the cardiovascular-cerebrovascular axis, particularly at the blood-brain barrier (BBB).[148]

Any perturbation in these domains could result in a consequential loss of BBB's integrity, ensuing damage to brain tissue and its functional cellular units. The structural integrity of the BBB relies on healthy luminal endothelial cells (ECs), maintenance of tight junctional (TJ) and adherin proteins, active pericytes (PCs), functional vascular smooth muscle cells (VSMCs), and astrocyte (AC) end feet, each playing a crucial role.[97,112,149,150] These components together with resident AC, microglia (MG), and neurons constitute the functional neurovascular unit (NVU).[151]

Proinflammatory events within luminal endothelial cells (EC), or aberrant changes to laminar blood flow, can dysregulate the protective function of the blood-brain barrier (BBB). This dysregulation of the BBB could cause neuroinflammation and uncoupling of the neurovascular unit (NVU), potentially leading to white matter hyperintensities (WMH) and/or cerebral microbleeds (CMB). Such vascular consequences have been suggested to pave the way for a neurodegenerative pathway that leads to vascular cognitive impairment (VCI).[150–152]

The “two-hit hypothesis” presents an intriguing model for neurodegenerative disease progression.[153] It postulates that a primary hit, originating from a vascular insult along the cardiovascular—neurovascular axis, instigates BBB dysfunction and/or oligemia (reduced blood volume), subsequently paving the way for the second hit: toxic protein accumulation and/or cerebral hypoperfusion (ischemia) resulting in subsequent neurodegeneration (cell death).

Hit 1: The progression of atherosclerosis triggers further vascular inflammation and irregular vascular remodeling along the cardiovascular—cerebrovascular axis.[97,154] Extracranial atherosclerosis exacerbates vascular inflammation promoting the upregulation of pro-inflammatory molecules and extracellular matrix (ECM) degrading proteins, such as matrix metallopeptidase proteins 3 and 9 (MMP3 and MMP9).[154,155] These ECM degrading proteins break apart the supporting fibrous cap that surrounds the underlying necrotic core within advanced atherosclerotic lesions. Reduced atherosclerotic lesion integrity increases the risk for rupture and the initiation of an ischemic event that can take place in either the heart or brain.[97,155] Additionally, ICAS instigates vascular inflammation within intracranial arteries and small cerebral vessels that could result in the upregulation of MMPs. Increased MMPs disrupt tight junction (TJ) proteins connecting the endothelial cells (ECs) of the blood-brain barrier (BBB).[156] The loss of TJ proteins enhances BBB permeability and leakage, paving the way for various neurovascular pathologies associated with cognitive impairment (CI).[150–152]

Hit 2: Increased BBB permeability and a milieu of inflammatory mediators have been shown to reduce the clearance of neurotoxic proteins such as amyloid beta from the brain. For instance, vascular inflammation at the BBB has been reported to reduce the expression

of membrane-bound low density lipoprotein receptor-related protein (LRP-1), while concurrently augmenting the release of advanced glycation end products (AGE).[157] Upon activation of AGE receptors (RAGE) and a reduction of LRP-1, a dysregulated clearance of amyloid beta (abeta) from the brain may occur.[157,158] The accumulation of abeta within the brain has been heavily implicated in Alzheimer's Disease (AD) neuropathology and CI.[159,160]

An alternate “two hit hypothesis” might originate from vascular remodeling caused by ICAS, specifically stenosis. Stenosis disrupts the laminar blood flow along the endothelium, leading to biomechanical processes that initiate pro-inflammatory EC signaling.[97,161] Concurrently, progressive stenosis of large cerebral vessels reduces cerebral blood flow (CBF),[161,162] resulting in oligemia across the BBB that has been associated with the uncoupling of the neurovascular unit (NVU).[158,163,164] Uncoupling of the NVU along with oligemia leads to reduce clearance of toxic metabolites and proteins from the brain,[158,159] resulting in the activation of brain resident glial cells.[165,166] The activated glial cells may quickly establish a localized neuroinflammatory environment that can instigate neurodegenerative pathologies associated to cognitive impairment (CI).[166]

1.5.3 Vascular Cognitive Impairment

Dementia, not being a disease but a syndrome characterized by a collection of symptoms, impacts cognitive domains including memory, reasoning, language, judgment, and behavior.[167] Major neuropathological contributors to dementia include Alzheimer's disease (AD), vascular dementia (VaD), Lewy body dementia (LBD), frontotemporal dementia (FTD), and mixed dementia.[168] Progressive and accumulative harm to neuronal function precipitates cognitive decline, complicating daily tasks like problem-solving and decision-

making. Simultaneously, unusual changes in mood, personality, and social behavior significantly affect a person's quality of life and their ability to live independently.[167,169]

The global prevalence of dementia, currently affecting over 55 million people, is estimated to triple by 2050 [WHO Fact sheets, 2023, accessed Apr 23]. The Alzheimer's Association reported a steady increase in dementia cases in America, with 6.5 million Americans living with some form of dementia in 2023, compared to 5.4 million in 2016.[170] It's approximated that around 1 in 9 individuals over 65 and nearly 1 in 3 over 85 are living with dementia.[170]

Dementia's treatment and care impose a large financial burden globally, with an annual global cost reaching $1314 billion in 2019. In the US, the annual care cost for dementia patients is approximately $23,796, which can impose a significant financial burden on the patient and their family.[171] At present, the available medical and palliative approaches offer limited ability to halt or reverse the progression of dementia. Instead, these approaches primarily focus on slowing down the disease's progression and alleviating associated symptoms. While these interventions can provide some relief and moderately improve the quality of life for individuals living with dementia, they do not offer a cure or complete resolution of the condition.[172,173]

AD, often linked with a buildup of improperly folded amyloid and tau proteins,[174] neuroinflammation,[175] neuronal dysfunction, and neuronal death,[176] is considered the primary contributor to dementia. AD accounts for 60-70 percent of dementia cases worldwide, ranking as the seventh leading cause of death in the United States [CDC Key Facts, 2023 march, accessed Apr 2023]. Vascular dementia, the second most common subtype, accounts for approximately 15%-20% of all dementia cases in North America and

Europe. With its incidence rate doubling every five years after the age of 65, VaD poses a significant concern for our aging population.[177]

For optimal brain function and health, the presence of healthy vessels supplying oxygen-rich blood and vital nutrients is indispensable. Any impairments to these vessels may precipitate neurodegenerative vascular pathologies, including cerebral infarcts, white matter lesions, microbleeds, and cerebral amyloid angiopathy (CAA). These are but a few of the adverse outcomes of vascular dysfunction that can contribute to vascular cognitive impairment (VCI).[178,179]

Within the array of potential vascular dysfunctions, intracranial atherosclerosis (ICAS) stands out as the most prevalent vascular lesion observed at autopsy in the aging brain, irrespective of dementia status.[180] The implications of ICAS are multifaceted, affecting cerebral blood flow (CBF), promoting ischemic events, exacerbating vascular inflammation, and amplifying the permeability of the blood-brain barrier (BBB). These cumulative effects may all precipitate neuroinflammatory events, ultimately tying ICAS to neurodegenerative processes and vascular-associated cognitive impairment.[158,159,161–166]

However, ICAS is not the only significant factor; other vascular risk factors such as obesity, smoking, diabetes, and hypertension, when persistent over a lifetime, can be equally deleterious to brain health and cognition. Notably, all these factors contribute to the pathological development and progression of atherosclerosis.[181] Despite the widespread co-occurrence of ICAS and VCI findings at autopsy, establishing the direct mechanisms that links ICAS to neurodegenerative pathology remains elusive, largely due to the scarcity of biomarkers for vascular/neurodegenerative disease, limitations in imaging techniques,

and the lack of animal models that develop both extracranial atherosclerosis (ECAS) and ICAS.[181–183]

The uncertainties of the direct impact of ICAS on cognition and brain function necessitates general recommendations for achieving optimal brain health. Promoted by the AHA and the American Stroke Association, preserving cardiovascular health is considered a salient factor for the enhancement of cognitive abilities, including memory, learning, judgment, and language proficiency. This translates into adopting a VCI risk reduction strategy identical to the guidelines for reducing the risk of cardiovascular disease, specifically atherosclerosis related.[181] Key preventive measures involve tobacco abstinence, maintaining a body mass index below 25 kg/m^2, increased physical activity, and a healthy diet abundant in fruits and vegetables with limited saturated fats and sugars. Further, achieving optimal blood pressure (120/80) mmHg), cholesterol (<200mg/dL), and fasting blood glucose levels (<100 mg/dL) are strongly recommended. The aforementioned interventions, that form the backbone for cardiovascular health, are supported by an array of evidence showing their potential in improving cerebrovascular health, potentially reducing the risk for stroke, VCI, and dementia.[181] However, it is important to note that none of these measures have been demonstrated to reverse the damage elicited by atherosclerotic vascular disease (AVD) on tissues, including the heart and brain. Consequently, further research is warranted to assess whether the regression of atherosclerotic lesions can improve downstream vasculopathies association with neurodegeneration, cognitive impairment, and dementia.

1.6 Mitigating Risk (Therapeutics)

The traditional first-line therapeutic approach to reduce the risk of atherosclerotic vascular diseases has been to lower circulating LDL-C levels. While statin therapy is widely and successfully used to reduce LDL-C and AVD events, a considerable population of patients remain either unresponsive or intolerant to statin therapies. Moreover, unrecognized hypercholesterolemia and late initiation of statin therapy fails to reverse established and advanced atherosclerotic lesions, thus leading to a residual risk for developing cardiovascular and cerebrovascular events.[184] Recognizing and addressing the residual risk that remains emphasizes the necessity for innovative therapeutic approaches.

PCSK9 inhibitors, such as Evolocumab and Alirocumab, have demonstrated effective LDL-C reduction and significant lowering of CV event risks.[185,186] Conversely, the efficacy of CETP inhibitors has been inconsistent despite their potential to reduce LDL-C and elevate HDL-C levels.[78,185]

RNA-based therapies are showing encouraging preclinical and clinical outcomes, causing improvements in lipid profiles and delays in atherosclerosis progression. Antisense oligonucleotides (ASOs) and siRNAs can target and inhibit pro-inflammatory genes involved in atherosclerosis, while targeting certain miRNAs, such as miR-143/145 and miR-27, can ameliorate vascular smooth muscle cell plasticity, reduce inflammation, and alleviate plaque burden.[185–187] Among the most widely studied miRNAs, miR-33 suppression has been associated with improvements in fatty acid oxidation, cholesterol efflux, and macrophage function, thereby indicating potential benefits for stabilizing or even regressing AVD. [188–191]

Concurrent research is also exploring alternative approaches including the targeting of long non-coding RNAs, messenger RNAs, macrophage-targeting nanoparticles, and liver X receptor (LXR) inhibitors.[192–194] As it stands, the challenge lies in fully understanding the effects and limitations of these emerging therapies. However, their promise lies in their potential to address the residual risk that remains with AVD. Expanding our understanding of these RNA-based therapies necessitates devising suitable experimental conditions to induce and monitor atherosclerotic progression. In parallel, developing animal models that exhibit both ECAS and ICAS is critical.

1.7 Animal Models of Atherosclerosis

The application of animal models has long been instrumental in elucidating the intricate mechanisms underlying atherosclerosis, as well as in the exploration of potential therapeutic interventions. Ranging from small-sized species such as mice to larger mammals like non-human primates (NHPs), each animal model provides distinct capabilities and insights into this multifaceted disease and its progression. However, leveraging these resources to their fullest potential requires a comprehensive understanding of their unique strengths, and perhaps more important, their limitations. Furthermore, an appreciation of the breadth of variations in AVD expression and progression across different models aids in contextualizing their respective findings and collectively enabling a more accurate translation to human pathophysiology. The complexity across these models underscores the importance of a rigorous approach to the selection and application of each, setting a firm foundation for insightful and translational research in atherosclerosis.

Mice have long been the preferred models in atherosclerosis research due to a combination of factors, including their adaptability for genetic manipulation, relatively low cost, and rapid breeding capabilities. The use of genetically modified strains, specifically ApoE−/− and LDLR−/− mice maintained on a high-fat/cholesterol diet may recapitulate aspects of human atherosclerosis. This model has been instrumental in shedding new light on the underlying molecular processes driving the disease.[195]

However, an important limitation exists in mouse models, particularly related to the spatial development of atherosclerosis. Mice, unlike humans, chiefly develop ECAS along the aorta not within coronary or intracranial arteries. This spatial limitation impedes the use of these models in fully understanding the impact that progression of both ECAS and ICAS has on the heart and brain.[195] Thus, while genetically modified mouse models have offered valuable insights into the mechanisms of atherosclerosis, caution must be exercised when extrapolating these findings to the human disease process due to the uniquely different spatial development of AVD.

Rabbits bring a unique niche to hypercholesterolemia research due to their human-like plasma lipid profile, a characteristic that offers a more reliable replication of human cholesterol dynamics and AVD development.[196,197] This is illustrated by WT rabbits' ability to develop rapid fibrofatty atherosclerotic lesions under diet-induced hypercholesterolemia, a process that closely mirrors key aspects of human AVD's initiation and progression, albeit at an accelerated level.[198] A pertinent distinction, however, arises in how cholesterol is transported, where humans predominantly employ LDL as their carrier, contrasting with rabbits' carrying the majority of their cholesterol on VLDL.[197,199] Despite the capability of rabbit models to develop both coronary and carotid

atherosclerosis, a significant constraint arises in their inability to consistently develop ICAS in the absence of additional proatherogenic stimuli, such as surgically induced hypertension.[197,200] These limitations continue to underscore the importance of careful animal model selection tailored to the specifics of each atherosclerosis research question.

Transitioning from smaller to larger animal models, pigs exhibit promise in atherosclerosis and ICAS research due to their size, genetic, anatomical, and physiological characteristics closely resembling those of humans.[201–203] These models not only develop coronary atherosclerosis spontaneously, but diet-induced porcine models can also mimic the human atherosclerotic progression pattern culminating in advanced atherosclerotic lesions that are both spatially and compositionally like that found in humans. This replication of disease progression underscores their value in ECAS studies, particularly those focused on surgical interventions for coronary atherosclerosis.[203,204] Their anatomical similarity to humans and extensive development of ECAS make porcine models fitting for surgical intervention and imaging studies.

Despite these advantages, porcine models present certain limitations that need to be considered. Their reasonably large size and considerable associated costs in terms of housing, feeding, and material needs has limited their widespread adoption.[204] Additionally, limited availability of immunologic tools, such as porcine-specific antibodies, present further challenges. Notably, while ICAS has been observed by our lab in a small cohort of LDLR -/- pigs (unpublished data), other porcine models may not consistently demonstrate similar ICAS development. Therefore, additional research is needed to determine if any neuropathological and/or cognitive changes occur in this model when ICAS is present.

Amongst these animal models, NHPs serve as a remarkable platform for research, primarily due to their significant genetic, anatomical, and physiological similarities to humans. These factors grant NHPs a unique standing among animal models, making them the 'gold standard' for preclinical efficacy and safety in pharmaceutical drug studies.[205] Nonhuman primates are responsive to diet, react to environmental and social stressors, have extended lifespans, develop neuropathological and cognitive changes with age, and are capable of developing complex ECAS and ICAS lesions, thereby enabling a comprehensive exploration of lifestyle and disease factors associated with vascular biology and pathology.[206–210] Nevertheless, NHP models are not without their unique complexities, which include variable responses to hypercholesterolemia and atherosclerosis, attributed to numerous genetic, metabolic, and environmental factors.[211,212] Ethical concerns, limited availability and skyrocketing cost must also be considered when using NHP models.

While the distinct advantages offered by each model - mice, rabbits, porcine, and NHPs - significantly advance our understanding of atherosclerosis, their inherent limitations remind us of the careful extrapolation needed when applying these findings to human disease progression. Despite their unique complexities, NHPs' close genetic and physiological similarities to humans make them a potent platform for studying atherosclerosis, particularly in the context of both ECAS and ICAS.

The selection of an animal model for atherosclerosis research demands thoughtful consideration, aligning study objectives with the specific strengths and inherent limitations of the chosen model. Each of these models, from the genetically tractable mice to the physiologically similar NHPs, can provide a unique set of data from which we can understand and further explore the complex pathophysiology of atherosclerosis.

Despite certain limitations, mice models, with their genetic tractability, have proven instrumental in illuminating a number of molecular mechanisms related to atherosclerosis [195]. Rabbit models, notable for their human-like plasma lipid profiles, have offered significant insights into hypercholesterolemia's role in ECAS lesion development, although their potential in ICAS development remains uncertain.[197,200]

The porcine models, in spite of their size and costs, offer a uniquely human-like model for studying atherosclerosis due to their genetic and physiological proximity to humans.[201–204] And then we have NHPs, which are genetically and physiologically most similar to humans, offering an unparalleled platform for studying vascular biology and pathology, despite the unique complexities they present.[206–212]

The use of animal models in atherosclerosis research is not just an optional approach, but rather a vital and essential one. Animal models provide researchers with a controlled experimental environment to explore the complex mechanisms involved in the development, progression, and regression of atherosclerosis. By studying animals that closely resemble humans in terms of genetics, physiology, and anatomy, researchers can gain valuable insights into the underlying disease processes. Animal models allow for the testing of novel therapeutic interventions and the evaluation of their effectiveness before progressing to clinical trials.

Among animal models, NHPs are particularly well-suited for investigating atherosclerosis regression. NHPs offer a unique research platform that closely replicates the progression of atherosclerotic vascular disease observed in humans. Their development of human-like atherosclerotic lesions provides a realistic benchmark to assess the efficacy of therapies

aimed at stabilizing and reversing established atherosclerotic lesions in both coronary and intracranial arteries.

Furthermore, NHPs present an opportunity to examine the potential impact of atherosclerosis regression, particularly in intracranial arteries, on clinical outcomes related to stroke and dementia. This expands the scope of atherosclerosis research to encompass a broader context of cerebrovascular and cognitive health and function. By utilizing NHP models, researchers can further our understanding of the complex interplay between atherosclerosis, stroke, and cognitive decline, leading to the development of more effective interventions and treatment strategies.

1.8 Closing Remarks

In the hope of providing a comprehensive overview, this chapter has delved into the intricate mechanisms of atherosclerosis development and its contemporary treatment strategies. The focus has been on the profound impact of this disease on both heart and brain health, and the indispensable role of animal models in enhancing our understanding of this multifaceted condition. The challenges and opportunities that pervade this field underscore the pressing need for sustained research and innovation.

This chapter has been planned and written to instill a robust foundational understanding of atherosclerosis, with a particular emphasis on its manifestation within intracranial arteries. The exploration of this specific aspect is of great interest to myself and others in the field, given its significant implications for cognitive health and overall brain function.

Moreover, this chapter is meant to serve as a springboard for the forthcoming section of this dissertation, which will delve deeper into the development, regression, and potential

treatment of ICAS using an NHP model. The insights gleaned from this chapter should provide a solid foundation for understanding the subsequent research findings and discussions within this dissertation.

In essence, this chapter was not just meant to be an isolated discussion on atherosclerosis, but a steppingstone towards a more profound understanding of the challenges faced when studying atherosclerosis, particularly ICAS. It is a journey that takes us from the broad landscape of atherosclerosis to the specific nuances of ICAS in NHPs. As we navigate through this journey, it is hoped that the knowledge and insights gained will contribute significantly to the field of vascular biology and beyond, ultimately leading to improved strategies for the prevention, diagnosis, and treatment of atherosclerosis.

CHAPTER 2. UNDERSTANDING INTRACRANIAL ATHEROSCLEROSIS: INSIGHTS FROM AN NHP MODEL AND THE ROLE OF MIR-33 ANTAGONISM

2.1 Introduction

Atherosclerosis, a chronic inflammatory vascular disease characterized by the accumulation of lipids and fibrous elements in the large arteries, has become a significant global health crisis. Its profound impact on cardiovascular and cerebrovascular health, especially among the aging population, calls for innovative strategies in prevention and treatment. With atherosclerosis contributing to over half of all heart disease and stroke-related deaths, it stands as a major cause of mortality worldwide.[3] However, the detrimental effects of atherosclerosis extend beyond mortality, as it directly affects the quality of life in aging populations.

Atherosclerosis within the intracranial arteries has long been considered a hallmark of vascular cognitive impairment (VCI) and vascular dementia (VD).[180,181] As the global population ages, the prevalence of neurocognitive disorders associated with cerebrovascular diseases, including VCI and VD, is increasing, posing a significant public health challenge.[171,179,180,213] Vascular dementia is the second most common cause of dementia after Alzheimer's disease in many developed countries.[170] The cognitive decline resulting from these disorders has a profound impact on individuals' daily functioning, leading to emotional distress and economic strain for patients, families, and healthcare systems.[171] Urgent action is needed to develop novel strategies that can stabilize or reverse atherosclerosis, with the potential to reduce the incidence of death and neurocognitive disorders. Such approaches hold the promise of improving brain health and function, ultimately enhancing the overall quality of life for our aging population.

For half a century, the cornerstone of extracranial atherosclerosis (ECAS) and intracranial atherosclerosis (ICAS) management has revolved around the regulation of circulating cholesterol levels.[4,214]. The introduction of statins, potent inhibitors of cholesterol biosynthesis, transformed the landscape of cardiovascular and cerebrovascular disease management, leading to marked reductions in low-density lipoprotein cholesterol (LDL-C) levels and subsequently, a steady decline in atherosclerosis-related mortalities.[215], [52,184,216]. Despite these significant advancements, an element of residual risk continues to haunt the therapeutic scenario. This persistent risk is attributed to a spectrum of factors beyond LDL cholesterol, including inflammation, oxidative stress, and other lipid abnormalities, such as elevated triglyceride levels and low high-density lipoprotein (HDL) cholesterol levels.[19,32,59,66,72,80] Addressing this residual risk has become a paramount concern for researchers and clinicians worldwide, prompting the need for innovative therapeutic strategies that comprehensively address the complex pathophysiology of atherosclerosis.

In recent years, there has been increasing attention in the field of cardiovascular research towards the therapeutic potential of microRNAs (miRNAs), particularly the miR-33 family, in the context of atherosclerosis. [187,217] MicroRNAs are small non-coding RNA molecules that play a significant role in regulating gene expression post-transcriptionally. They achieve this by binding to the 3' untranslated regions of multiple messenger RNAs (mRNAs) involved in specific biological pathways, leading to the degradation of the target mRNA or the suppression of its translation.[218]

Among the multitude of identified microRNAs, miR-33a and miR-33b have drawn significant attention due to their profound influence on cellular lipid homeostasis.[191] These

microRNAs, embedded within the sterol regulator element-binding protein (SREBF 1/2) host genes, suppress the expression of numerous genes involved in fatty acid oxidation and cholesterol efflux.[189,190] This unique regulatory function places miR-33a and miR-33b at the intersection of lipid metabolism and inflammation, exerting a significant influence on the progression of atherosclerotic disease.

Earlier studies have provided compelling evidence that inhibition of miR-33 leads to enhanced macrophage cholesterol efflux, autophagy, and efferocytosis. [188,219] These cellular processes are believed to promote a shift in macrophage polarization towards an anti-inflammatory, pro-resolving phenotype, thereby modulating the inflammatory environment within atherosclerotic lesions. Building upon these findings, in vivo studies using mouse models of atherosclerosis have demonstrated that suppression of miR-33a expression results in a reduction in aortic atherosclerosis.[220] This beneficial outcome, attributed to the combination of improved macrophage function and a decreased inflammatory milieu, highlights the potential therapeutic value of miR-33 antagonism in the treatment of atherosclerosis.

It is important to note that humans and other primates express two isoforms of miR-33, miR-33a and miR-33b, unlike mice that only express miR-33a. This distinction is significant because therapeutic implications derived from models expressing only miR-33a, such as mice, may not fully translate to humans. However, preliminary investigations conducted in healthy African green monkeys, a non-human primate (NHP) species that naturally expresses both miR-33a and miR-33b, have yielded promising insights. In these studies, treatment with anti-miR-33 antisense oligonucleotides (ASOs) resulted in increased hepatic ABCA1 expression, circulating HDL-C levels, and HDL efflux capacity

in these NHPs.[221] These encouraging findings in a dual miR-33 isoform model provide a compelling basis for considering the potential therapeutic implications of miR-33 antagonism in human atherosclerotic lesions.

Based on the promising initial findings in healthy African green NHPs, we aimed to investigate the potential of miR-33a and miR-33b antagonism in regressing atherosclerotic lesions using a more susceptible NHP model of atherosclerosis. The cynomolgus monkey (Macaca fascicularis), an old-world primate, was chosen for its well-documented susceptibility to diet-induced extracranial atherosclerosis (ECAS), particularly in males.[212] This model closely recapitulates the pathogenesis of human ECAS, enhancing the potential translational relevance of our study findings.

To simulate the progression phase of ECAS, a total of 61 NHPs were subjected to a high fat/cholesterol diet for a period of 20 months, resulting in the development of hypercholesterolemia. Following this progression phase, we initiated a dietary shift to a standard NHP "chow" diet. Concurrently, the animals were divided into two groups and received either anti-miR-33 ASO or a saline vehicle for either 6 weeks or 24 weeks. This intervention aimed to correct hypercholesterolemia while investigating the potential benefits of miR-33 antagonism on the morphometric and compositional measures of atherosclerosis regression in this non-human primate model.

During our research on ECAS in this NHP model, we unexpectedly made a remarkable discovery: the presence of pronounced ICAS within the large intracranial vessels of the Circle of Willis (COW). While it is known that old world NHPs can develop measurable atherosclerosis within the COW under specific conditions, previous studies have not extensively characterized the progression of ICAS or examined the potential for

regression of established ICAS lesions.[209,222] This serendipitous finding presented us with a unique and invaluable opportunity: the chance to investigate whether a translational therapeutic approach using anti-miR-33 ASO could regress ICAS lesions. This unexpected dimension added significant importance and novelty to our atherosclerosis research, highlighting the critical role and potential impact of our study in addressing the challenging issue of ICAS.

By exploring the potential of anti-miR-33 ASO in stabilizing or regressing ICAS lesions, our study aims to fill the gap in knowledge regarding the therapeutic possibilities for this complex form of atherosclerosis. We hypothesized that by antagonizing miR-33, a pivotal regulator of lipid metabolism and inflammation, we could induce the regression of ICAS. Our hypothesis was based on the rationale that targeting miR-33 would result in measurable changes in vessel morphology and composition, ultimately providing novel therapeutic avenues for the management of this debilitating condition.

Our findings have far-reaching implications for understanding the complex pathophysiology of ICAS and provide a solid foundation for future investigations into innovative therapeutic strategies. By shedding light on the underlying mechanisms of ICAS and exploring novel treatment approaches, our study contributes to the advancement of medical knowledge and brings us closer to effective interventions for this challenging condition.

2.2 Methods

2.2.1 Animals and Treatment Groups

A total of 61 young adult male cynomolgus monkeys (Macaca fascicularis) were included in this nonhuman primate study. The monkeys were captive-bred and sourced from Mauritius, and at the start of the study, they had an average age of 5.2 years (range: 4.2-6.7). The NHPs were housed in an AAALAC-accredited facility under the direct care of the University of Kentucky (UK) Division of Laboratory Animal Resources (DLAR) and were maintained under climate-controlled conditions with a 12-hour light and dark cycle. Initially, the NHPs were fed a standard nonhuman primate diet (Teklad 2050) ad libitum. Subsequently, the NHPs were singly housed from approximately 08:00 to 15:00 each day and provided weighed portions of a semi-synthetic atherogenic diet. The atherogenic diet composition is provided in appendix **Table 1**. The average daily caloric intake from the atherogenic diet was 72 kcal/kg body weight. After 20 months on the atherogenic diet, the monkeys were switched back to the standard diet and treated for either 6 weeks or 24 weeks with either vehicle (USP grade saline) or miR-33a/b antagonist RG428651, a 2'-fluoro/methoxyethyl-modified, phosphonothioate (PS)-backbone-modified, antisense oligonucleotide (ASO).[221] The dosing regimen involved subcutaneous injections of the vehicle or 5 mg ASO/kg body weight. During the treatment period, the NHPs were singly housed from approximately 08:00 to 15:00 each day and received a standard nonhuman primate diet, providing an average of 64 kcal/kg body weight/day.

2.2.2 Plasma Lipid and Lipoprotein Cholesterol Concentrations

After an overnight fast, monkeys were sedated with ketamine (10 mg/kg, IM), body weights were recorded, and blood was collected from the femoral vein into EDTA-

containing or serum separation vacutainers. Plasma and serum were isolated by centrifugation at 1,500xg for 30 min at 4°C. Enzymatic assays were used to measure plasma total cholesterol (C7510, Pointe Scientific) and triglyceride (T2449 & F6428, Sigma). The plasma cholesterol distribution among lipoprotein classes was determined after separation by gel filtration chromatography based upon the method described previously[2]. An aliquot of plasma was diluted to 0.5 µg total cholesterol/µL in 0.9% NaCl, 0.05% EDTA/NaN_3 and centrifuged at 2000xg for 10 minutes to remove any particulate debris. The supernatant was transferred to a glass insert contained in a GC vial.

After loading the vial into an autosampler set at 4°C (Agilent Technologies, G1329A), 40 µL of sample was injected onto a Superose 6 10/300 or Superose 6 Increase 10/300 (GE Healthcare Life Sciences) chromatography column. Under the control of an isocratic pump (Agilent Technologies, G1310A/B), the sample was separated at a flow rate of 0.4 ml/min with eluent containing 0.9% NaCl, 0.05% EDTA/NaN3. The column effluent was mixed with total cholesterol enzymatic reagent (C7510, Pointe Scientific) running at a flow rate of 0.125 mL/min and the mixture was passed through a knitted reaction coil (EPOCOD, Aura Industries Inc.) in a 37°C H_2O jacket. The absorbance of the reaction mixture was read at 500 nm using a variable wavelength detector (Agilent Technologies, G1314F). The signal was subsequently integrated using Agilent OpenLAB Software Suite (Agilent Technologies). VLDL-C, LDL-C, and HDL-C concentrations were determined by multiplying the TPC concentration by the cholesterol percentage within the elution region for each lipoprotein class.

2.2.3 Tissue Collection and Preparation

At the end of the treatment period, the NHPs underwent specific procedures for tissue collection. The monkeys were fasted overnight and then sedated with ketamine (25 mg/kg, IM) and isoflurane (3-5% induction, 1-2% maintenance). To minimize potential post-mortem tissue damage caused by delay between brain and cerebrovascular tissue collection that was preceded by cardiovascular tissue collection, a cold pack was applied to the head of the animal. This approach aimed to induce cerebral hypothermia and lower the brain temperature, preserving tissue integrity and minimizing cellular degradation during subsequent brain tissue collection. Following collection of the cardiovascular tissue, the skin and muscle from the superior aspect of the skull was dissected to expose the calvarium from the orbits and ears to the atlantooccipital area. The calvarium was then carefully removed using a bone saw (Mopec 810 Autopsy Saw, 115 volts/60hz/IPH, with a standard Mopec blade part# BD101, Madison Heights, MI 48071). Caution was used to avoid damaging underlining parenchyma and full removal of the calvarium was aided with the use of a Virchow Skull Breaker (Mopec, Madison Heights, MI 48071). The falx cerebri, a portion of the dura that descends and extends down the longitudinal fissure between the cerebral hemispheres, was initially removed to prevent sheering or tissue damage upon removal of the brain. Following dissection of the falax cerebri, the cranial nerves and spinal cord were dissected proximal to floor of the cranial cavity exercising caution so as to not damage the brain or vascular tissue. The brain and cerebrovascular tissue were then immediately removed and submerged in a chilled saline solution for 10 minutes. This process was meant to firm the tissue and preserve its integrity for subsequent removal of the circle of Willis (COW) and sectioning of brain tissue.

The brain was then placed with its superior surface down into a chilled dish with chilled saline solution. The entire COW was carefully excised from the brain starting caudally at the vertebral arteries (VA) moving rostrally to the anterior cerebral artery (ACA). Once removed, the intact COW was placed in a 15 mL glass container filled with 10% neutral buffered formal (NBF) and stored at room temperature for later dissection. The brain was then placed with the superior surface down into a chilled stainless steel coronal slicing matrix (Ted Pella, INC., Redding, CA, 96049-2477) and dissected into thirteen 4 mm coronal sections using a modified tissue blade (Thomas Scientific, Cat # NC9436795). The coronal sections were then imaged for cataloging before being further dissected into left and right hemisphere at the longitudinal fissure. The left hemisphere was snap frozen using powdered dry ice, then wrapped in aluminum foil sachets before being stored at -80˚C for future study while sections from the right hemisphere were placed in 10% NBF for future histology studies.

Following fixation, the ACAs, internal carotid arteries (ICAs), medial cerebral artery (MCAs), posterior cerebral artery (PCAs), basilar artery (BA) and vertebral artery (VA) were dissected from the COW. The BA was further dissected into three sections BA1 (proximal to the VA), BA2 (a central section marked as waste) and BA3 (proximal to the PCA). All COW vessels were then marked with blue tissue marking dye (Mark-It™, ThermoFisher Scientific, Cat # 5000, Waltham, MA 02451) for tissue embedding orientation before being placed into nylon tissue bags (Fisherbrand™ Nylon Biopsy Bags, small, Cat # 15-182-500, Fisher Scientific) that were then secured in tissue processing cassettes (VWR, Histology Cassettes, VWR, Cat # 18000-244, Radnor, PA 19087-8660) and submerged into 10% neutral buffered formalin. The ACA, BA1, and BA3 were then

processed for tissue embedding at the University of Kentucky Pathology Research Core D (University of Kentucky, Lexington, KY 40536-0200). The embedded ACA, BA1, and BA3 were then cut using a microtome (Shandon Finesse, 77500102 Issue 9, Thermo Fisher Scientific, Waltham, MA, USA) into 5μm cross sections and mounted onto glass slides for chemical and immunohistochemical staining.

2.2.4 Histology for Morphometric Measurements and Collagen Composition

The paraffin-embedded ACA, BA1 and BA3 sections collected were first deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol before being subjected to routine histological staining procedures for morphological analysis and collagen composition. In brief:

1. Hematoxylin and Eosin (H&E) staining for visualization of cytoplasmic structures and the internal elastic lamina (IEL) for morphology measures: Slides were incubated with hematoxylin (Harris Hematoxylin, Cat# HHS16, Thermo Fisher Scientific, Waltham, MA, USA) for 7 minutes for nuclear staining, followed by counterstaining with eosin (Cat# E6003, Sigma-Aldrich, St. Louis, MO, USA) for 45 seconds before being rinsed, dehydrated through increasing concentrations, cleared in xylene and cover slipped in DPX (Cat# 13515, Electron Microscopy Sciences, Hatfield, PA, USA).

2. Picrosirius Red/Fast Green Staining (PSRFG) for visualization of collagen within the atherosclerotic lesion: Slides were stained with Weigert's hematoxylin (Thermo Fisher Scientific, Waltham, MA, USA) for 8 minutes, followed by incubation in a solution containing Fast Green FCF (F7258; Sigma-Aldrich, St. Louis, MO) and Sirius Red F3B (Cat #365548, Sigma-Aldrich, St. Louis, MO) in

saturated picric acid ((1.3% saturated Picric acid, Cat#: P6744, Sigma-Aldrich, St. Louis, MO) for 60 minutes at room temperature. After staining, the slides were rinsed with acidified water, dehydrated through increasing concentrations, cleared in xylene and cover slipped in DPX.

2.2.5 Immunohistochemistry for Vascular Smooth Muscle Cells and Inflammatory Macrophages

For immunohistochemical (IHC) analysis, the ACA, BA1 and BA3 sections were deparaffinized with xylene, and rehydrated with decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the slides in citrate buffer (pH 6.0) (Declere, Cell Marque, CA, USA, Cat# 921P-06) for 25 minutes, then cooled to 50°C.

Slides were incubated with 3% H2O2 in methanol for 5 minutes at room temperature, blocking endogenous peroxidase activity. Slides were then washed with TBST (Tris-buffered saline with 0.1% Tween 20) and blocked with 5% goat serum (Thermo Fisher Scientific, Waltham, MA, USA) in TBST for 1 hour at room temperature. Primary antibodies, including mouse anti-human alpha actin (1:500 in TBST; Biolegend, CA, USA, Cat # 904601) and mouse anti-human CD-68 antibody (1:500 in TBST; Biolegend, CA, USA, Cat# 916104) were incubated with the tissue sections overnight at 4°C.

Following overnight incubation with primary antibody slides were washed with TBST and then incubated with biotinylated secondary antibodies: goat anti-mouse IgG (H+L), 1:200 in TBST with 5% goat serum, Cat# BA9200; goat-anti-rabbit IgG (H+L), 1:200 in TBST with 5% goat serum, Cat# BA1000, Vector laboratory, Burlingame, CA) for 1 hour at room temperature before being washed and incubated with the ABC-HRP

(Avidin-Biotin Complex-Horseradish Peroxidase) amplification and detection system (Vecta stain Elite ABC-HRP [peroxidase, standard], Cat# PK-6100, Vector Laboratory, Burlingame, CA). The activity of horseradish peroxidase was visualized using the Nova red substrate kit (Cat# SK4800, Vector laboratory, Burlingame, CA, USA), resulting in red precipitate at the site of the antigen following approximately 1 minute of incubation. The Nova Red enzymatic reaction was halted in a PBS wash, counterstained with hematoxylin, dehydrated through increasing concentrations of ethanol, cleared in xylene, and cover slipped in DPX.

2.2.6 Imaging and Image Processing

Images of chemical and IHC stained ACA, BA1, and BA3 were collected using the Zeiss Axio Scan slide scanner at 20x magnification. To assess vessel morphology and atherosclerotic lesion collagen matrix and cellular composition we first established binary regions of interest (ROI) using Elements AR Image Analysis System (Nikon Instruments, Melville, NY).

All morphological area measurements were calculated using hand drawn binaries in Elements AR. Lumen area was set at the surface of the endothelial cell layer. The intima, or lesion area, was defined as the space between the internal elastic lamina (IEL) and endothelial surface area. Media area encompassed the entire tunica media, from the external elastic membrane to the IEL. Additional morphological calculations included percent stenosis, wall area, percent lesion, and total vessel area as follows:

1. Percent stenosis: (intima area / (lumen area + intima area)) x 100
2. Wall area: (intima area + media area)
3. Percent lesion: (intima area / wall area) x 100

4. Vessel area: (lumen area + intima area + media area)

Binary thresholds for positive stained collagen, CD-68 positive cells (macrophages), and alpha-actin positive cells (vascular smooth muscle cells) were set on a subset of images (20 slides per stain) to train a working threshold. All quantification was then performed using batch analysis on each category of staining, ensuring a blinded and standardized approach using Elements AR General Analysis 3 package (GA3, Nikon Instruments, Melville, NY). Total positive binary area measurements from positive thresholding were then used to calculate percent collagen, CD-68, and alpha-actin positive matrix and cellularity within the lesion.

2.2.7 Cholesterol Measurements in the External Carotid Arteries

Frozen left external carotid artery (ECA) was ground to powder using a Freezer/Mill® Cryogenic Grinder (SPEX, Model # 6775, Metuchen, NJ 08840). The conditions used for grinding were 1 minute precool, run time 30 seconds, cool time 1 minute, cycle 3, rate 15 cps. A portion of the ECA powder (15-20 mg) was transferred into a tared 16x100mm glass screw top tube containing 50 µg of 5-alpha cholestane (Steraloids, C3300-000), and the tube was weighed using an analytical balance to determine the exact wet weight of the ECA powder. To extract total lipids from the ECA powder, 2 ml 2:1 chloroform: methanol was added to the tube, which was then sealed with a Teflon-lined cap and incubated at 60˚C for 3 hours. Using a glass Pasteur pipet, the lipid extract was transferred to a new 16x100mm glass screw top tube along with two subsequent 1 ml 2:1 chloroform: methanol washes of the extracted ECA powder. After evaporation of the solvent at 55˚C under a stream of nitrogen gas, the lipid was dissolved in 4 ml 2:1 chloroform: methanol and 0.8 ml 5M NaCl was then added. The tube was sealed with a

Teflon-lined cap, vortexed for 20 seconds, and centrifuged at room temperature at 1500xg for 10 minutes. After aspirating the upper, polar phase, ~4 ml of the lower, nonpolar, lipid-containing phase was transferred using a glass Pasteur pipet to a new 16x100mm glass screw top tube. After evaporation of the solvent as described above, the lipid was dissolved in 200 µL hexane and transferred to a 12x32 mm gas liquid chromatography vial (Sun Sri #500300) containing a 350 µL cylinder insert (Thermo Fisher #200668). The extracted lipid was analyzed for free cholesterol by injecting 1 µL of sample onto a 250˚C ZB-50 gas-liquid chromatography column (0.53 mm inner diameter × 15 m × 1 µm, Phenomenex, Part # 7HM-G004-17, Torrance, CA 90501-1430), which was installed in an Agilent Technologies 7890B gas chromatograph equipped with an Agilent Technologies 7693 autosampler using on-column injection and a flame ionization detector. The amount of free cholesterol in the sample was calculated using the following equation:

µg cholesterol = AUC cholesterol/AUC 5-alpha cholestane x 50 µg 5-alpha cholestane/0.86

Where 0.86 equals the determined relative response factor of cholesterol vs 5-alpha cholestane.

The lipid-containing hexane was returned to a 16x100mm glass screw top tube and the hexane was evaporated as described above. To saponify the lipids, 2 ml 100% ethanol and 0.2 ml 50% KOH was added to the tube, which was sealed with a Teflon-lined cap, vortexed for 20 seconds, and incubated at 60□C for 3 hours. After allowing the tube to cool to room temperature, 2 ml hexane and then 2 ml water were added to the tube with 20 seconds of vortexing between additions. The polar and nonpolar phases were separated by centrifuging the tube at room temperature at 1500xg for 10 minutes. The upper hexane

phase was transferred to a clean glass tube, the hexane was evaporated as described above, and the non-saponifiable lipid was dissolved in 500 μL hexane. The hexane was transferred to a 12x32 mm gas liquid chromatography vial and total cholesterol was analyzed by gas chromatography as described above. The amount of total cholesterol in the sample was calculated using the same equation for determining free cholesterol. Esterified cholesterol was calculated as the difference between total cholesterol and free cholesterol.

2.2.8 Bulk RNA Sequencing of External Carotid Arteries

Carotid arteries were pulverized as described above in the carotid cholesterol analysis section. Following the manufacturer's instructions, RNAzol RT (#RN190, Molecular Research Center, Cincinnati, OH) was used to extract RNA from 50-80 mg of fine carotid powder from 7-8 randomly selected animals per treatment group. RNA quality and concentration were assessed by the University of Kentucky Genomics Core Laboratory using an Agilent 4150 TapeStation. RNA samples (n=7 for 6wk vehicle, 24wk vehicle, 6wk anti-miR-33 and n=8 for 24wk anti-miR-33) that had the required quality and quantity were then shipped to Novogene (CA) for bulk mRNA sequencing. cDNA library was generated from total mRNA (1 μg) using NEBNext UltraTM RNA Library Prep Kits for Illumina (New England BioLabs). cDNA libraries were sequenced by NovaSeq 6000 (Illumina) in a paired-end fashion to reach more than 1.5M reads. Reads were removed with adapter contamination, when uncertain nucleotides constituted more than 10 per cent of either read (N > 10%), and when low-quality nucleotides (Base Quality less than 5) constituted more than 50 per cent of the read. Paired-end reads were mapped to the cynomolgus reference genome (Macaca_fascicularis_5.0) using HISAT2 (v2.0.5) and quantified using FeatureCounts (v1.5.0-p3).

2.2.9 Data Analysis

Data will be presented as either mean ± standard deviation (SD) or median with interquartile range (IQR) and will be noted as so in the text. Statistical analysis was performed using GraphPad Prisim analysis software program (GraphPad Software, Boston, MA 02110). Plasma lipids, vessel morphology, lesion composition, and carotid cholesterol levels were first tested for normality using Shapiro-Wilk test (SWT) then analyzed by either ordinary one-way analysis of variance (ANOVA) with post-hoc Tukey's test or Kruskal-Wallis test (KWT) with post-hoc Dunn's test, dependent on results of SWT. Statistical significance was assigned when the *P*-value was <0.05. Bulk RNA sequencing data were analyzed using "edgeR" (v3.36.0) Bioconductor package on R (v4.1.0) with R studio (2022.12.0). Dispersion parameter estimates and normalization factor calculation using the trimmed mean of M-value (TMM) method were performed. FDR-adjusted P<0.05 was considered statistically significant.

2.3 Results

2.3.1 The Model, the Treatment Groups, and the Plasma Lipid Response Between Groups

In this multifaceted study, sixty-one male cynomolgus monkeys (Macaca fasicularis) were fed a high fat/high cholesterol diet (38% calories from fat, 0.4% cholesterol w/w) for 20 months to induce hypercholesterolemia and atherosclerosis in both extra- and intracranial arteries. After 20 months on atherogenic diet, a fifth of the animals (n = 12) were euthanized and designated as the progression group. The remaining animals (n = 49) entered the regression phase, during which they were fed a standard NHP diet (14% calories from fat) to reduce plasma lipids, and were divided into four treatment

groups. Approximately half of the animals entered a 6-week regression arm, where they received weekly subcutaneous injections of either saline vehicle (n = 13) or 5 mg/kg of the miR-33 targeting ASO RG428651 (n = 12). The rest of the animals (n = 24) were part of a 24-week regression group, receiving either the saline vehicle (n = 12) or the anti-miR-33 ASO treatment (n = 12) as described for the 6-week regression groups. Figure 1 provides a graphical representation of the experimental design.

Blood plasma from several time points was collected and analyzed to determine a general lipid profile that included total cholesterol (TC), very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), [Figure 2]. For reference, the median baseline lipid profile for all animals (n = 61) on standard NHP diet at the start of the study was as follows: TPC = 85 mg/dL, VLDL-C = 2.1 mg/dL, LDL-C = 30 mg/dL, HDL-C = 54 mg/dL, and TG = 37 mg/dL.

2.3.2 Frequency and Spatial Distribution of Atherosclerotic Lesion within the Circle of Willis (COW)

The COW [Figure 3b] of each animal was grossly inspected at necropsy and later inspected histologically for the presence or absence of atherosclerotic lesions. Observations of one or more lesions within the COW classified the animal as being a lesion animal. For simplicity we will refer to lesion animals and normal animals as simply ‘lesion’ or ‘normal’.

Gross and histological inspection of the COW revealed one or more lesions among: 10 animals within the progression group (n = 12; 83% with lesion), 11 animals within the 6 wk vehicle group (n = 13; 85% with lesion), 8 animals within the 6wk anti-miR-33 group

(*n* = 12; 67% with lesion), 11 animals within the 24wk vehicle group (*n* = 12; 91% with lesion), and 7 animals within the 24wk anti-miR-33 group (*n* = 12; 58% with lesion). Total lesion animals across all groups were 47 (*n* = 61; 77% with one or more lesions). A graphical representation of the frequency distribution of the 61 animals across their perspective treatment groups can be observed in Figure 3a. There was no significant difference observed in lesion frequency across the animal groups.

Vessel segments from the COW that were found to have the greatest frequency of lesions were the anterior cerebral artery (ACA) and the basilar artery (BA) Figure 3c. All animals presented with only a single distal ACA, azygous presentation of the ACA is common among cynomolgus monkeys[223] and can be observed in Figure 3c (red dot). The single distal ACA was dissected proximal to bifurcation at the left and right ACA branches. Given the extended length of the BA it was dissected into 3 segments, and only segments BA1 and BA3 were analyzed due to their frequent appearance of lesions at their point of bifurcation with the posterior cerebral artery (PCA) and with vertebral artery (VA) respectively.

A total of 180 vessel segments (*n* = 61 ACA, *n* = 60 BA1, and *n* = 59 BA3) were ultimately selected for morphometry and compositional measurements [Figure 3d]. A total of 87 vessel segments (48% of total vessel segments) were found to have lesions (*n* = 24 ACA; 39%, *n* = 31 BA1; 52%, and *n* = 32 BA3; 54%). A greater frequency of lesions was observed across the BA segments [Figure 3d], while the most occlusive or stenotic lesions with decreased lumen area were generally observed across the ACA segments [Figure 3e].

2.3.3 Morphometric Analysis of ACA, BA1, and BA3 Vessel Segments Independent of Lesion

Following gross observations, the ACAs (n =61), BA1s (n = 60) and BA3s (n = 59) were embedded (FFPE), sectioned (5µm), stained (HE), and imaged. Binarized ROIs were set labeling the lumen, intima, and media for area measurements. The lumen [Figure 6b, binary ROI in Blue] was defined at the level of the endothelial layer inward. The intima [Figure 7a, binary ROI in yellow] was defined to be between the internal elastic lamina (IEL) and the endothelial cell (EC) layer. The media [Figure 8b, binary ROI in red] was defined as being between adventitia and IEL.

Raw morphological area measurements including lumen area [Figure 4a-c], intima area [Figure 4d-f.], media area [Figure 4g-i], and vessel area [Figure 4j-l] were collected inside Nikon Elements AR software. We then used raw area data to calculate additional morphology measures including percent stenosis [Figure 5a-c], percent lesion [Figure 5d-f], and wall area [Figure 5g-i]. The calculations for percent stenosis, percent lesion, and wall area can be found within the methods section. Statistical analysis using KWT and Dunn's test for multiple comparison was performed on all raw and calculated morphometric data using GraphPad Prism.

Initially each vessel segment (ACA, BA1, BA3) independent of the presence or absence of a lesion was analyzed between treatment groups. The only statistically significant difference for the morphological measures was a decrease in total vessel area of the ACA for the 24wk anti-miR-33 versus progressions group [Figure 4j]. While not backed by statistical analysis, some trends were observed in the data. The ACAs from the progression group had among the smallest lumen areas [Figure 4a] while also having the

greatest percent stenosis [Figure 5a] when compared to other vessel segments and treatment groups. The ACAs from the progression group also were observed to have a greater median intimal area [Figure 4d] and percent lesion when compared to BA segments and other treatment groups. The BA1 segments compared to all treatment groups and vessel segments had the largest median media area [Figure 4g-i]. Total median vessel area [Figure 4j-l] and wall area [Figure 5g-i] from both BA1 and BA3 vessel segments, independent of treatment groups, was larger than those of ACA segments.

2.3.4 Morphometric Analysis of Vessels Only from Animals with Lesions

A majority of animals were identified as having a lesion across one or more of the vessel segments (47 of 61 animals with lesions; 77% total animals) [Figure 3a]. If an animal did not present with a lesion across one of the three vessel segments, then all their vessel segments were removed from further analysis (n = 14 animals free of lesion; n = 42 vessel segments removed from analysis). For clarity, only the vessels from those animals with lesions were analyzed (n = 3 vessel segments from each lesion animal) for both morphometric and compositional measures.

Figures 6 thru 8 represent the three independent vessel morphometry measures from each lesion animal and therefore the sample counts for each of the treatment groups is as follows: progression (n = 30), 6wk vehicle (n = 33), 6wk anti-miR-33 (n = 24), 24wk vehicle (n = 33), and 24wk anti-miR-33 (n = 21). The median lumen area of the progression group was larger than the other treatment groups with notable significance (p = 0.034*) between the progression and 6wk anti-miR-33 groups [Figure 6a]. Conversely to a larger lumen area, the progression group also tended to have a higher median percent stenosis when compared to the other treatment groups. Stenosis was highly variable among vessels,

representative degrees of stenosis (25%, 50%, 75%, 100%) can be observed in Figure 6d-g. A total of 7 animals had >98% stenosis in their ACAs (n = 5 from progression, n = 1 from 6wk vehicle, and n = 1 from 24wk vehicle) [Figure 6c]. There were no significant differences for percent stenosis across the treatment groups.

The median intima area in the progression group tended to be greatest amongst the groups and was significantly larger compared to the 24wk vehicle ($p = 0.0128$) and 24wk anti-miR-33 ($p = 0.0031$) groups [Figure 7a]. No significant differences were observed between groups when percent wall is intima was calculated. Instead, we observed a similar trend in percent wall is intima as we did with intima area, with progression tending to be higher than the other treatment groups [Figure 7c]. Examples of percent wall is intima for lesion containing vessels are presented in Figure 7e-f.

The progression group's median vessel area was significantly ($p = 0.0358$) larger than that of the 24wk vehicle treated group [Figure 8e] but there were no significant differences across groups in median media area [Figure 8a] and median wall area [Figure 8c]. The median wall area in the progression and 6wk vehicle groups tended to be slightly larger than that observed for the 6wk vehicle and both 24wk regression groups [Figure 8c]. Similarly, the progression and 6wk vehicle groups tended to have a slightly higher median vessel area when compared to remaining groups [Figure 8e].

2.3.5 Compositional Analysis of Vessels Only from Animals with Lesions

Figures 9 thru 11 represent the three independent vessel measures for lesion composition from each lesion animal and therefore the sample counts for each of the treatment groups is as follows: progression ($n = 30$), 6wk vehicle (n = 33), 6wk anti-miR-

33 (n = 24), 24wk vehicle (n = 33), and 24wk anti-miR-33 (n = 21). Lesions features such as foam cells, lipid pools, cholesterol crystals and clefts, necrotic cores, and inter plaque hemorrhages could be observed under routine HE histology [Figure 7d-f]. Lesion composition measure however were split into 3 domains using additional approaches to staining: collagen content as measured by Picro sirius red/fast green (PSRFG) chemical staining [Figure 9b], vascular smooth muscle cell (VSMC) migration/proliferation as measured by α-actin IHC [Figure 10b], and macrophage (0M) infiltration as measured by CD-68 IHC [Figure 11b]. Of importance, all lesion compositional measures were performed only within the intimal area [Figure 7b, ROI/binarization of intima in yellow].

To determine the amount of positive staining within the lesion, ROI binarization of the lumen, intima, and media was first performed within Nikon Elements AR. A full description of image processing and analysis can be found in the methods section. In brief, the image pre-processing pipeline involved white balance and automated contrast being applied before image analysis was conducted. A subset of pre-processed images was chosen (~25 tissue sections for each stain) for threshold training within Nikon Elements AR. Initial thresholds were set across training slides for each stain then tested manually against another set of slides (~15 slides for each stain). If needed, refinements were made to training threshold before setting up images from each stain for automated image analysis and quantification of PSRFG, α-actin, and CD68.

We observed positive PSRFG staining within all lesions and even small traces of positive staining within the intimal space of lesion free vessels. Figure 9b-e illustrates a vessel with positive PSRFG staining within the lesion as well as the binarization and positive labeling of PSRFG for collagen. The median positive PSRFG area was

significantly greater in the progression group compared to the 6wk vehicle ($p = 0.0052$) and 24wk anti-miR-33 ($p = 0.0053$) groups [Figure 9a]. The collagen positive area also tended to be larger in the progression group compared to the other regression groups. When percent positive PSRFG was calculated, there were no significant differences between groups, but this parameter for both the 6wk anti-miR-33 group and 24wk vehicle group tended to be greater than the other groups [Figure 9f]. Figure 9g-j depicts four lesion vessels and their relative distribution of percent positive PSRFG. Figure 9g shows the ACA of an animal with a full occlusion, note the size of the lesion and lack of PSRFG positive red staining underline that lesion size (raw intima area) was not always an accurate predictor of increased positive PSRFG staining. Indeed, the percent positive PSRFG staining across lesion animals was highly variable.

Similar to PSRFG, we observed positive α-actin staining within the intima of all vessels, normal and lesion. Figure 10b-e illustrates a lesion vessel with positive α-actin staining within the intima as well as the binarization and positive labeling of α-actin for detection of VSMC in the lesion. The median positive α-actin area was significantly greater in the progression group compared to that observed among the 6wk anti-miR-33 ($p = 0.0487$), 24wk vehicle ($p = 0.0012$), and 24wk anti-miR-33 groups ($p < 0.0003$) [Figure 10a]. In contrast, percent positive α-actin was not significantly different between groups, although there was a trend towards a stepwise reduction from progression to 6 wk regression to 24 wk regression [Figure 10f]. Figure 10g-j depicts four lesion vessels and their relative distribution of percent positive α-actin staining. Figure 10g captures the ACA of animal with a near full occlusion but only 6% positive actin staining that appears

proximal to the lumen whereas a smaller eccentric lesion is observed in a BA1 segment [Figure 10i] but contains a much larger percentage of positive α-actin staining (43%).

CD68 positive staining for macrophages was highly variable in the ACA, BA1, and BA3 lesions [Figure 11g-j] and was completely absent within the intimal space of normal vessels. Figure 11b-e illustrates a lesion vessel with positive CD68 staining within the intima as well as the binarization of the staining. There was additional background from the CD68 antibody in large occlusive lesions such as those in Figure 11b and Figure 11i. Positive CD68 staining was highly variable among the progression, 6wk vehicle, and 6wk anti-miR-33 groups [Figure 11a]. The median positive CD68 area was significantly greater for the progression group compared to the 24wk vehicle ($p = 0.0011$) and 24wk anti-miR-33 groups ($p = 0.0008$) [Figure 11a]. Unlike PSRFG and α-actin staining, percent positive CD68 area showed significance differences across several groups: progression/24wk vehicle ($p = 0.012$), progression/24wk anti-miR-33 ($p = 0.075$), 6wk vehicle/24wk vehicle ($p < 0.0089$), and 6wk vehicle/24wk anti-miR-33 ($p < 0.0063$) [Figure 11f].

2.3.6 Atherosclerosis in External Carotid Artery Assessed by Cholesterol Content

To assess atherosclerosis in an extracranial artery that can directly impact blood flow to the brain, cholesterol levels in the external left carotid artery (CA) were measured by GC following lipid extraction from the tissue [Figure 12a]. Esterified cholesterol (EC) and free cholesterol (FC) are established markers of atherosclerosis since these lipids accumulate at high levels in foam cells and necrotic cores of lesions.

The EC in the progression group was significantly ($p < 0.0001$) higher than the 24wk vehicle and 24wk anti-miR-33 groups [Figure 12h]. Additionally, we observed

significantly higher EC in the 6wk vehicle group when compared to the 24wk vehicle and 24wk anti-miR-33 groups ($p < 0.05$) [Figure 12h]. Similarly, EC in the 6wk anti-miR-33 group was significantly higher ($p < 0.05$) when compared to the 24wk vehicle and 24wk anti-miR-33 groups [Figure 12h].

The FC in the progression group was significantly ($p < 0.0001$) higher than the 6wk vehicle, 24wk vehicle and 24wk anti-miR-33 groups [Figure 12i]. Additionally, we observed significantly higher FC in the 6wk vehicle group when compared to the 24wk vehicle group ($p < 0.05$) [Figure 12i]. Similarly, FC in the 6wk anti-miR-33 group was significantly higher ($p < 0.05$) when compared to both the 24wk vehicle and 24wk anti-miR-33 groups [Figure 12i].

In summary, transitioning animals from an atherogenic diet (promoting progression of the disease) to a standard diet (promoting regression of hypercholesterolemia) over periods of 6 weeks or 24 weeks resulted in a progressive decrease in EC or FC in the CA. However, no significant differences in these lipids were discernible between the group treated with the vehicle and the group receiving anti-miR-33 treatment, regardless of whether atherosclerosis regression was evaluated at the 6-week or 24-week mark.

2.3.7 Analysis of miR-33 Target Gene Expression in External Carotid Artery

Because there were no significant differences in ICAS and ECAS between the anti-miR-33 and vehicle treated groups, RNAseq was used to assess in the left CA the expression of experimentally verified miR-33 targets (Table X). This analysis was limited to comparison between the vehicle and anti-miR-33 groups at either 6wk or 24 wk treatment. Based upon the trimmed mean of M-values (TMM) [Figure 11a-b], the miR-33

targets had a broad expression range in the CA. Based upon fold change (log2), anti-miR-33 treatment for 6wk or 24 wk appeared to derepress the expression of few of the miR-33 targets [Figure 11c-d]. Importantly, when considering false discovery rate (FDR), no significant differences in the expression of miR-33 targets was found between the anti-miR-33 and vehicle groups [Figure 11e-f].

2.4 Discussion

In this study, we leveraged a cohort of 61 cynomolgus monkeys with diet-induced atherosclerosis to evaluate the influence of miR-33 antagonism on the regression of ICAS lesions. Upon necropsy, we found that 77% of animals developed ICAS, though the prevalence of lesions did not significantly vary across different treatment groups. Furthermore, when assessing various morphological metrics, no significant differences were found between the vehicle and anti-miR-33 groups, regardless of whether the assessment was conducted at the 6 or 24-week mark.

The dichotomy between animals with and without lesions was considered, but even when only vessels from animals with lesions were analyzed for parameters such as lumen area, stenosis, intima area, wall is intima, media, wall area, and vessel area, the findings remained unchanged. Likewise, we detected no significant variations concerning intimal collagen, vascular smooth muscle cells (VSMC), or macrophage content between the vehicle and anti-miR-33 treatment groups at both 6 and 24-week intervals.

These consistent observations extended to the analysis of external CA tissues where EC, FC, or miR-33 target genes showed no significant shifts between the vehicle and anti-miR-33 groups. Collectively, our findings suggest a lack of treatment effect on vessel

morphology, composition, cholesterol content, or miR-33 target genes. As such, the null hypothesis remains unchallenged by our data.

The NHP model demonstrates remarkable similarities to humans in terms of diet-induced atherosclerosis, lipid metabolism, and cardio/cerebrovascular physiology.[212] Therefore, the NHP serves as an exceptional model for studying atherosclerosis. A notable advantage of this model is its ability to develop atherosclerotic lesions within intracranial arteries, providing a unique opportunity to explore the pathology of ICAS progression. Additionally, these conditions allowed for testing and monitoring the efficacy of miR-33 antagonism aimed at promoting the stabilization and/or regression of ICAS under controlled experimental conditions.

To induce hypercholesterolemia, all animals were fed a high-fat (38% fat) and high-cholesterol (0.4% w/w) diet for a duration of 20 months, resulting in elevated cholesterol levels (median TC = 562 mg/dL). The majority of this cholesterol originated from LDL-C (368 mg/dL), which is known to play a causal role in the development of atherosclerotic lesions.[224] These levels of hypercholesterolemia closely resemble those observed in humans with untreated heterozygous familial hypercholesterolemia (FH), where TC ranges from 350 to 550 mg/dL and LDL-C exceeds 160 mg/dL.[225] Such elevated cholesterol levels are strongly associated with an increased risk of coronary artery disease and stroke.[215] Notably, FH patients often exhibit cutaneous bumps or lumps called xanthomas, which are visible manifestations of extreme hypercholesterolemia. Xanthomas form as a result of excess cholesterol deposition over tendons or beneath the skin.[226] Perhaps not surprisingly, at the conclusion of the progression arm, we observed multiple cutaneous xanthomas in the vast majority of animals (data not shown). The elevated plasma TC, LDL-C, and the

presence of cutaneous xanthomas indicated that our animals exhibited a phenotype resembling heterozygous FH.

Based on this observation, we hypothesized that our NHPs fed atherogenic diet for 20 months would have both ECAS and ICAS. As anticipated, we observed extracranial atherosclerotic lesions within the coronary arteries of all animals and intracranial atherosclerosis in the COW of 47 out of the 61 animals (77%). To the best of our knowledge, there is no existing study that has demonstrated diet-induced intracranial atherosclerosis across such a large number of animals. In fact, only a few reports have mentioned diet-induced intracranial atherosclerosis in non-human primates (NHPs), and those studies often focused on surgical models with hypertension or brain ischemia.[209,227] The presence of ICAS in our study underscores the invaluable role of NHPs as study models, given the significant contribution of intracranial atherosclerosis to ischemic stroke and vascular cognitive impairment (VCI) in humans.[142,180]

One of the key genes targeted by miR-33 is ATP-binding cassette transporter A1 (ABCA1), which plays a crucial role in HDL formation and reverse cholesterol transport (RCT).[189] ABCA1 facilitates the export of cholesterol and phospholipids from cells to lipid poor apolipoproteins such as apoAI and apoE. Additional cholesterol is then collected by the HDL and is transported to the liver for excretion.[220] By repressing ABCA1, miR-33 reduces the amount of cholesterol that can be effluxed from cells, resulting in a decrease in HDL-C levels. Therefore, part of our hypothesis was that antagonizing miR-33 would alleviate its repression on ABCA1, enhancing cholesterol efflux and leading to an increase in HDL-C levels. Indeed, our findings validated this hypothesis, as we observed significant increases in HDL-C among the groups treated with anti-miR-33 compared to those treated

with a saline vehicle (Table 1). These results are in line with previous studies in NHPs, supporting the role of miR-33 antagonism in elevating HDL-C levels.[221,228]

The COW is a crucial arterial anastomotic system located at the base of the brain. It serves as a safeguard, providing collateral blood flow (CBF) and ensuring continuous cerebral perfusion, especially during instances of arterial occlusion.[229] This ring-like structure was initially described by the 17th-century British physician Thomas Willis.[230] The paramount importance of the COW in maintaining cerebrovascular health cannot be overstated.

However, the COW is susceptible to the effects of atherosclerosis. Atherosclerotic vascular remodeling leads to stenosis, hindering CBF and challenging the compensatory capabilities of the COW under ischemic conditions. This can result in severe clinical manifestations, such as ischemic stroke or vascular cognitive impairment (VCI).[231] Recognizing the impact of atherosclerosis in the COW on cerebrovascular disease and brain health is crucial for the development of diagnostic and therapeutic strategies.

Investigating the COW ICAS poses historical challenges due to its vulnerable spatial location within the cranial vault. Conventional imaging modalities, although effective in measuring stenosis, often lack the necessary high-resolution images required for a detailed evaluation of atherosclerosis to assess the efficacy of therapeutic strategies aimed at stabilizing or regressing ICAS.[232] Furthermore, while some imaging modalities offer better resolution, they may be considered invasive, carrying inherent risks and ethical considerations.[232]

One of the challenges in investigating ICAS within the COW is the limited availability of animal models that spontaneously develop COW ICAS or exhibit it under dietary conditions. Mouse and Rat models, anatomically lacking the complexity of the human COW, do not naturally nor experimentally develop ICAS in the COW, limiting their suitability for ICAS research.[233] NHPs have been suggested as a more suitable model due to their anatomical similarities to humans in terms of the COW structure and potential for ICAS development.[234] However, few studies have successfully replicated ICAS in the COW solely through non-invasive means, with most reports relying on surgical interventions to simulate an occlusive atherosclerotic lesion and subsequent ischemic events.[209]

In our study, gross observations of atherosclerotic lesions were made in over half of the animals (77%) during necropsy, providing us with ample vascular tissue for investigation. The majority of gross atherosclerotic lesions were observed in the ACA and BA segments of the COW, with less frequent observations in the middle cerebral artery (MCA) and internal carotid artery (ICA) vessels. These findings somewhat differ from observations in humans, where occlusive ICAS is predominantly seen in the MCA, BA, and ICA segments, with less involvement of the ACA.[235]

Interestingly, our histological examinations revealed that the ACA segment exhibited the most occlusive and complex lesions. This discrepancy may be attributed to slight anatomical differences between humans and NHPs in the ACA structure. Humans have two distal ACAs joined by a communicating arterial segment, whereas NHPs have a single distal ACA.[223] This anatomical variation of lacking two distal ACAs may have broader implications in ICAS, potentially affecting the regulation of CBF due to the

absence of collateral blood flow facilitated by parallel distal ACAs. Additionally, the occurrence of ACA lesions among our animals was most frequent at the point where the proximal right and left ACAs converge into the distal ACA, the bifurcation site known for early complex lesion development associated with shear stress mechanisms.[46]

While the ACA exhibited the most occlusive and complex lesions, the highest frequency of lesions was found in the BA segment proximal to the point of bifurcation with the posterior cerebral artery (PCA) and vertebral artery (VA) segments. Given the length of the BA, we further divided it into BA1, BA2, and BA3 sections. The BA1 and BA3 sections were then chosen to assess the lesions frequently observed proximal to the two bifurcation points. Unlike the large occlusive concentric lesions observed in the ACA, the BA segments mostly presented with eccentric lesions. The concentricity or eccentricity of atherosclerotic lesions have been associated with varying risks for plaque stability and rupture. Concentric lesions are thought to be more stable but more occlusive, while eccentric plaques are thought to be less stable and more vulnerable to rupture, particularly around the shoulder region in thin cap fibroatheromas (TCFA).[122] However, we did not observe TCFA in any of the BA or ACA segments.

Morphometric measurements of the ACA and BA segments revealed a notable trend: the BA segments (BA1/BA3) tended to have larger vessel/wall areas compared to the ACA [Figure 4J-l and Figure 5g-i]. This difference in size between the anterior (ACA) and posterior (BA) segments of the COW has also been observed in humans.[236] However, it is important to note that within the BA segments, the BA3 segment exhibited a smaller media compared to both the ACA and BA1 segments. This difference in vessel size (vessel/wall area) is likely attributed to BA3 segments having a slightly larger lumen area

than those of the BA1 [Figure 4b-c], as the overall vessel area is calculated by summing the media, intima, and lumen areas together.

Overall, there were no significant changes in raw area measurements (lumen, intima, media) when comparing treatment groups. It is important to note that these initial morphometric measures aimed to determine the heterogeneity between treatment groups, independent of the presence or absence of atherosclerotic lesions. Therefore, the variability observed in Figure 4 and Figure 5 is likely influenced by lesion vessels, which may skew the median area of the collective vessel segments. This should be considered when interpreting these figures. Thus, while ACA vessel area was significantly higher in the progression compared the 24wk anti-miR-33-treated animals, this observation may be skewed due to the mixture of lesion and non-lesion vessels in the analysis.

To address the aforementioned issue, we categorized the vessels into two groups: those with lesions and those without lesions. Lesion vessels were identified by an expanded intimal space, presence of cellular bodies, accumulation of lipid, and positive staining for PSRFG, α-actin, or CD68. Normal vessels, on the other hand, showed no signs of intimal expansion, cellular bodies, or lipid accumulation within the intima. It is worth noting that even some normal vessels exhibited small amounts of positive PSRFG or α-actin within the intima. In such cases, the presence or absence of actual cellular bodies was used to confirm the absence of a lesion.

After separating the animals with lesions vessels from the animals without lesions, we conducted the same morphometric measurements, this time only on lesion animals. With the exception of raw media area, all raw and calculated morphometric measures tended to be highest in the progression group [Figures 6-8]. The vessels from the

progression animals were significantly larger than those in the 24wk anti-miR-33 treated group. However, lumen area does not necessarily correlate to lumen occlusion. Therefore, we calculated a percent stenosis score, and the significance between the progression and 24wk anti-miR-33 groups was lost after this calculation, instead the median percent stenosis between groups became more similar [Figure 6c].

Since stenosis often results from atherosclerotic lesions obstructing the luminal space, we shifted our focus to assessing the intima area of vessels across the different treatment groups. Similar to our findings in luminal area measurements, we observed the largest intima to be within the progression group, which were significantly higher than those in both 24wk regression groups. The significance between these groups was lost when we calculated the percent wall is intima, although the trend in the progression group being higher than the other sets persisted. It is worth noting that the progression group showed a wider spread of sample data, as evidenced by the distribution of points being distant from the median in the violin plots of Figure 7. This spread could be attributed to the large number of occlusive ACA vessels in the progression group. Large ACA occlusions (as observed in Figures 6g, Figure 7f, and Figure 9g) were most frequently observed in the progression vessels and accounted for the 7 data points in the fourth quartile of the progression group, as seen in the violin plots in Figure 7.

Additional morphometric measures did not reveal much beyond significantly larger median vessel areas in the progression set compared to the 24wk vehicle-treated set. Collectively, the morphometric data indicated that larger and more stenotic lesions were most frequently observed in the progression set of vessels compared to the other treatment sets. However, there was no significant difference in the median morphometric data

measured between the 6wk and 24wk treatment groups. Moreover, there appeared to be no significant difference in median raw area and calculated measures between the vehicle and anti-miR-33 groups. This suggests that miR-33 antagonism did not have a treatment effect on morphometric measure of lesion vessels.

By targeting ABCA1, the autophagy pathway, and other proteins involved in intracellular cholesterol trafficking, miR-33 in macrophage foam cells can have the adverse effect of inhibiting cholesterol efflux [Ouimet, M., 2017, ATVB]. Moreover, during the progression of atherosclerosis, activated lipid laden macrophages have been shown to polarize towards a proinflammatory phenotype contributing to further dysfunction and formation of the necrotic core.[188] Conversely, when miR-33 is inhibited, macrophages can efficiently offload excess cholesterol onto HDL, resulting in a shift from a proinflammatory state to a pro-resolving state.[188] Based on this understanding, we hypothesized that antagonizing miR-33 would improve lesion composition by reducing proinflammatory or total macrophage content in the lesion.

Moreover, a reduction in inflammatory cells and their proinflammatory mediators has been associated with improvements in extracellular matrix proteins such as collagen, which is synthesized by VSMCs within the lesion.[129] Therefore, our hypothesis also included that there would be both a reduction in macrophages and an increase in synthetic VSMCs along with more collagen present, all suggestive indicators of plaque stability and regression.

By targeting miR-33 and promoting a shift in macrophage phenotype and modulation of extracellular matrix proteins, we aimed to promote a favorable environment within the lesions, ultimately contributing to plaque stability and regression. Understanding

the impact of miR-33 antagonism on these cellular and molecular processes provides valuable insights into potential therapeutic strategies for atherosclerosis management and prevention of disease progression.

The compositional characteristics of the lesions were assessed independently from morphometric changes. Collagen, VSMCs, and macrophages were stained to evaluate lesion composition. Initial attempts to quantify collagen using picro sirius red or Mason Trichrome staining were limited by the lack of collagen resolution for brightfield imaging. As a result, we employed picro sirius red with fast green (PSRFG) counterstaining for quantification. Contrary to expectations, we observed more raw area collagen staining in the progression group compared to the other treatment groups. This increase in raw collagen area could be due to the notably larger intima in the progression group [Figure 7a and Figure 9a]. To improve data resolution, we calculated the percent area of positive staining, which brought the median values of all groups closer together.

Similar trends were observed in lesions stained for α-actin. The raw median positive area of α-actin staining was largest in the progression group, likely influenced by the larger-sized lesions in that group [Figure 7a and Figure 10a]. The calculation of percent positive α-actin area within the intima did not reveal any notable differences among the groups. While there was a significant decrease in the total positive α-actin-stained area between the progression group and the 6-week vehicle, 24-week vehicle, and 24-week anti-miR-33 treatment groups, there was no significant difference between the vehicle and anti-miR-33 groups.

The last compositional measure involved staining the samples for CD68 to assess the presence of macrophages. It is also possible that CD68 stained a subset of VSMCs as

these cells are known to undergo phenotypic switching under inflammatory atherosclerotic conditions [Miano, J., 2021, Circulation]. However, the results of the CD68 staining were not as informative as expected, with most lesions showing minimal or no CD68-positive areas [Figure 11a]. Similar to the findings from PSRFG and α-actin staining, the majority of positive CD68 staining was observed in the progression group, where larger lesions were more common.

To further analyze the CD68 staining, we calculated the percentage of positive CD68 area and found significant differences between the progression group and both the 24-week regression groups, with the progression group exhibiting a higher percentage of positive staining [Figure 11f]. Furthermore, there was a significantly higher percent positive CD68 area when comparing the 6-week vehicle group to both the 24-week vehicle and anti-miR-33 groups. However, no significant differences were observed between the regression groups (vehicle/anti-miR-33) at either 6 or 24wk of treatment. Overall, the staining with CD68 did not provide substantial insights into the macrophage presence in the lesions. Although there were some differences observed between the progression group and the 24-week groups, the overall impact of miR-33 antagonism on the presence of inflammatory cells was not significant when comparing the different regression groups (vehicle/anti-miR-33).

These findings, along with those observed from the PSRFG and α-actin staining, suggest that miR-33 antagonism did not change the composition of the lesions beyond what was observed with vehicle treatment. Therefore, miR-33 antagonism in our NHP model did not appear to enhance the stability of the atherosclerotic lesions by increasing collagen and VSMCs and decreasing macrophages.

In our study, the anti-miR-33 treatment did not exhibit a significant effect on both intracranial and extracranial atherosclerosis, as reflected by morphometry, composition, cholesterol levels, and miR-33 gene expression. Several hypotheses may shed light on this unexpected result. One plausible explanation could be related to cholesterol efflux from plaque foam cells. Although anti-miR-33 treatment resulted in elevated circulating high-density lipoprotein (HDL), it may not have been sufficient to significantly increase cholesterol efflux from these cells. This observation underscores that HDL elevation alone may not be enough to stimulate effective cholesterol efflux, suggesting a need for supplementary interventions.

Another possible explanation pertains to the delivery of the anti-miR-33 treatment. It is conceivable that the treatment did not reach a concentration within the atherosclerotic lesions high enough to substantially inhibit miR-33, thereby derepressing the expression of its target genes. This hypothesis highlights an ongoing challenge in atherosclerosis treatment: ensuring that therapeutics efficiently reach their target location within atherosclerotic plaques.

Furthermore, the cynomolgus monkey model might exhibit too low expression levels of miR-33a/b to significantly impact target gene expression and, consequently, the size or composition of atherosclerotic lesions. This suggests that the effectiveness of anti-miR-33 treatment could hinge on the baseline levels of miR-33 in the specific animal model used. Finally, the high degree of variability in intracranial atherosclerotic lesions might have masked any potential treatment effects. This complexity of atherosclerosis as a disease necessitates larger-scale studies. Future research involving a more substantial number of

animals may yield more definitive insights. Each of these hypotheses merits further investigation to continue exploring the role of miR-33 in atherosclerosis.

The variability in lesion presentation, with some lesions showing signs of progression while others did not, may be attributed to the inter/intra- species variability of the response to an atherogenic diet observed in cynomolgus monkeys. Previous studies have noted that under the same proatherogenic conditions, some NHPs develop robust progressive extracranial atherosclerotic lesions while others do not.[237] Unfortunately, the limited macrophage involvement and other compositional measures within the lesions may have prevented us from accurately assessing the efficacy of miR-33 antagonism on lesion stabilization or regression.

It is important to address the limitations of our study. Firstly, we focused on only three segments of the COW, which limited the generalizability of our conclusions to the entire COW and reduced the total number of lesion vessels for assessment. A more comprehensive approach would include analysis of all major vessel segments of the COW (ACA, MCA, ICA, PCA, BA, etc.). Additionally, the relatively short regression periods may have been sufficient for significant changes in lesion size or composition. However, extending the treatment duration could further compromise the already limited compositional measures observed in the study.

Furthermore, the scarcity of viable antibodies for use in NHPs restricted our ability to investigate other markers associated with miR-33, such as autophagy and efferocytosis. Lastly, A significant limitation of this study lies within the inherent constraints of its experimental design. Specifically, our ability to control or manipulate variables was largely confined to the stage of intracranial vessels and brain tissue collection, aimed at assessing

the impact of miR-33 antagonism on ICAS. The experimental design of this study was largely shaped by the requirements of the parent study, which primarily focused on evaluating the effect of miR-33 antagonism on ECAS.

Despite the limitations and the lack of supporting evidence for the efficacy of miR-33 antagonism on ICAS regression, this study has several noteworthy strengths. Firstly, it was built upon a larger atherosclerosis study that specifically investigated miR-33 antagonism on ECAS. While we acknowledged this as a limitation, it is important to highlight the value of utilizing this valuable NHP model to delve into uncharted territory. By successfully addressing the formidable challenge of studying diet induced ICAS in an experimental model, this study achieved a significant feat.

The key strength of our study lies in the successful induction of ICAS in a non-human primate model without resorting to hypertensive manipulations, a strategy often employed in stroke models. This achievement, to the best of our knowledge, is a first in atherosclerosis research, thus filling a critical gap in our understanding of ICAS within an atherogenic context.

This notable breakthrough offered a unique opportunity to explore the effects of miR-33 antagonism on ICAS regression, shedding light on the intricate mechanisms underpinning this disease. These findings not only hold significant potential for advancing our understanding of ICAS but also for spearheading the development of innovative therapeutic strategies. Moreover, this model paves the way for evaluating the efficacy of other potential treatments for ICAS, thereby facilitating the effective translation of preclinical findings into clinical applications.

2.4.1 Conclusion

Our study, conducted as part of a larger atherosclerosis investigation in nonhuman primates, presented a serendipitous opportunity to delve into the intricacies of intracranial arteries and explore the formation of atherosclerotic plaques within the Circle of Willis. Through comprehensive analysis, we have provided compelling evidence to support the notion that persistent exposure to a high-fat, high-cholesterol diet, coupled with hypercholesterolemia, can indeed stimulate the development and progression of intracranial atherosclerosis in an animal model. This groundbreaking achievement represents a significant advancement in the field, as prior establishment of intracranial atherosclerosis in an animal model had remained elusive.

The discovery of intracranial atherosclerosis in nonhuman primates opens a realm of possibilities for future research endeavors. One promising avenue lies in the exploration of advanced imaging modalities to monitor the dynamic progression or regression of these atherosclerotic plaques under experimental conditions. By honing our understanding of the factors influencing plaque stability and regression, we can lay the groundwork for the development of innovative treatment strategies aimed at reducing the burden of intracranial atherosclerosis.

Moreover, our study holds immense potential for unraveling the intricate mechanisms underlying intracranial atherosclerosis and its consequential impact on stroke and vascular cognitive impairment. By delving into the complexities of intracranial atherosclerosis within a controlled experimental setting, we gained valuable insights that may ultimately translate into effective interventions to mitigate the deleterious effects of intracranial atherosclerosis on brain health and function.

In conclusion, our investigation not only established the presence of ICAS in a carefully controlled NHP model but also shed light on the pathogenesis and progression of this condition. This pivotal achievement paves the way for future studies to further explore the nuances of intracranial atherosclerosis, devise novel imaging techniques, develop targeted therapeutic interventions, and ultimately alleviate the burden of ICAS-related complications on individuals' lives.

Table 2.1 Macronutrient Distribution Across Diets

Macronutrient distribution of Progression and Regression diets for comparison. (a) Represents house made Western-type atherogenic diet. (b) Represents commercial monkey diet (https://www.inotivco.com/primate-natural-ingredient-diets)

a. Western-type "Progression Atherogenic Diet" Macronutrient Composition

Macronutrient			Calories (%)
Fat			**38**
Carbohydrate			46
Protein			16
Cholesterol	mg/kcal	% dry weight	
	1.0	**0.4**	

b. Teklad 2050™ "Regression Chow Diet" Macronutrient Composition

Macronutrient	Calories (%)
Fat	**14**
Carbohydrate	57
Protein	29

Table 2.2 MicroRNA-33 Target Genes

Gene	Gene Name	Pathway
ABCA1	ATP binding cassette subfamily A member 1	cholesterol efflux
NPC1	NPC intracellular cholesterol transporter 1	intracellular cholesterol trafficking
CPT1A	carnitine palmitoyltransferase 1A	fatty acid oxidation
HADHB	hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta	fatty acid oxidation
CROT	carnitine O-octanoyltransferase	fatty acid oxidation
ATG5	autophagy related 5	autophagy
ATG12	autophagy related 12	autophagy
LAMP1	lysosomal associated membrane protein 1	autophagy
SIRT6	sirtuin 6	histone deacetylase; FA and glucose metabolism
PRKAA1	protein kinase AMP-activated catalytic subunit alpha 1	fatty acid oxidation
PPARGC1A	PPARG coactivator 1 alpha	mitochondrial function
PDK4	pyruvate dehydrogenase kinase 4	mitochondrial function
CDK6	cyclin-dependent kinase 6	cell cycle
CCND1	cyclin D1	cell cycle
ABCB11	ATP binding cassette subfamily B member 11	PL secretion into bile
ATP8B1	ATPase phospholipid transporting 8B1	PL secretion into bile
NSF	N-ethylmaleimide sensitive factor, vesicle fusing ATPase	vesicular trafficking, VLDL secretion
OSBPL6	oxysterol binding protein like 6	intracellular cholesterol trafficking
PCK1	phosphoenolpyruvate carboxykinase 1	glucose metabolism
G6PC1	glucose-6-phosphatase catalytic subunit 1	glucose metabolism
NCOA1	nuclear receptor coactivator 1	glucose metabolism
RORA	RAR related orphan receptor A	glucose metabolism
CREB1	cAMP responsive element binding protein 1	glucose metabolism

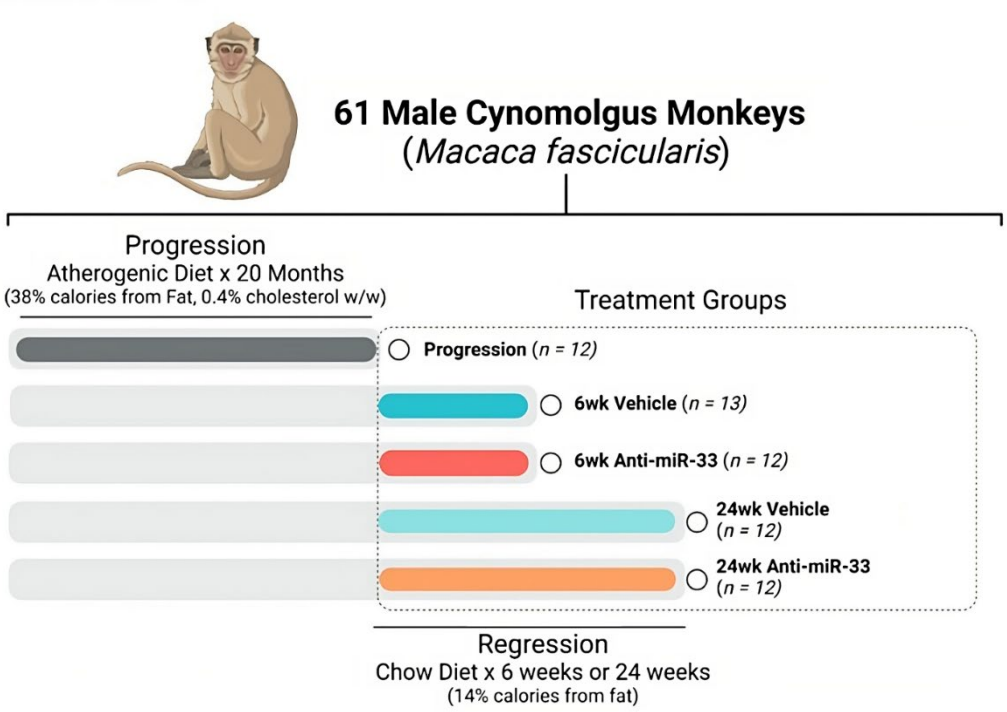

Figure 2.1: Study Design
Illustration of study design, including diet, number of animals, and treatment groups.

Figure generated with BioRender.com.

	Progression	6wk Vehicle	6wk Anti-miR-33	24wk Vehicle	24wk Anti-miR-33
Cholesterol (mg/dl)	562^{a}	$134^{b,c}$	$156^{a,b}$	82^{c}	$113^{b,c}$
VLDL-C (mg/dl)	148^{a}	4.2^{b}	$6.9^{a,b}$	3.0^{b}	3.1^{b}
LDL-C (mg/dl)	368^{a}	63^{b}	$64^{a,b}$	25^{c}	$26^{b,c}$
HDL-C (mg/dl)	42^{a}	48^{a}	74^{b}	$55^{a,b}$	81^{b}
Triglyceride (mg/dl)	10^{a}	16^{a}	$20^{a,b}$	$45^{a,b}$	43^{b}

Figure 2.2: Median Plasma Lipids Across Treatment Groups
Changes in plasma lipid levels across different time points. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Each panel represents median plasma lipid levels for the respective animal groups: those with n = 12 are denoted by *, and those with n = 13 are denoted by #. Different superscript letters within each row denote statistically significant differences ($p < 0.05$) between treatment groups at each respective time point.

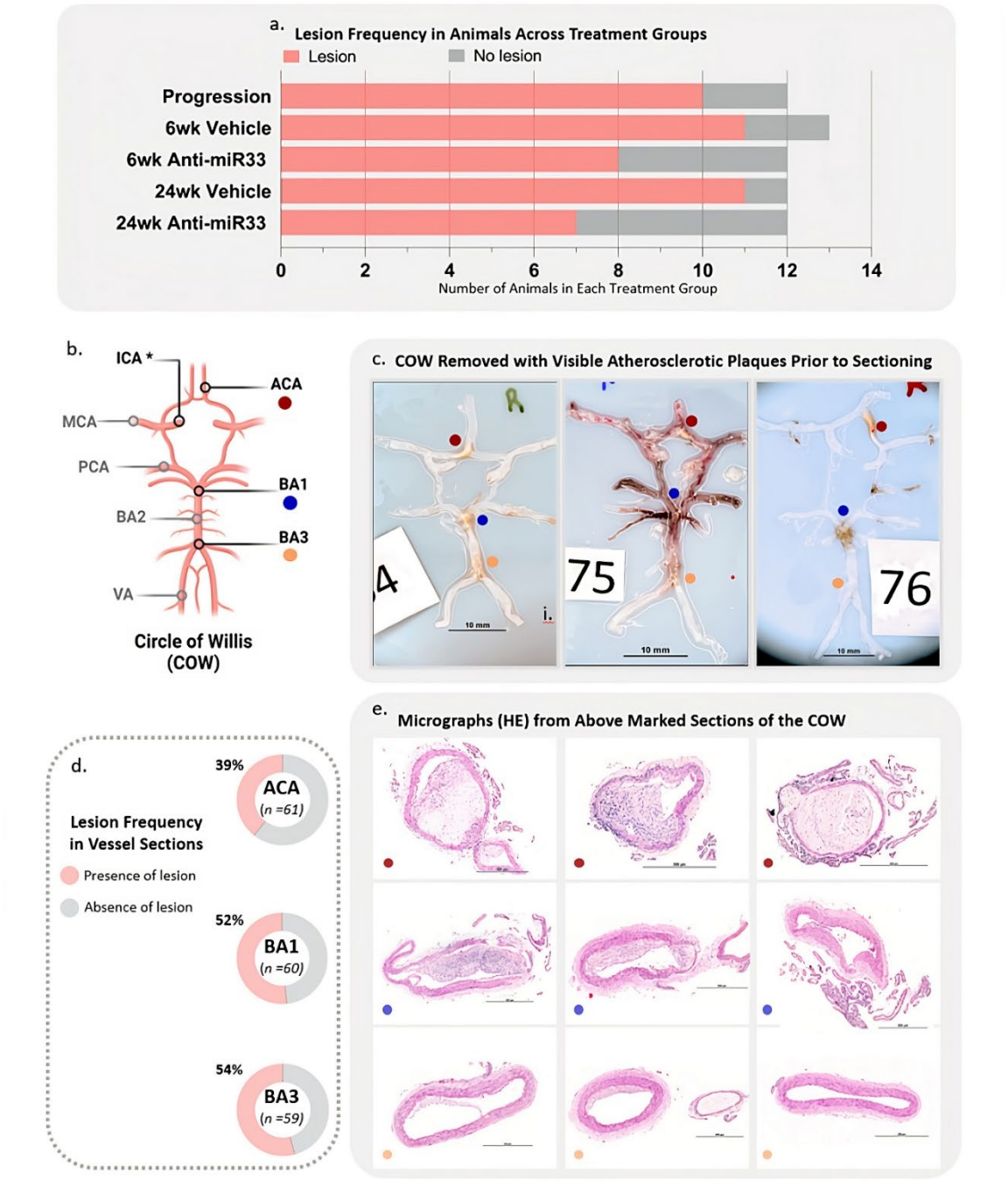

Figure 2.3: Gross and Microscopic Observations of Lesion Frequency and Spatial Distribution of Lesions within the Circle of Willis (COW)

(a) Frequency of lesions observed in animals across treatment groups. (b) Graphical representation of the Circle of Willis (COW). (c) Photographs of extracted COW with one or more gross atherosclerotic lesions. Small color dots align with ACA, BA1, BA3

(Continued Figure 2.3) segments pictured in (b). (d) Illustrates frequency of lesions within each vessel segment, percentages based of total animals in study (N=61). (e) Example micrographs of vessels with and without lesions aligned with each of the above COW (c) and vessels segments to the left (d).

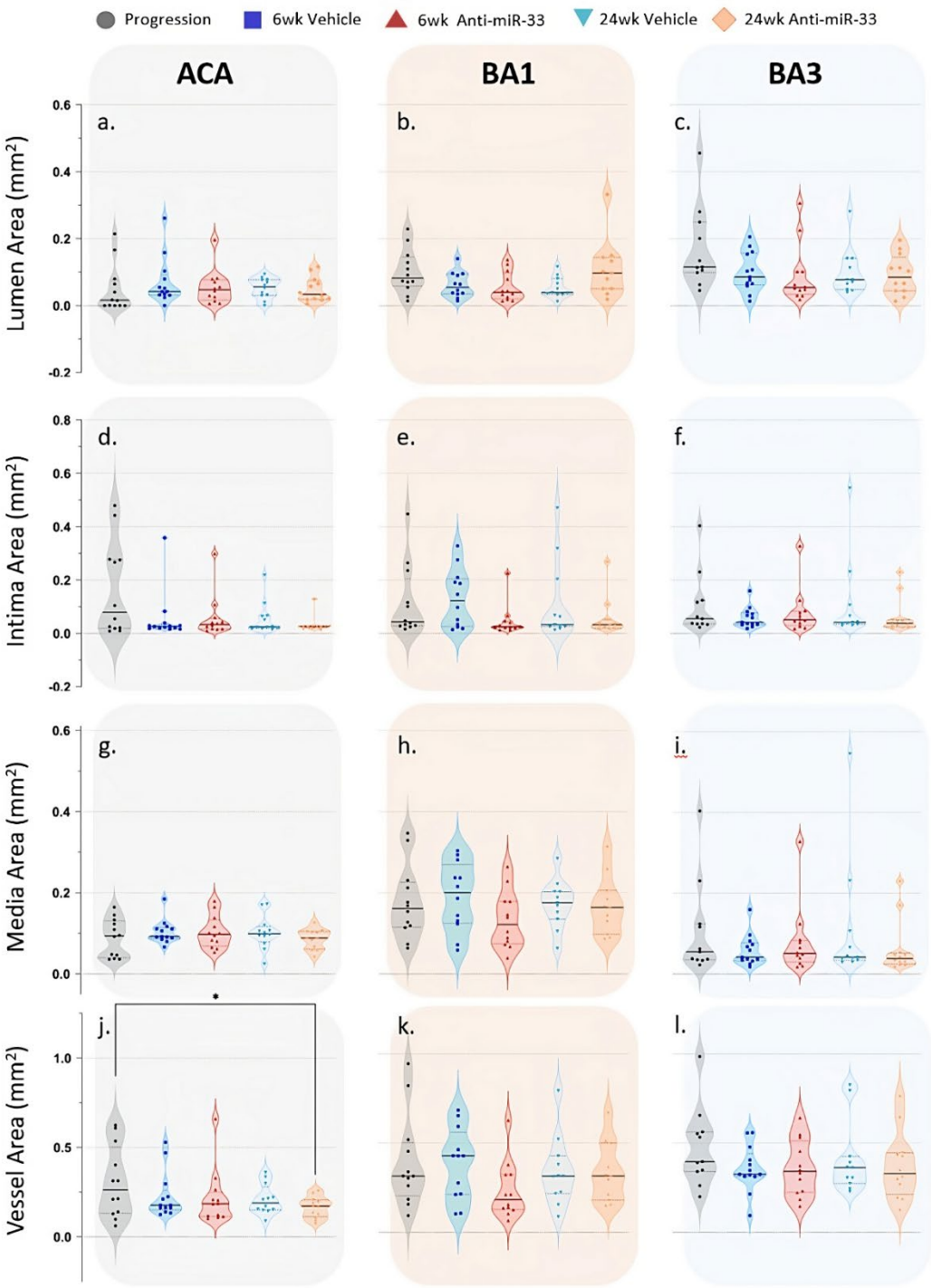

Figure 2.4: Quantification of Morphometrical Raw Area (mm²) Binarization from Vessel Segments Observed Among all Animals Across Treatment Groups.
Vessel images were binarized to establish ROI for each of the following row measures

raw (a-c) lumen area, (d-f) intima area, (g-i) media area, (j-l) vessel area. Due to a non-

(Continued Figure 2.4) normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01. The three columns represent the median area (mm^2) for each measure across vessel segments ACA, BA1, BA3 (N=183 vessels) and treatment groups. Total ACA vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33 (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Total BA1 vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33 (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Total BA3 vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33 (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Treatment groups are indicated in the top legend.

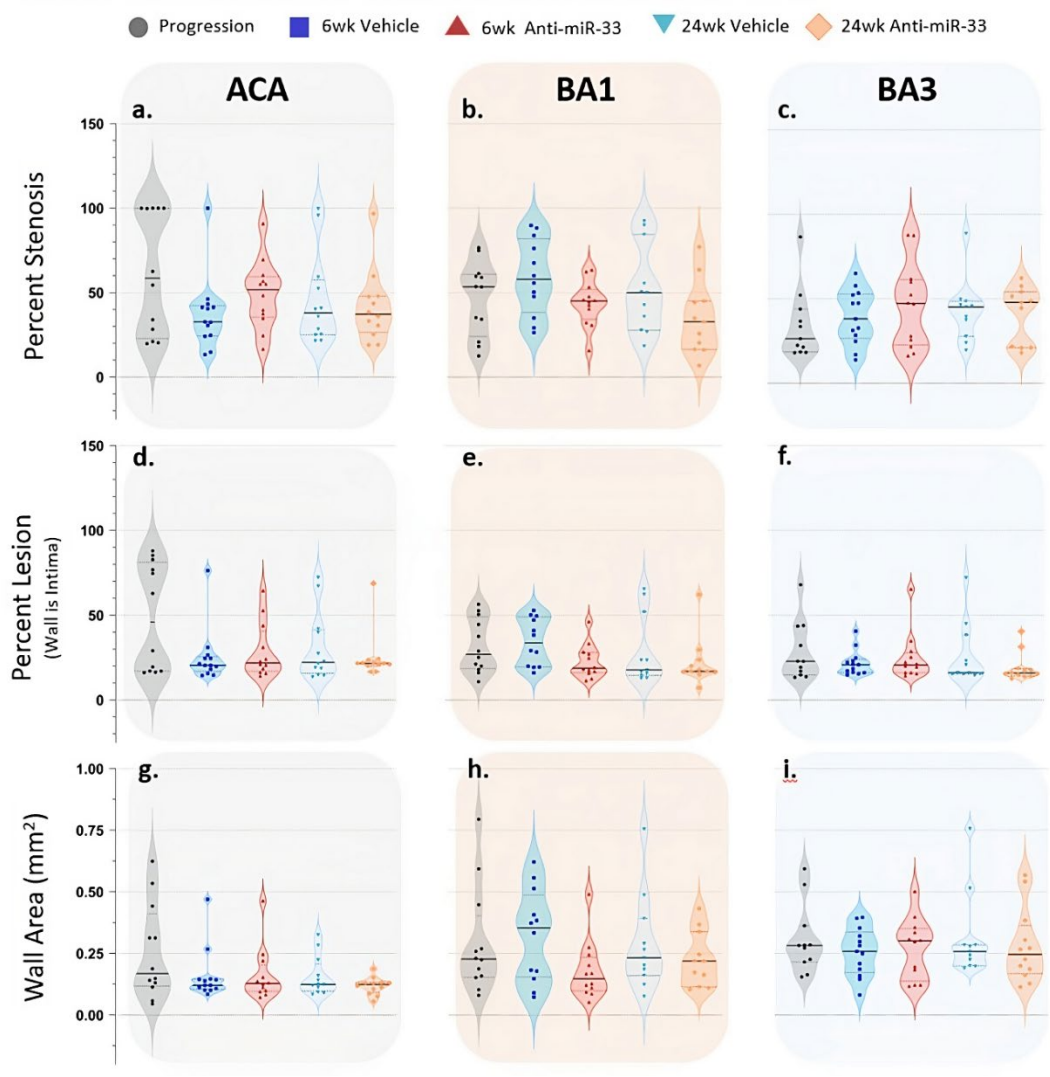

Figure 2.5: Quantification of Calculated Morphometry Measures from Raw Area (mm2) Binarization of Vessel Segments Observed Among all Animals Across Treatment Groups.

Calculated morphometry measures from vessel raw area data in Figure 2.4 (a-c) percent stenosis, (d-f) percent wall is intima, (g-i) wall area (mm2). Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01. The three columns represent the median area (mm^2) for each measure across vessel segments ACA, BA1, BA3 (N=183 vessels) and treatment groups. Total ACA vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33

(Continued Figure 2.5) (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Total BA1 vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33 (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Total BA3 vessels in progression (n=12), 6wk vehicle (n=13), 6wk anti-miR-33 (n=12), 24wk vehicle (n=12), 24wk treatment (n=12). Treatment groups are indicated in the top legend.

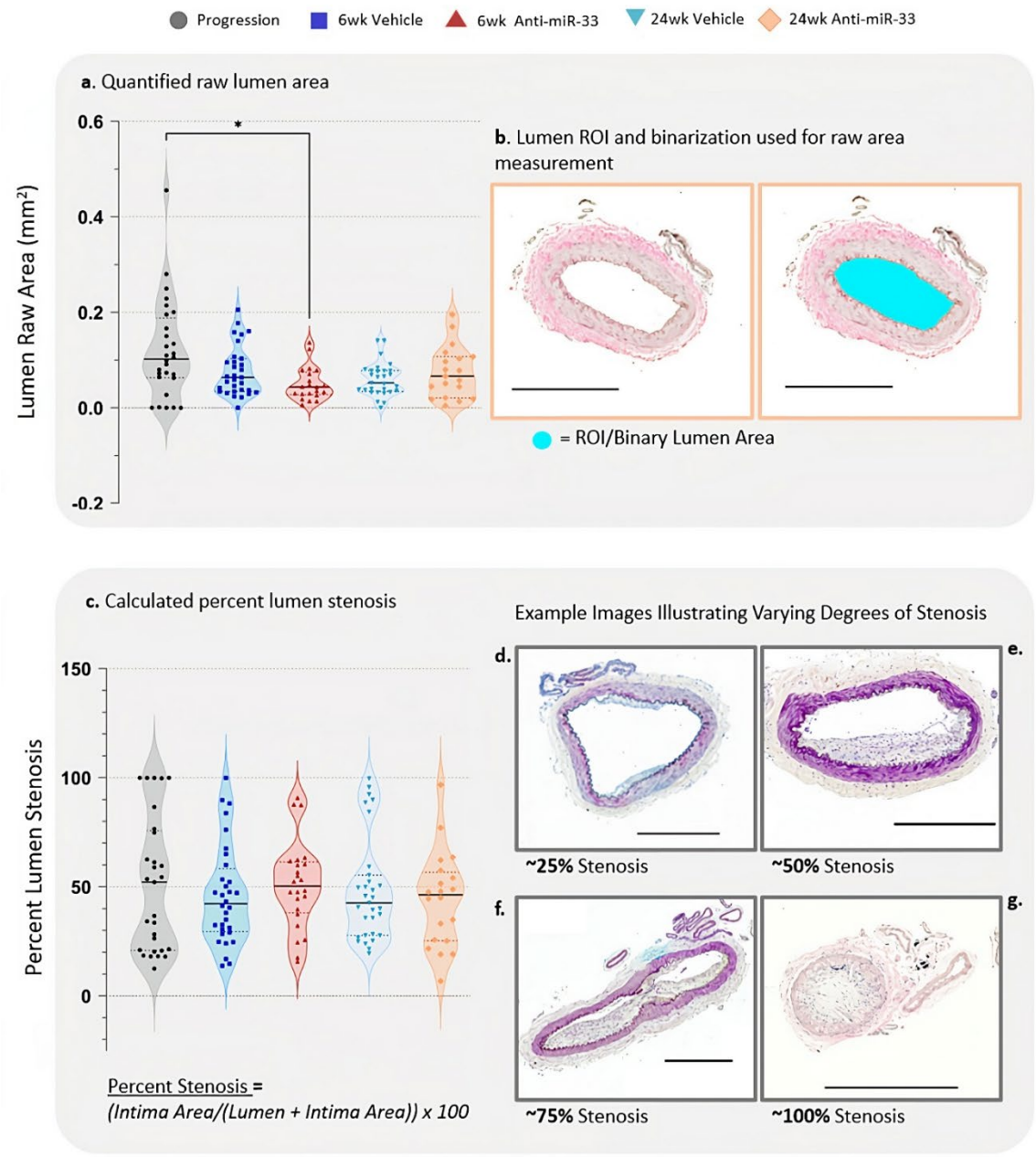

Figure 2.6: Quantification and Micrographs of Lumen Measures from Lesion Animals Only Across Treatment Groups

(a, c) Analysis of lumen measures, raw area and percent stenosis, in only those vessels from lesion animals. The ACA, BA1, and BA3 segments from each lesion animal were clustered into each of their perspective treatment groups. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis

(Continued Figure 2.6) test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01. (b) Example of lumen binarization for raw and calculated lumen measures. (d-g) Representative images of different degrees of stenosis, actual percent stenosis measures are bolded below images. Scale bar represents 500μm.

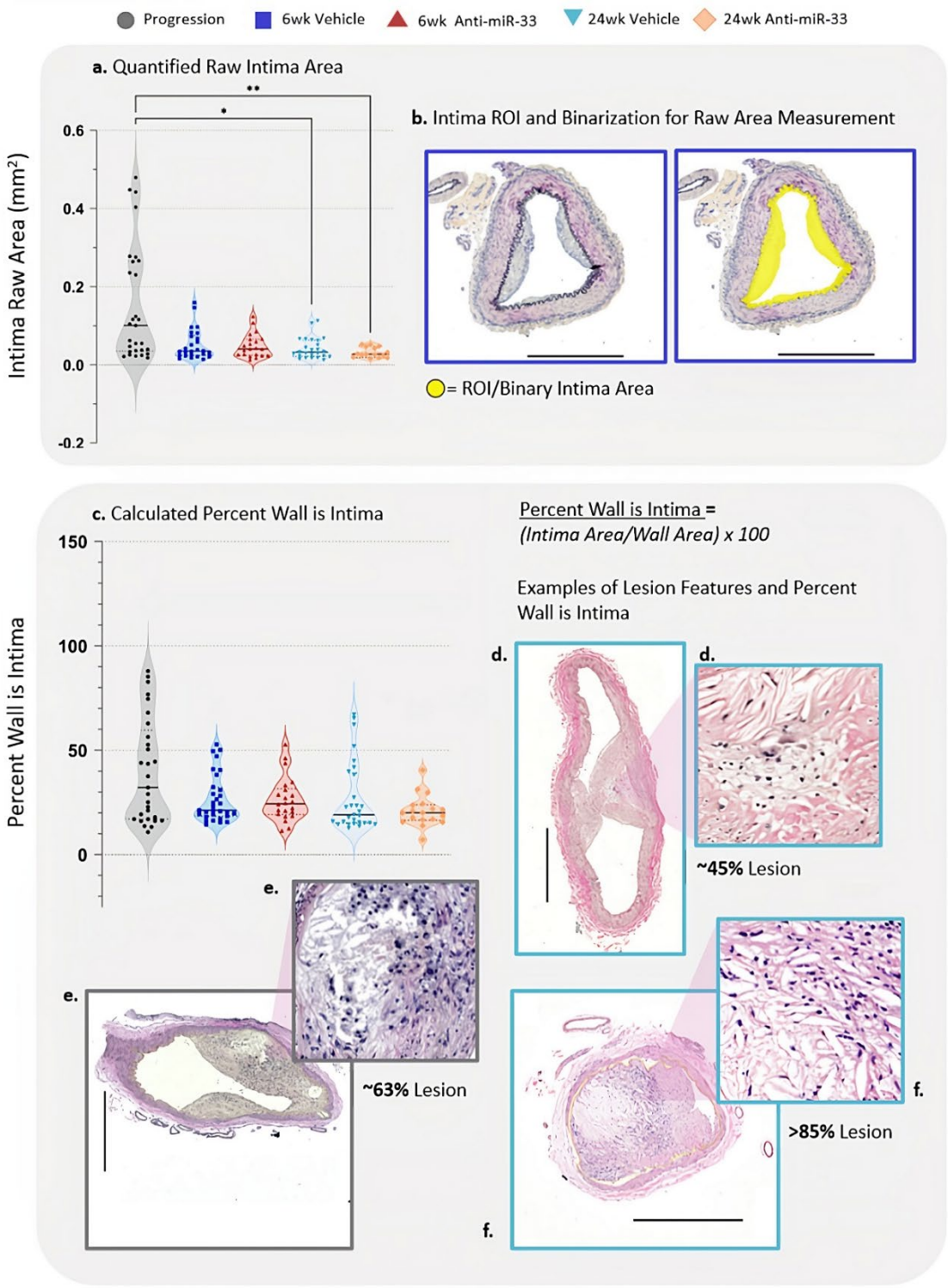

Figure 2.7: Quantification and Micrographs of Intima (Lesion) Measures from Lesion Animals Only

(a, c) Analysis of intima measures, raw area and percent wall is intima, in only those vessels from lesion animals. The ACA, BA1, and BA3 segments from each lesion animal were

(Continued Figure 2.7) clustered into each of their perspective treatment groups. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01, ** p=0.01-0.001. (b) Example of intima binarization for raw and calculated intima measures. (d-f) Representative images of lesions within the intima, actual percent wall is intima measures are bolded next to images. (e-f) Digital zoom of lesion features (e) necrotic core with inflammatory cells on the periphery, (d) large foam cells clustered together, (f) cholesterol clefts observed within a necrotic core. Scale bar represents 500μm.

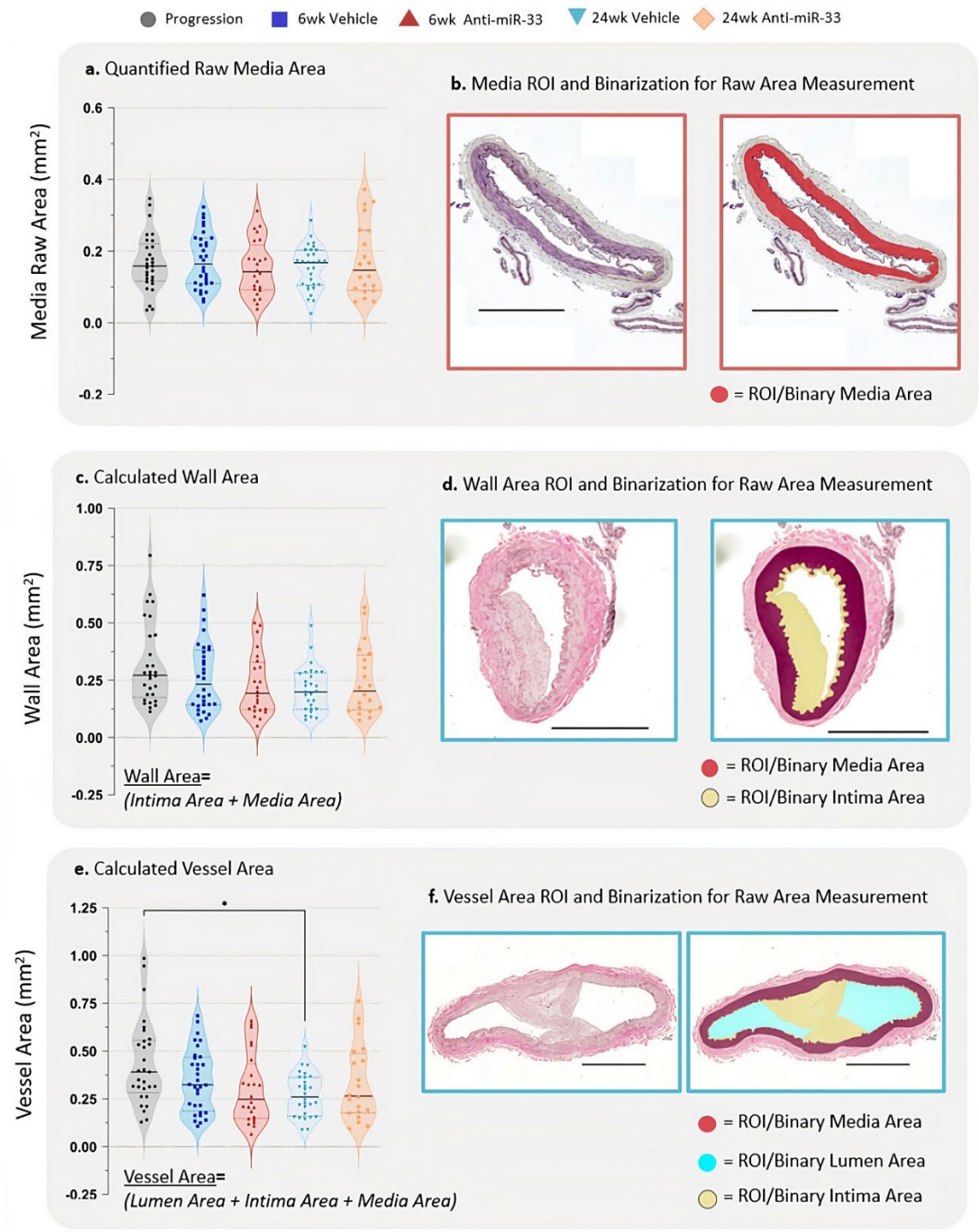

Figure 2.8: Quantification and Micrographs of Additional Morphometrical Measures from Lesion Animals Only Across Treatment Groups
(a, c,) Analysis of calculated measures (mm^2) in only those vessels from lesion animals.

The ACA, BA1, and BA3 segments from each lesion animal were **clustered** into each of

(Continued Figure 2.8) their perspective treatment groups. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01. (b) Example of media binarization for raw media area. (d) Example of media and intima binarization for calculation of wall area. (f) Example of media, intima, and lumen binarization for calculation of total vessel area. Scale bar represents 500μm.

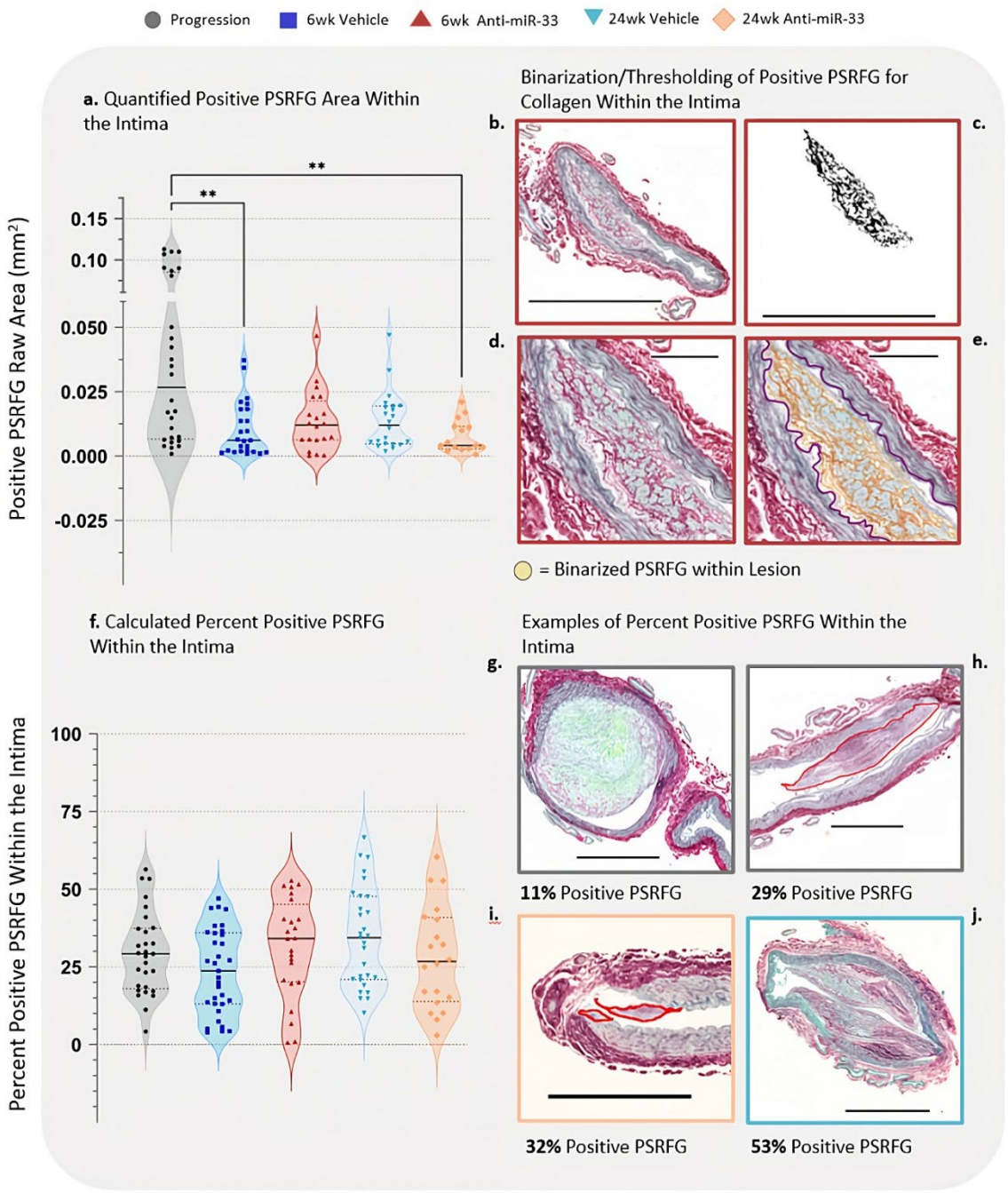

Figure 2.9: Quantification and Micrographs from the Binarization of PSRFG Positive Staining for Collagen within the Intima Measured Only from Lesion Animals Across Treatment Groups

(a, f,) Analysis of positive picro sirius red fast green (PSRFG) within the intima, raw area and percent positive intima area. The ACA, BA1, and BA3 segments from each lesion animal were clustered into each of their perspective treatment groups. Due to a non-normal

(Continued Figure 2.9) distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: ** p=0.01-0.001. (b-e) Example of positive PSRFG binarization within the intima for collagen raw area and percent area measures. (c) Masked binary area of positive stained PSRFG in intima. (g-j) Representative images of different degrees of percent positive PSRFG within intimal lesions, actual percent PSRFG measures are bolded below images. (b,c, g-j) Scale bar represents 500μm. (d-e) Scale bar represents 100 μm.

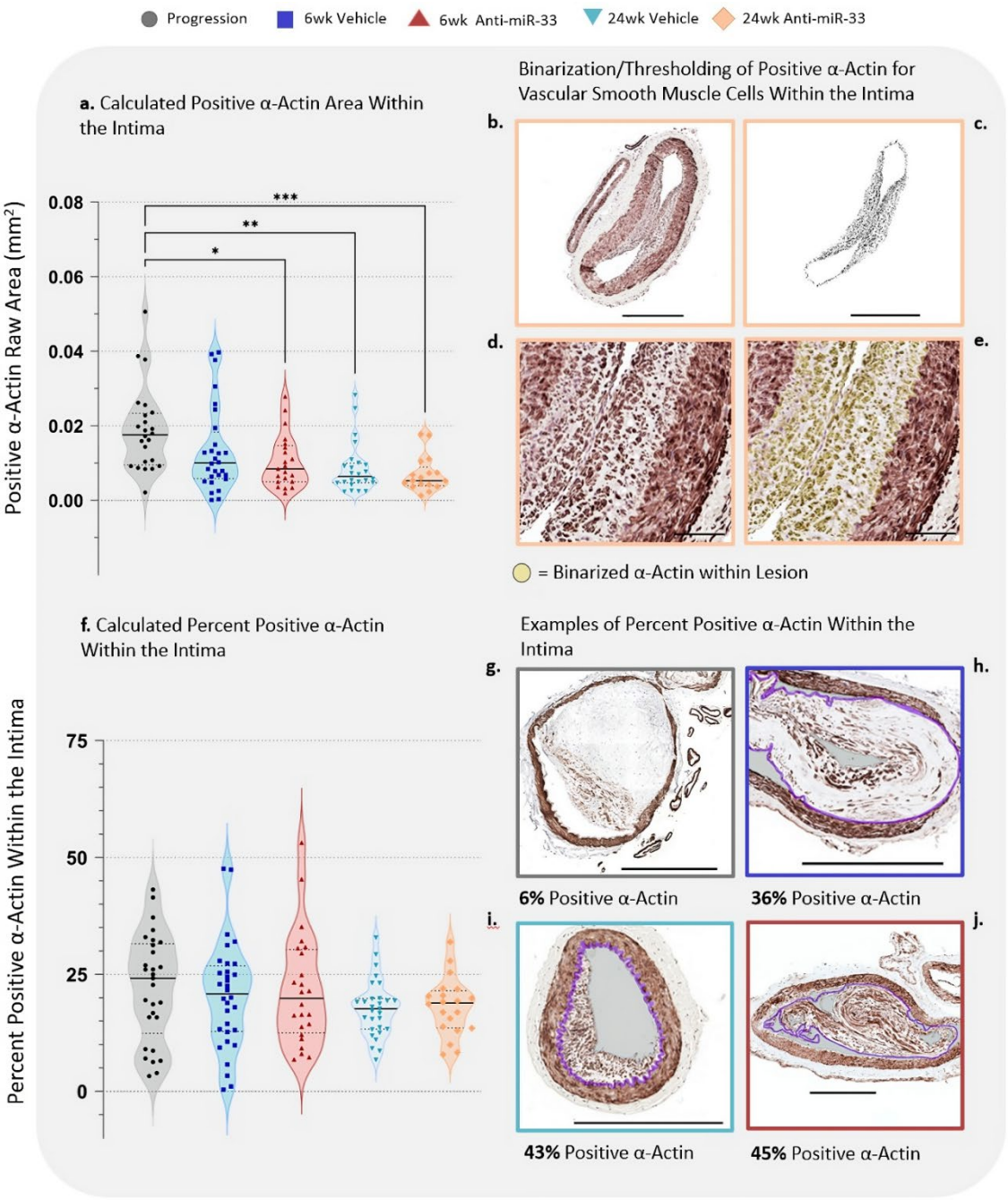

Figure 2.10: Quantification and Micrographs from the Binarization of α-Actin Positive Staining for Vascular Smooth Muscle Cells within the Intima Measured Only from Lesion Animals Across Treatment Groups

(a, f,) Analysis of positive alpha-actin (α-actin) within the intima, raw area and percent positive intima area. The ACA, BA1, and BA3 segments from each lesion animal were clustered into each of their perspective treatment groups. Due to a non-normal distribution

(Continued Figure 2.10) of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01 ** p=0.01-0.001, ***p=0.001-0.0001. (b-e) Example of positive α-actin binarization within the intima for vascular smooth muscle cell raw area and percent area measures. (c) Masked binary area of positive stained α-actin in intima. (g-j) Representative images of different degrees of percent positive α-actin within intimal lesions, actual percent α-actin measures are bolded below images. (b,c, g-j) Scale bar represents 500μm. (d-e) Scale bar represents 100 μm.

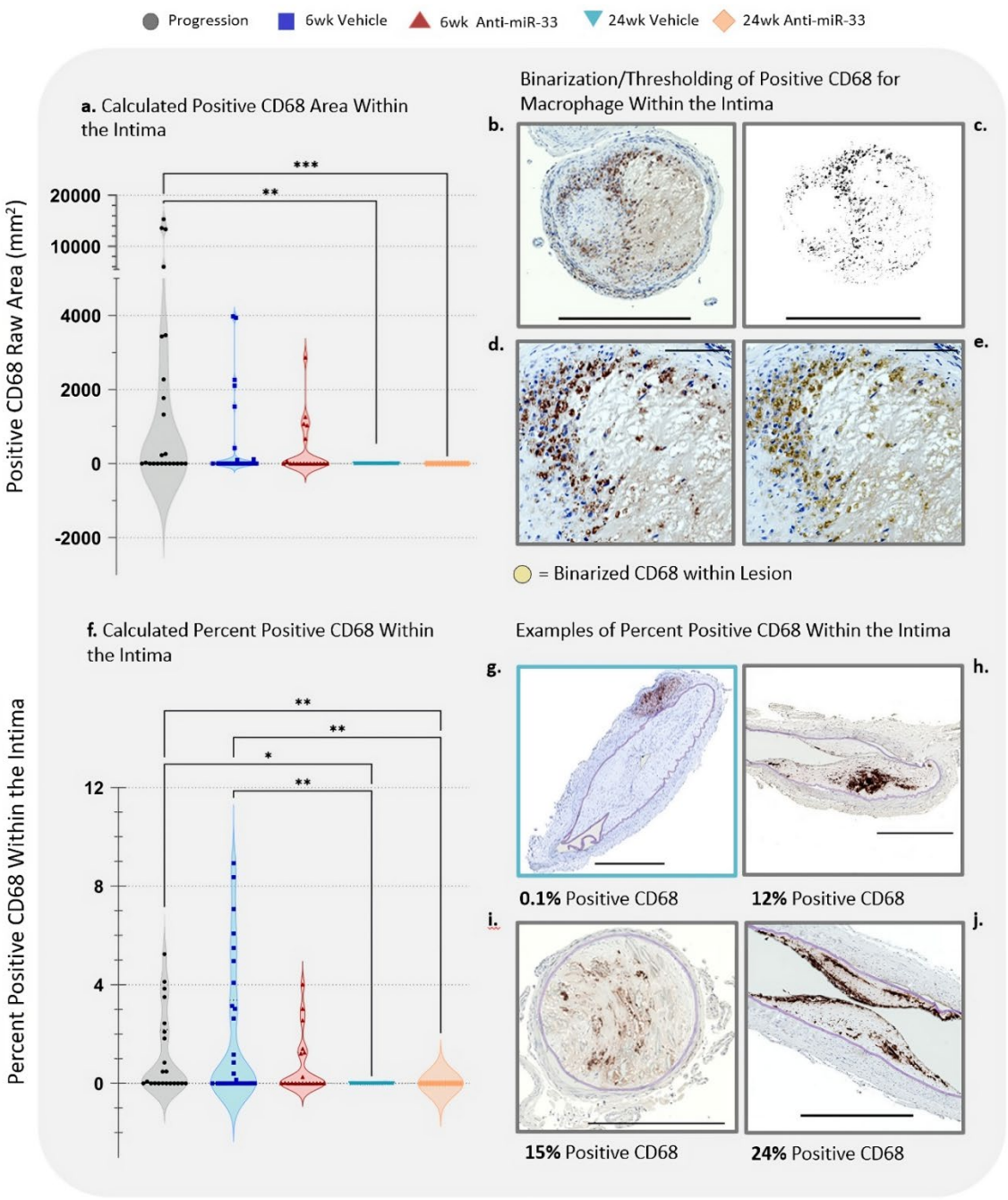

Figure 2.11: Quantification and Micrographs from the Binarization of CD68 Positive Staining for Inflammatory Macrophage within the Intima Measured Only from Lesion Animals Across Treatment Groups

(a, f,) Analysis of positive CD68 within the intima, raw area and percent positive intima area. The ACA, BA1, and BA3 segments from each lesion animal were clustered into each

(Continued Figure 2.11) of their perspective treatment groups. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01 ** p=0.01-0.001, ***p=0.001-0.0001. (b-e) Example of positive CD68 binarization within the intima for macrophage raw area and percent area measures. (c) Masked binary area of positive stained CD68 in intima. (g-j) Representative images of different degrees of percent positive CD68 within intimal lesions, actual percent CD68 measures are bolded below images. (b,c, g-j) Scale bar represents 500μm. (d-e) Scale bar represents 100 μm.

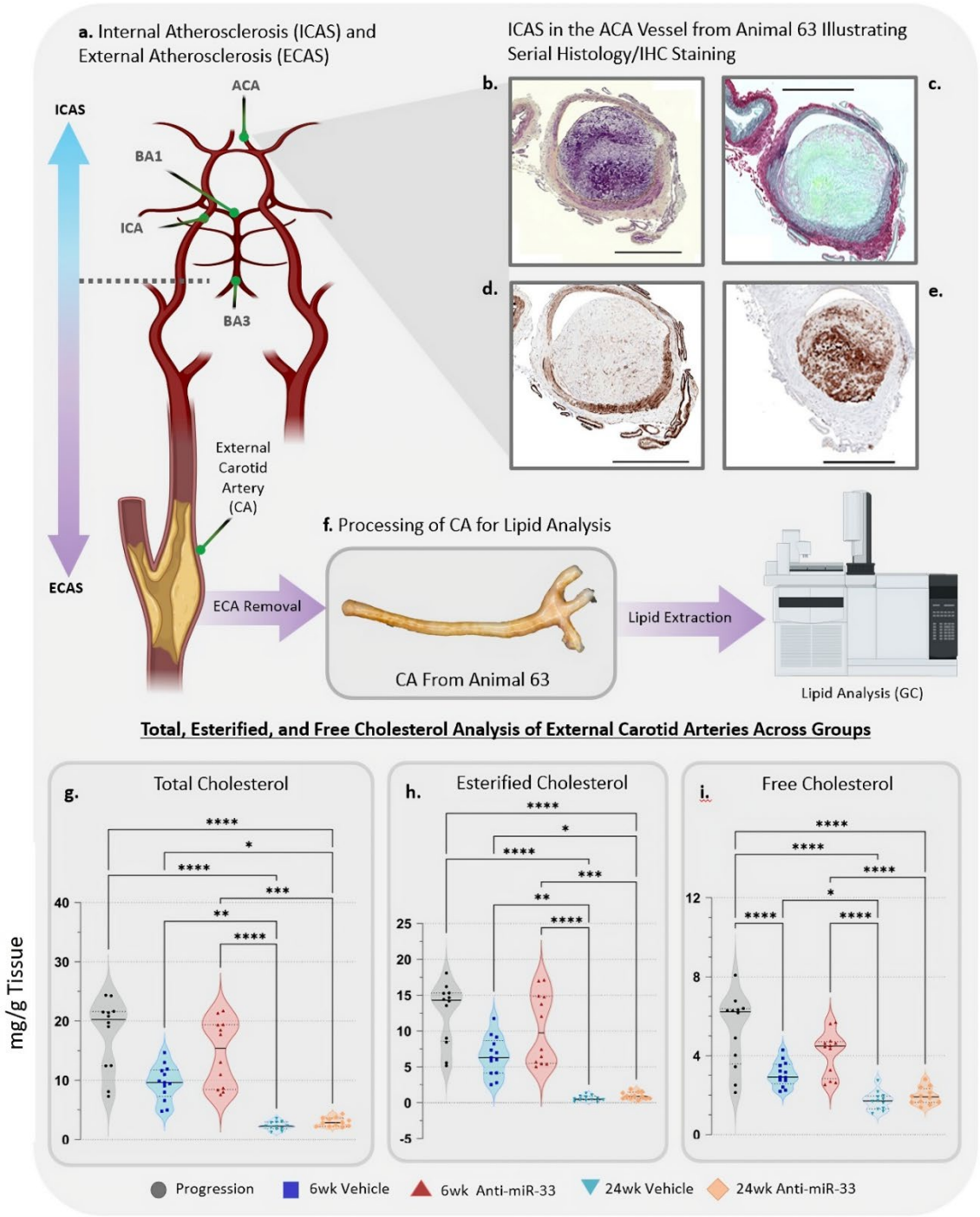

Figure 2.12: Graphical Abstract Illustrating the Removal and Processing of the External Carotid Artery for Lipid Analysis and Quantification

(a) Relationship of external carotid artery to intracranial vessels. (b-e) Sample micrographs depicting serial sections of an ACA vessel segment. (b) Movat staining where intraplaque hemorrhage can be observed. (c) PSRFG staining for collagen where intraplaque

(Continued Figure 2.12) hemorrhage from (b) can be observed as light green area. (d) α-actin staining for vascular smooth muscle cells shows little positive staining within the lesion. (e) CD68 staining for inflammatory macrophage shows heavy positive staining within the lesion. (g-i) Analysis of total, esterified, and free cholesterol (TC, EC, FC) measures from processed external carotid tissue (f) across treatment groups. Due to a non-normal distribution of data, as determined by the Shapiro-Wilks test (SWT), we employed the Kruskal-Wallis test (KWT) followed by Dunn's post hoc test for multiple comparisons. Significant markers: * p=0.05-0.01, ** p=0.01-0.001, *** 0.001-0.0001, **** $p < 0.0001$. Scale bar represents 500μm.

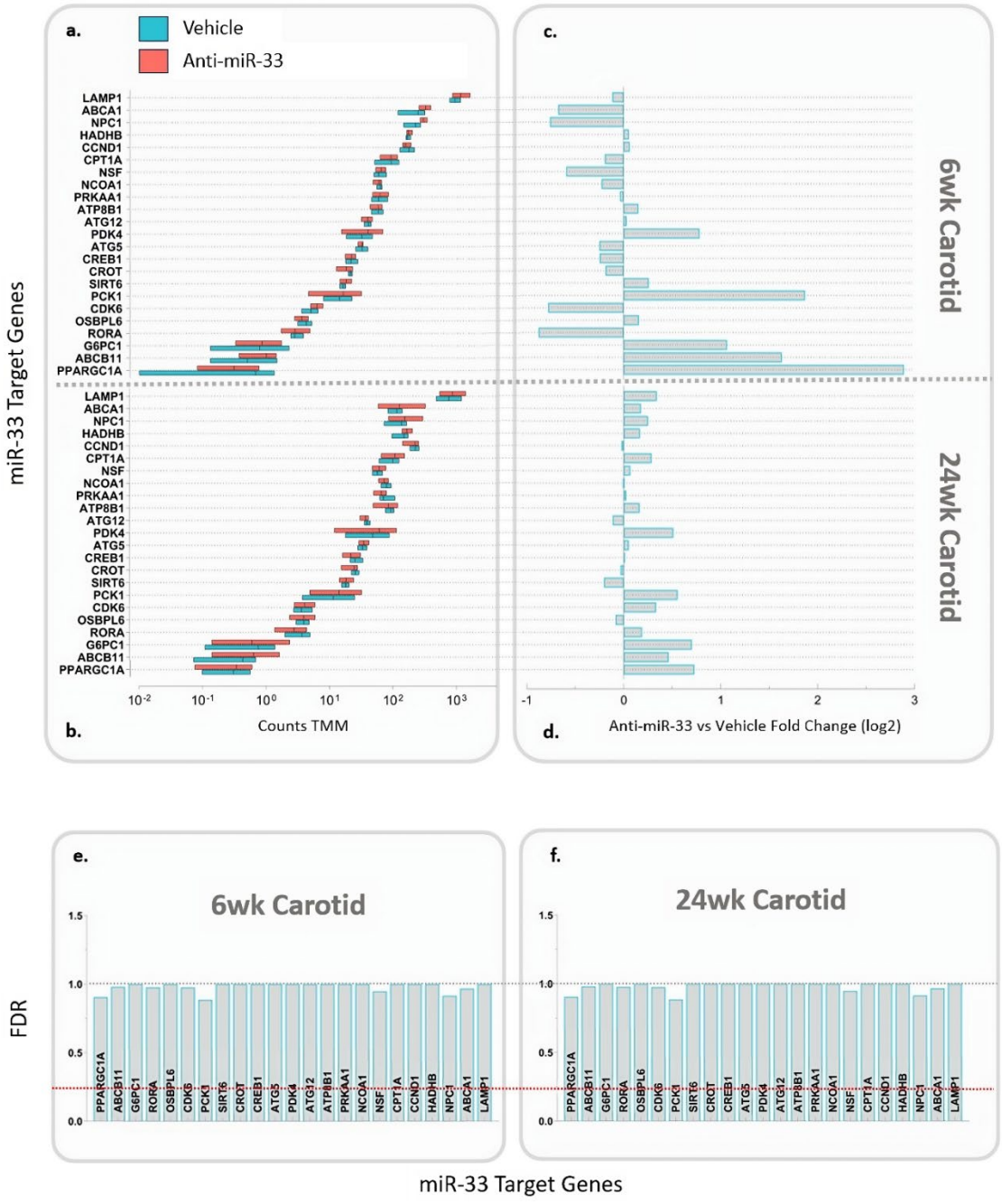

Figure 2.13: RNAseq Gene Expression Analysis of External Carotid Arteries from 6wk and 24wk vehicle and anti-miR-33 Treated Animals

(a) Trimmed mean of M-values (TMM) from bulk RNAseq filtered for miR-33 target genes in animals treated with vehicle or anti-miR-33 for 6wks. (b) TMM for miR-33 target genes

(Continued Figure 2.13) in animals treated with vehicle or anti-miR-33 for 24wks. (c,d) Fold change (FC; log2) between anti-miR-33 vs Vehicle treated animals, (c) 6wk FC aligned with (a) and (d)24wk FC aligned with (b) target genes. (e-f) False discovery rate (FDR) for each of the miR-33 target genes.

CHAPTER 3. DISCUSSION

3.1 Discussion

Atherosclerosis, a chronic inflammatory disease characterized by the accumulation of lipids and the remodeling of the vascular wall of large arteries, is a leading cause of morbidity and mortality worldwide. The study of atherosclerosis, particularly its manifestation within intracranial arteries, has been a focal point of cardiovascular and cerebrovascular research due to its profound impact on heart and brain health. The manifestation of intracranial atherosclerosis (ICAS) is particularly concerning due to its association with stroke and vascular cognitive impairment. Despite the significant health burden posed by ICAS, our understanding of its pathophysiology and our ability to effectively treat it remain limited. The residual risk associated with current treatments, the lack of suitable animal models for studying ICAS, and limitations in imaging techniques for diagnosing and monitoring ICAS have all posed significant hurdles in the path of progress. This dissertation aimed to address some of these challenges by exploring the role of miR-33 in atherosclerosis and developing a non-human primate model for diet induced ICAS.

In the first chapter of this dissertation, we provided a comprehensive overview of atherosclerosis, with a particular emphasis on ICAS. We discussed the impact of atherosclerosis on heart and brain health and highlighted the crucial role of animal models in advancing our understanding of this complex disease. We then set the stage for the subsequent chapter by outlining the need for further research into the development, regression, and potential treatment of ICAS.

In the second chapter, we presented our original research on the efficacy of miR-33 antagonism in promoting regression of ICAS in a non-human primate model. The findings from these chapters have not only contributed to our understanding of the efficacy of miR-33 antagonism on atherosclerotic lesions, but also served as a formal introduction to ICAS in a non-human primate (NHP) model. The mere opportunity to study a therapeutic thought to stabilize and/or regress atherosclerosis should not be understated. For the first time, we were able to reliably recapitulate the conditions for, and manifestation of, human-like ICAS in an experimental animal model, underscoring the enormous value NHPs offer for the study of ICAS while laying the foundation for future therapeutic approaches for the treatment of this disease.

In this discussion chapter, we will synthesize the findings from the first two chapters, situate them within the broader context of the field, and discuss their implications for future research. We will also address the limitations of our study and propose future directions for ICAS research. The ultimate goal is to highlight the potential impact of our work on improving brain health and function and reducing the risk of stroke and vascular cognitive impairment.

3.1.1 Historical Challenges in Atherosclerosis Research

The treatment of extracranial atherosclerosis (ECAS) and ICAS has seen significant advancements over the years, leading to a reduction in the global burden of these diseases. However, despite these advances, a significant residual risk remains from the complex and multifactorial nature of atherosclerosis, which involves various cellular and molecular processes, such as inflammation, lipid metabolism, and vascular remodeling.[73] Additionally, complex interactions between genetic, environmental, and lifestyle factors,

presents a significant challenge for patients to overcome and clinicians to control for.[73] Furthermore, current treatment strategies primarily target systemic risk factors, such as hypertension and dyslipidemia, but do not directly address the local pathological processes within atherosclerotic lesions.[238]

The lack of suitable animal models for studying ICAS has also been a significant challenge in the field. While various animal models have been used to study ECAS (mice, rabbits, pigs), these models have not been shown to accurately replicate the development and complex pathophysiology of human ICAS.[195,233] In parallel to a lack of animal models, limitations in imaging techniques for diagnosing and monitoring ICAS have posed significant challenges. While advancements in imaging technologies have improved our ability to visualize atherosclerotic extracranial plaques, these techniques often lack sufficient resolution to accurately assess, beyond stenosis and gross lesion size, the cellular and molecular composition of intracranial atherosclerotic plaques.[232,239] This has limited not only our understanding of the disease but also our ability to noninvasively monitor the efficacy of treatment strategies to reduce residual risk through promoting the stabilization and/or regression of both ECAS and ICAS.

3.1.2 Development and Validation of the Nonhuman Primate Model for ICAS

The establishment of a robust and reliable animal model for ICAS arose from our original investigation into the effectiveness of anti-miR-33 ASO in promoting the regression of cardiovascular atherosclerotic plaques in NHPs. The unexpected and serendipitous discovery of diet induced ICAS in this model marks a significant milestone in the field of cerebrovascular atherosclerosis research, with far-reaching implications.

Non-human primates serve as valuable models for studying many human diseases due to their genetic, physiological, and anatomical similarities with humans. These animals have been extensively utilized in research focusing on ECAS. However, limited evidence exists regarding their potential as models for ICAS. While it has been reported that NHPs naturally develop ICAS, the reliable induction of ICAS in this model remains relatively unexplored in the existing literature.

Only one group has demonstrated the presence of large atherosclerotic plaques within the COW of cynomolgus monkeys. This pioneering research conducted by Prusty et al. in 1988 involved studying two animals and provided evidence that an atherosclerotic diet combined with surgically induced hypertension (coarctation of thoracic aorta) resulted in the development of occlusive atherosclerosis in multiple segments of the COW.[209] Subsequent work by Hollander et al. in 1993 further investigated the induction of ICAS, comparing the effects of diet alone (n=16) versus diet combined with surgically induced hypertension (n=16). Their findings revealed that animals treated solely with an atherogenic diet had smaller lesion scores and lower lesion frequency (n=5; 31%), whereas a higher frequency of complex occlusive lesions was observed in animals (n=15, 94%) treated with both the atherogenic diet and surgically induced hypertension.[222]

The combination of diet and surgically induced hypertension was shown to have a significant impact on promoting severe ICAS development, highlighting the role of blood pressure in the progression of plaque formation. Interestingly, the occurrence of ICAS was found to be only 31% in animals treated with diet alone, which was relatively low compared to the much higher incidence of ICAS (94%) in animals treated with both diet and surgically induced hypertension. It is important to acknowledge that the use of surgical

means to induce hypertension may not fully replicate the natural progression of hypertension and atherosclerosis. However, these initial findings provided early support for the potential of diet induced ICAS in NHPs, a concept that had not been further explored until now.

In our research, we implemented a hypercholesterolemic NHP model that allowed us to study both ECAS and ICAS. Unlike the previous studies mentioned, which utilized a 12-month atherogenic diet feeding period, we opted for 20-month of high fat/high cholesterol diet feeding. This extended duration of dietary intervention had a significant impact on the lipid profile of our animals. Within the first month of being on the diet, we observed a nearly six-fold increase in plasma total cholesterol (TC) levels, primarily driven by proatherogenic low-density lipoprotein cholesterol (LDL-C). These elevated lipid levels closely resembled those typically observed in individuals with heterozygous familial hypercholesterolemia.[66]

There are several factors that may explain the contrasting results between our study and the work of Hollander's group. Firstly, it is worth noting that the diet provided to Hollander's animals was indeed capable of inducing hypercholesterolemia, as evidenced by a mean plasma TC level of 658±52 mg/dL.[222] However, the specific level of LDL-C achieved in their animals is unclear from their report. While sufficient hypercholesterolemia alone was not enough to induce ICAS, one potential explanation for the discrepancy in lesion frequency could be the duration of exposure to the diet or the time spent under hypercholesterolemic conditions. Hollander's study had a duration of 12 months, whereas our study extended to 20 months. The additional eight months of diet and

elevated TC/LDL-C exposure in our study might have played a notable role in promoting the development of ICAS.

However, it is also important to consider the variability observed in primates, both humans and monkeys, in their response to atherogenic diets. This variability is often characterized by hypo- and hyper-responders, reflecting the complex interplay between genetic, environmental, and lifestyle factors in the development of atherosclerotic vascular disease.[237] It is possible that the cohort of animals used in Hollander's 1993 study had a higher proportion of hypo-responders, which could explain why 69% of their animals did not develop ICAS, while in our study, 77% of the animals did.

Hollander et al. (1993) concluded that hypertension played a role in the development and severity of lesions within intracranial arteries.[222] It is feasible given the diet and lack of physical activity that our animals developed hypertension during the course of our study, whereas the animals in Hollander's study not subjugated to aortic coarctation did not. However, we lack any supporting evidence to definitively claim that our animals were hypertensive.

In addition to hypertension, psychosocial stress and depression are known to impact cardiovascular and cerebrovascular health in both humans and NHPs.[206,240]. It is plausible that the environment and social interactions experienced by Hollander's animals were more favorable compared to those in our study, which could have influenced the frequency of ICAS development.

Another factor that may have influenced the disparity in ICAS frequency between our studies is the age of the animals at the beginning and end of the experiment.[208] Our

animals were relatively young (5.2 years) and had reached sexual maturity at the start of the study, but we do not have information on the age of Hollander's animals.

Collectively these observations underscore the multifactorial nature of atherosclerosis, particularly ICAS, and highlight the need for further research to better understand the individual factors that contribute to its development and progression. Moreover, these observations highlight some of the complexity of working with NHPs and the importance of considering variables such as species, diet, lipids, blood pressure, environment, psychosocial factors, and age in the conception and design of future NHP studies. Taking these factors into account can help provide a more comprehensive understanding of the development and progression of atherosclerotic vascular disease in NHP models.

In our study, a total of 47 animals showed gross measures of ICAS upon necropsy, which were further confirmed through histological and immunohistochemical (IHC) analyses. The findings from our IHC studies revealed striking similarities between ICAS lesions in NHPs and humans. While many lesions exhibited characteristics of fibroatheromas, there were also notable instances of advanced atherosclerosis, including the presence of inflammatory foam cells, loss of collagen matrix, initiation of necrotic cores (cholesterol crystals/clefts), intraplaque hemorrhages, and severe to fully occlusive stenosis.[102]

The successful production of a reliable ICAS model in NHPs allowed us to test the efficacy of our anti-miR-33 treatment and evaluate its impact on the cellular and molecular components within intracranial lesions. The model also holds significant promise for studying the mechanisms and potential therapeutic interventions for ICAS, as well as

assessing the effects of targeted treatments on the complex pathology of ICAS in a preclinical model that closely resembles the human condition.

. Despite having a substantial numberDespite having a substantial number of animals with lesions (n=47) and a considerable number of measurable vessels with lesions (n=89) evenly distributed across treatment groups, we did not observe any significant treatment effect with miR-33 antagonism. Contrary to our hypothesis, we did not observe the anticipated signs of plaque stabilization, such as increased collagen matrices, nor did we observe a reduction in inflammatory cells as an indication of plaque regression.[220,241] However, with a return to baseline lipid levels, we noted a general reduction in collagen between the progression and regression arms of the study, with no discernible treatment effect on either collagen or CD68-positive cells between the vehicle and anti-miR-33 treatment groups. These findings suggest that miR-33 antagonism did not have the intended impact on the cellular and molecular components of ICAS lesions in our NHP model.

To assess the cholesterol burden within the arterial wall of the external carotids in all 61 animals, we employed biochemical measures to evaluate free cholesterol (FC) and esterified cholesterol (EC) similar to that performed by Hollander et al. (1993). FC refers to unesterified cholesterol that has not been bound to fatty acids and is believed to directly contribute to the development of atherosclerosis. Elevated levels of FC within the vessel wall may indicate increased inflammation. On the other hand, EC is cholesterol that has been esterified with fatty acids and stored within cells, such as foam cells, playing a role in the progression of atherosclerosis. Higher levels of EC may indicate lesion progression, while decreased EC levels may suggest an improvement in the lipid status within the lesion.[216]

The measurements of different forms of cholesterol within the vascular tissue provided valuable insights into the progression and severity of atherosclerosis, as well as the potential impact of miR-33 antagonism on relieving cholesterol burden. We observed significant increases in FC and EC during the progression period, indicating the accumulation of cholesterol within the arterial wall. The reduction in tissue cholesterol levels was found to be associated with the duration of time on the cholesterol-lowering diet, with levels returning to baseline after 24 weeks. However, no significant differences were observed between the vehicle and anti-miR-33 treated groups, suggesting that miR-33 antagonism did not have a measurable effect on cholesterol reduction within the lesions or vessel wall. These findings are consistent with the results of our histology and immunohistochemistry studies, providing no clear evidence to support the hypothesis that miR-33 antagonism promotes the regression of either carotid or intracranial atherosclerosis.

In our efforts to assess the effectiveness of miR-33 antagonism, we also studied changes in the expression of miR-33 target genes through bulk RNA sequencing of carotid tissue. Contrary to our expectations, none of the miR-33 targets displayed significant changes in expression between the vehicle and anti-miR-33 treated groups, as determined by false discovery rate (FDR) analysis. We had hypothesized that the miR-33 ASO treatment would have boosted the expression of numerous target genes, yet this wasn't the case.

The lack of influence on target gene expression could be due to several factors that warrant further research. For instance, it is possible that the dosage of the ASO administered was not sufficient to effectively inhibit miR-33 or, alternatively, that miR-33

was not expressed at levels high enough to notably affect target gene expression in the cynomolgus monkey model. Understanding these intricacies is crucial, as they could potentially influence the role of miR-33 in the context of atherosclerotic plaque formation and progression in this model. This is an important area of focus for future investigation in the quest for improved therapeutic strategies against atherosclerosis.

Although not specifically addressed in chapter 2, we did make efforts to validate the effectiveness of the anti-miR-33 treatment. For instance, early analysis of liver tissue using Western blotting revealed an increase after 24 weeks of anti-miR-33 treatment in ABCA1 expression, a primary target of miR-33. This increase was also reflected in a significant elevation of HDL-C levels in the plasma of anti-miR-33 treated animals compared to those treated with the vehicle. These findings were aligned with past observations using the miR-33 antagonist in mice and NHPs.[188,221] Additionally, preliminary IHC analysis of vessel cross sections indicated the presence of the anti-miR-33 antisense oligonucleotide (ASO) in vascular tissue, suggesting its localization in atherosclerotic lesions. However, despite these preliminary observations, the overall evidence in the end was insufficient to support our hypothesis, leading us to conclude that miR-33 antagonism had no direct effect on the regression of ICAS in NHPs.

Our study has successfully established a reliable animal model of atherosclerosis with both ECAS and ICAS l. This comprehensive representation of atherosclerosis along the cardiovascular-cerebrovascular axis is a significant achievement. Furthermore, our comprehensive histological studies characterizing ICAS in this unique model have provided compelling evidence that a healthy diet and maintenance of optimal plasma cholesterol levels has the potential to modify ICAS lesion composition and cholesterol

levels in as little as 6 weeks. These findings highlight the importance of a healthy diet in reducing the risk of ICAS, stroke, and VCI.

While our =study of miR-33 antagonism in an NHP model did not yield the expected result, our study still contributes valuable insights to the field. It emphasizes the significance of diet and plasma cholesterol in ICAS progression, and our unique model opens additional avenues for further research to develop targeted interventions and preventive strategies for managing ICAS and its associated complications.

3.1.3 Trends in Current Therapeutics for the Treatment and Management of ICAS and Associated Complications

The therapeutic landscape for ICAS consists of two major categories: pharmacological and non-pharmacological approaches. By combining both pharmacological and non-pharmacological approaches, comprehensive management strategies can be developed to address the multifactorial nature of ICAS and optimize patient outcomes.

Pharmacological approaches include medications such as antiplatelet therapies (aspirin, clopidogrel), anticoagulants, and lipid-lowering agents (statins). These medications aim to reduce thrombus formation, slow down the progression of atherosclerosis, and stabilize existing plaques.[242–244] Blood pressure control is also crucial in ICAS management, and medications like angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), or calcium channel blockers may be prescribed to achieve optimal cerebral perfusion and lower the risk of stroke.[245,246] Individualized treatment plans may include additional medications based on specific

patient needs, such as glycemic control agents for diabetes management, and symptomatic treatment options for headache or dizziness.

Non-pharmacological interventions focus on lifestyle modifications. These include adopting a balanced diet, regular physical exercise, and quitting tobacco use.[181,238] Lifestyle changes also target the management of co-existing conditions like hypertension, diabetes, and obesity, aiming to address the underlying causes of atherosclerosis and reduce overall cardiovascular and cerebrovascular risk. These interventions target fundamental etiological factors contributing to ICAS and aim to improve long-term outcomes.

While therapeutic strategies aimed at addressing the underlying causes of ICAS show promise, there is still a residual risk associated with the development of stroke and other cardiovascular events, even with optimal treatment adherence. This residual risk can be attributed to various factors, including the progression of atherosclerosis, the variability in patient response to therapy, and potential complications arising from concurrent medical conditions.

Despite efforts to slow down or halt the progression of atherosclerosis, the disease may continue to advance in some individuals, leading to the development of new lesions or the worsening of existing ones. This ongoing progression can increase the risk of plaque rupture or thrombosis, ultimately leading to adverse events with long term consequences, such as stroke with diminished motor and cognitive capacity.

Additionally, individual patients may exhibit variations in their response to therapy. Factors such as genetic predisposition, underlying comorbidities, and lifestyle choices can influence how a patient's body interacts with the prescribed treatment. This interindividual

variability can contribute to differences in clinical outcomes and the residual risk of an atherosclerotic related event.

Furthermore, concurrent medical conditions can complicate the clinical management of ICAS, contributing to the residual risk. Conditions such as hypertension, diabetes, and renal disease can interact with ICAS, exacerbating its progression and increasing the risk for vulnerable plaques to rupture. Additionally, the presence of comorbidities may require complex treatment strategies, increasing the potential for medication interactions and non-compliance contributing to undesired outcomes.

Addressing the residual risk associated with ICAS requires a comprehensive approach that encompasses ongoing monitoring of disease progression, personalized treatment strategies, and the management of comorbidities. Further research is needed to better understand the underlying mechanisms contributing to this risk and to develop targeted interventions that can effectively mitigate it.

3.1.4 Emerging Therapeutics for the Treatment of Atherosclerosis

Emerging therapies for the treatment of atherosclerosis aim to address the residual risk that remains from traditional common therapies used today. For example, we previously discussed in chapter 1 the use of PCSK9 inhibitors that further lower LDL cholesterol levels beyond what can be achieved with statins alone. These inhibitors work by targeting and inhibiting PCSK9, a protein that plays a role in the regulation of LDL receptor recycling and degradation, thereby increasing the number of LDL receptors available to clear LDL cholesterol from the bloodstream. Clinical trials have demonstrated

that PCSK9 inhibitors can significantly reduce LDL cholesterol levels and cardiovascular events in high-risk patients.[247]

Similarly, the development of anti-inflammatory therapies, including targeted therapies against specific inflammatory pathways, aims to address the inflammatory component of atherosclerosis. Chronic inflammation plays a critical role in the initiation, progression, and destabilization of atherosclerotic plaques.[248] By targeting specific inflammatory pathways, such as interleukin-1β (IL-1β) or the NLRP3 inflammasome, these therapies seek to reduce plaque inflammation and stabilize vulnerable plaques.

Other emerging therapies, such as miRNAs and antisense oligonucleotides (ASOs), offer potential avenues to modulate key molecular targets involved in lipoprotein metabolism and plaque regression. ASOs targeting apolipoprotein B (apoB) have shown promise in reducing plasma LDL cholesterol levels and atherosclerosis development in hyperlipidemic mice.[249] Mipomersen, an ASO inhibitor of apoB, has been approved for treating hypercholesterolemia in individuals with familial hypercholesterolemia (FH).[250] Clinical trials using Mipomersen have demonstrated notable reductions in apoB-containing lipoproteins and plasma LDL-C in FH patients.[251,252] Further research is needed to determine the efficacy of apoB suppression in regressing atherosclerotic lesions in humans and to address safety concerns.[253]

The main treatment strategy presented in this dissertation involved the use of ASOs to inhibit miRNAs, specifically miR-33, as a potential approach for regressing atherosclerosis. I would like to briefly revisit the rationale behind the proposal of ASOs that antagonize miR-33 as a promising treatment strategy for promoting the regression of atherosclerosis. The miR-33 family consists of two members, miR-33a and miR-33b,

encoded within intron 16 of sterol regulatory element binding factor 2 (SREBF2) and intron 17 of SREBF1.[189,254]

MicroRNA-33, specifically miR-33a and miR-33b, play a significant role in regulating cellular lipid homeostasis. These microRNAs bind to the 3' untranslated region of target mRNAs, leading to their degradation or inhibition of translation.[186,217] By doing so, miR-33a and miR-33b function in coordination with their host genes to suppress the expression of important genes involved in fatty acid oxidation and cholesterol efflux. This regulatory mechanism has widespread effects on maintaining lipid balance within cells.

It has previously been demonstrated that antagonizing miR-33 in mice can effectively reduce the burden of aortic atherosclerosis by promoting regression or stabilization of existing plaques.[220] This favorable outcome is closely associated with the regulation of ATP binding cassette transporter A1 (ABCA1), a target of miR-33. ABCA1 plays a crucial role in the formation of nascent high-density lipoprotein (HDL) particles and the efflux of cellular cholesterol from foam cells onto discoidal HDL particles.[90,255] Maintaining proper expression of ABCA1 at both the liver and vessel wall is believed to facilitate the removal of cholesterol from atherosclerotic plaques by promoting the efflux of cholesterol from foam cells to circulating HDL, ultimately facilitating its return to the liver.

It has been established that miR-33 binds to ABCA1 mRNA, thereby repressing its anti-atherogenic function.[186] Genetic deletion or inhibition of miR-33 in mice has been shown to enhance hepatic ABCA1 expression and increase circulating HDL-C levels by 25-30%.[189–191] Additionally, antagonizing miR-33 promotes fatty acid oxidation and induces M2 macrophage polarization, promoting an anti-inflammatory and pro-resolving

environment within atherosclerotic lesions.[188] Moreover, miR-33 antagonism has been shown to reduce plaque necrosis by upregulating key autophagy proteins in macrophages and enhancing phagocytic efferocytosis for the clearance of apoptotic debris.[191,219]

It is important to highlight that these studies demonstrating the therapeutic potential of miR-33 antagonism were primarily conducted in mice, which naturally express only miR-33a. However, in primates, including humans and monkeys, both miR-33a and miR-33b isoforms are expressed.[189,254] Having both miR-33a and miR-33b, NHPs are well-suited for investigating the full effectiveness of miR-33 antagonism in stabilizing and/or regressing atherosclerotic plaques due to their closer resemblance to the human physiological context. Our group and others have utilized anti-miR-33 ASO treatment to inhibit both miR-33a and miR-33b in healthy NHPs.[221,228]

Compared to healthy control animals treated with saline vehicle, inhibition of both isoforms of miR-33 using the anti-miR-33 ASO (Regulus [RG428651]) resulted in increased hepatic ABCA1 expression and circulating HDL-C levels, which remained elevated even after the animals were exposed to a high carbohydrate/moderate cholesterol diet.[221,228] Furthermore, animals treated with the ASO exhibited a more favorable lipoprotein profile, characterized by HDL with an enhanced capacity for cholesterol efflux when compared to the vehicle treated groups.[221]

In our study presented in this dissertation, we observed an increase in hepatic ABCA1 expression and circulating plasma HDL-C levels following miR-33 antagonism. However, despite these effects, we did not observe any additional benefits in terms of reducing lesion size or altering plaque composition. These findings were inconsistent with previous studies in mice that supported the role of miR-33 antagonism in promoting the

regression of atherosclerotic lesions and resolving plaque inflammation. A potential limitation of our study lies in the infrequency of macrophage-laden lesions and necrotic core formations within the ICAS lesions. This infrequency limited our ability to fully assess other markers of regression that are associated with improvements in efferocytosis and apoptosis. Understanding these biological processes in greater depth is critical, as they play integral roles in the development, progression, and potential regression of atherosclerotic disease. Future studies with an increased prevalence of such lesions could offer further insight into the role of miR-33 antagonism in the context of these processes.

By addressing the underlying mechanisms of atherosclerosis, including lipid metabolism, inflammation, and plaque stability, emerging therapies have the potential to further reduce the residual risk that remains despite traditional therapies. However, challenges exist in the development and clinical translation of these novel therapies, including the need for effective strategies to assess their efficacy in both experimental models and humans. Collaborative efforts among academia, industry, and regulatory agencies are crucial to address these challenges and expedite the development and implementation of these therapies into clinical practice.

By employing improved methodologies and assessing a broader range of technologies to assess cellular and molecular indicators of plaque progression and regression we can gain a more comprehensive understanding of the mechanisms underlying atherosclerosis and test the efficacy of novel strategies for targeted intervention, ultimately aiming to reduce the burden of ECAS and ICAS and improve patient outcomes.

3.2 Closing Remarks

This dissertation has provided a detailed characterization of intracranial atherosclerosis (ICAS) within a diet-induced atherosclerosis model in NHPs. The mere discovery of ICAS in an animal model represents a significant advancement in cerebrovascular research, offering a framework for further investigations into the underlying mechanisms of ICAS and the evaluation of potential therapeutic interventions. Additionally, this dissertation has shed light on the potential links between ICAS and vascular neuropathologies associated with stroke and vascular cognitive impairment (VCI).

However, our findings also highlight the multifaceted nature of ICAS and emphasize the need for a comprehensive approach in its study. Despite initial hypotheses, we did not observe regression of ICAS with miR-33 antagonism, underscoring the complexity of the disease and the importance of further research into the role of miR-33 and other factors in ICAS progression.

To advance further understanding of ICAS for the development of treatment strategies, it is imperative to consider various factors behind atherosclerosis progression, including lipid metabolism, inflammation, and plaque stability. Assessing these factors will rely heavily on new technologies that allow us to monitor the progression and regression of atherosclerosis in situ. Promising advancements in current imaging technologies, such as intravascular ultrasound (IVUS), optical coherence tomography (OCT), and molecular imaging techniques, hold the potential to revolutionize our ability to visualize and characterize atherosclerotic plaques at a cellular and molecular level in living organisms.

In addition to the aforementioned in vivo imaging advancements, the fields of microscopy, digital pathology, and machine learning hold great potential for accelerating the

visualization of whole vessel morphology and composition ex vivo. These technologies offer promising solutions to address current limitations by providing a wider spatial view of the atherosclerosis burden.

Microscopy techniques, such as multiphoton microscopy and confocal microscopy, allow for high-resolution imaging of vascular tissue, enabling researchers to examine the nuances of cellular and subcellular structures within atherosclerotic lesions. These approaches have the potential to provide valuable insights into the spatial distribution of plaque components, including lipid deposits, inflammatory cells, and extracellular matrix proteins.

Digital pathology leverages digital imaging and computer vision to transform traditional histopathology slides into archivable and widely assessable digital formats. This enables remote access to pathological data and facilitates the integration of quantitative image analysis algorithms across collaborators. Moreover, by leveraging machine learning and artificial intelligence, digital image formats allow for automated segmentation, identification, and classification of plaque features, providing a more efficient and standardized approach to atherosclerotic plaque characterization.

Machine learning is exciting, and algorithms have shown enormous promise in the analysis of large-scale datasets, aiding in the identification of patterns and predictive modeling previously unseen. By training deep learning algorithms on annotated datasets, machine learning algorithms can learn to recognize and quantify specific plaque characteristics, such as macrophage burden, fibrous cap thickness, and lipid content. This rapidly growing technology has the potential to enhance our understanding of plaque composition and vulnerability and assist in predicting the response to therapeutic interventions.

By harnessing the power of both in vivo and ex vivo imaging technologies, researchers can gain a more comprehensive understanding of atherosclerosis by examining plaque burden and composition across the entire vessel, rather than relying solely on localized assessments. This broader spatial view can provide valuable insights into the distribution and heterogeneity of plaques within the vascular system, aiding in risk stratification of atherosclerotic plaques and the development and monitoring of targeted therapies.

Furthermore, rapid advancements in molecular biomarkers, spatial genomics, metabolomics, and proteomics provide exciting opportunities to explore the intricate molecular pathways involved in both the progression and regression of atherosclerosis. By studying gene expression profiles, protein markers, and cellular signaling pathways within atherosclerotic plaques, we may identify potential targets for therapeutic interventions and assess the efficacy of novel treatment strategies.

The complexity of atherosclerosis necessitates an integrative approach that combines various imaging modalities, computer-based technologies, molecular techniques, and preclinical models. Furthermore, translating these findings will require a collaborative effort among academia, industry, and regulatory agencies to address these complexities and expedite the development and implementation of these therapies into both clinical trials and practice.

A long road remains but despite these challenges, we remain optimistic. With continued research and collaborative efforts, we are confident that we can gain a more comprehensive understanding of ICAS, develop effective strategies for targeted interventions, and ultimately improve patient outcomes. The discovery and characterization of ICAS in NHPs presented in this dissertation serve as a steppingstone for future research in the field of

ICAS, contributing to the collective effort to alleviate the burden of this disease and enhance the quality of life for individuals worldwide.

REFERENCES

1. Roth GA, Mensah GA, Johnson CO, et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019: Update From the GBD 2019 Study. *Journal of the American College of Cardiology*. Published online 2020. doi:10.1016/j.jacc.2020.11.010

2. Brown JC, Gerhardt TE, Kwon E. Risk Factors For Coronary Artery Disease. *null*. Published online 2020. doi:null

3. Tsao C, Aday A, Almarzooq ZI, et al. Heart Disease and Stroke Statistics—2023 Update: A Report From the American Heart Association. *Circulation*. Published online 2023. doi:10.1161/cir.0000000000001123

4. Klimchak AC, Patel MY, Iorga ŞR, Iorga ŞR, Kulkarni N, Wong ND. Lipid treatment and goal attainment characteristics among persons with atherosclerotic cardiovascular disease in the United States. *American Journal of Preventive Cardiology*. Published online 2020. doi:10.1016/j.ajpc.2020.100010

5. Vaduganathan M, Mensah GA, Turco JV, Fuster V, Roth GA. The Global Burden of Cardiovascular Diseases and Risk: A Compass for Future Health. *Journal of the American College of Cardiology*. Published online 2022. doi:10.1016/j.jacc.2022.11.005

6. Kannel WB, Dawber TR, Kagan A, Kagan A, Revotskie N, Stokes J. Factors of Risk in the Development of Coronary Heart Disease—Six-Year Follow-up Experience: The Framingham Study. *Annals of Internal Medicine*. Published online 1961. doi:10.7326/0003-4819-55-1-33

7. Keys A, Menotti A, Mj K, et al. The diet and 15-year death rate in the seven countries study. *American Journal of Epidemiology*. Published online 1986. doi:10.1093/oxfordjournals.aje.a114480

8. Hu FB, Rimm EB, Stampfer MJ, Ascherio A, Spiegelman D, Willett WC. Prospective study of major dietary patterns and risk of coronary heart disease in men. *The American Journal of Clinical Nutrition*. Published online 2000. doi:10.1093/ajcn/72.4.912

9. Arnold MJ, Harding MC, Conley AT. Dietary Guidelines for Americans 2020-2025: Recommendations from the U.S. Departments of Agriculture and Health and Human Services. *American Family Physician*. Published online 2021. doi:null

10. Haskell WL, Lee IM, Pate RR, et al. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Circulation*. 2007;116(9):1081-1093. doi:10.1161/CIRCULATIONAHA.107.185649

11. Manson JE, Greenland P, LaCroix AZ, et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women. *The New England Journal of Medicine*. Published online 2002. doi:10.1056/nejmoa021067

12. Sesso HD, Paffenbarger RS, Lee IM. Physical activity and coronary heart disease in men : The Harvard Alumni Health Study. *Circulation*. Published online 2000. doi:10.1161/01.cir.102.9.975

13. Ford ES, Ford ES. Does exercise reduce inflammation? Physical activity and C-reactive protein among U.S. adults. *Epidemiology*. Published online 2002. doi:10.1097/00001648-200209000-00012

14. Wang Y ting, Wang Y, Xu D. Effects of aerobic exercise on lipids and lipoproteins. *Lipids in Health and Disease*. Published online 2017. doi:10.1186/s12944-017-0515-5

15. Franczyk B, Gluba-Brzózka A, Ciałkowska-Rysz A, Ławiński J, Rysz J. The Impact of Aerobic Exercise on HDL Quantity and Quality: A Narrative Review. *International Journal of Molecular Sciences*. Published online 2023. doi:10.3390/ijms24054653

16. Kodama S, Kodama S, Kodama S, et al. EFFECT OF AEROBIC EXERCISE TRAINING ON SERUM LEVELS OF HIGH-DENSITY LIPOPROTEIN CHOLESTEROL: A META-ANALYSIS. *JAMA Internal Medicine*. Published online 2007. doi:10.1001/archinte.167.10.999

17. Hruby A, Hu FB. The Epidemiology of Obesity: A Big Picture. *PharmacoEconomics*. Published online 2015. doi:10.1007/s40273-014-0243-x

18. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of Obesity and Severe Obesity Among Adults: United States, 2017-2018. *NCHS data brief*. Published online 2020. doi:null

19. Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. *Journal of Clinical Investigation*. Published online 2017. doi:10.1172/jci92035

20. Després JP. Health consequences of visceral obesity. *Annals of Medicine*. Published online 2001. doi:10.3109/07853890108995963

21. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. Published online 2006. doi:10.1038/nature05485

22. Kershaw. Adipose Tissue as an Endocrine Organ. *American Family Physician*. Published online 2005. doi:10.1210/jc.2004-0395

23. Thethi TK, Kamiyama M, Kobori H. The link between the renin-angiotensin-aldosterone system and renal injury in obesity and the metabolic syndrome. *Current Hypertension Reports*. Published online 2012. doi:10.1007/s11906-012-0245-z

24. Adiels M, Olofsson SO, Taskinen MR, Nielsen J, Borén J. Overproduction of Very Low–Density Lipoproteins Is the Hallmark of the Dyslipidemia in the Metabolic Syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2008. doi:10.1161/atvbaha.107.160192

25. Klop B, Elte JWF, Cabezas MC. Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients*. Published online 2013. doi:10.3390/nu5041218

26. Saeedi P, Saeedi P, Saeedi P, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Research and Clinical Practice*. Published online 2019. doi:10.1016/j.diabres.2019.107843

27. Canto ED, Ceriello A, Rydén L, et al. Diabetes as a cardiovascular risk factor: An overview of global trends of macro and micro vascular complications. *European Journal of Preventive Cardiology*. Published online 2019. doi:10.1177/2047487319878371

28. Nishikawa T, Edelstein D, Du XL, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. Published online 2000. doi:10.1038/35008121

29. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. Published online 2001. doi:10.1038/414813a

30. Goldin A, Beckman JA, Schmidt AM, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation*. Published online 2006. doi:10.1161/circulationaha.106.621854

31. Souilhol C, Serbanovic-Canic J, Fragiadaki M, et al. Endothelial responses to shear stress in atherosclerosis: a novel role for developmental genes. *Nature Reviews Cardiology*. Published online 2020. doi:10.1038/s41569-019-0239-5

32. Taskinen MR. Diabetic dyslipidaemia: from basic research to clinical practice*. *Diabetologia*. Published online 2003. doi:10.1007/s00125-003-1111-y

33. Golbidi S, Laher I. Exercise Induced Adipokine Changes and the Metabolic Syndrome. *Experimental Diabetes Research*. Published online 2014. doi:10.1155/2014/726861

34. Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. *The Lancet*. Published online 2017. doi:10.1016/s0140-6736(17)30058-2

35. Kakouros N, Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet Function in Patients with Diabetes Mellitus: From a Theoretical to a Practical Perspective. *International Journal of Endocrinology*. Published online 2011. doi:10.1155/2011/742719

36. DeFronzo RA. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia*. Published online 2010. doi:10.1007/s00125-010-1684-1

37. Wang L, Li X, Wang Z, et al. Trends in Prevalence of Diabetes and Control of Risk Factors in Diabetes among US Adults, 1999-2018. *JAMA*. Published online 2021. doi:10.1001/jama.2021.9883

38. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart Disease and Stroke Statistics—2016 Update: A Report From the American Heart Association. *Circulation*. Published online 2016. doi:10.1161/cir.0000000000000350

39. Zhou B, Bentham J, Cesare MD, et al. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *The Lancet*. Published online 2017. doi:10.1016/s0140-6736(16)31919-5

40. Whelton PK, Carey RM, Aronow WS, et al. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Hypertension*. doi:10.1161/hyp.0000000000000066

41. Fuchs FD, Whelton PK. High Blood Pressure and Cardiovascular Disease. *Hypertension*. Published online 2020. doi:10.1161/hypertensionaha.119.14240

42. Law M, Wald NJ, Morris JK, Jordan R. Value of low dose combination treatment with blood pressure lowering drugs: analysis of 354 randomised trials. *BMJ*. Published online 2003. doi:10.1136/bmj.326.7404.1427

43. Davignon J, Davignon J, Ganz P. Role of Endothelial Dysfunction in Atherosclerosis. *Circulation*. Published online 2004. doi:10.1161/01.cir.0000131515.03336.f8

44. Chiu JJ, Chiu JJ, Chien S. Effects of Disturbed Flow on Vascular Endothelium: Pathophysiological Basis and Clinical Perspectives. *Physiological Reviews*. Published online 2011. doi:10.1152/physrev.00047.2009

45. Topper JN, Cai J, Falb D, Gimbrone MA. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proceedings of the National Academy of*

Sciences of the United States of America. Published online 1996. doi:10.1073/pnas.93.19.10417

46. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA*. Published online 1999. doi:10.1001/jama.282.21.2035

47. Chistiakov DA, Orekhov AN, Bobryshev YV. Effects of shear stress on endothelial cells: go with the flow. *Acta Physiologica*. Published online 2017. doi:10.1111/apha.12725

48. Provost P, Tremblay J, Merhi Y. The Antiadhesive and Antithrombotic Effects of the Nitric Oxide Donor SIN-1 Are Combined With a Decreased Vasoconstriction in a Porcine Model of Balloon Angioplasty. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 1997. doi:10.1161/01.atv.17.9.1806

49. SenBanerjee S, Lin Z, Atkins GB, et al. KLF2 is a novel transcriptional regulator of endothelial proinflammatory activation. *Journal of Experimental Medicine*. Published online 2004. doi:10.1084/jem.20031132

50. Cunningham KS, Cunningham KS, Gotlieb AI, Gotlieb AI. The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation*. Published online 2005. doi:10.1038/labinvest.3700215

51. Vardell E. Global Health Observatory Data Repository. *Medical Reference Services Quarterly*. Published online 2020. doi:10.1080/02763869.2019.1693231

52. Ference BA, Ginsberg HN, Graham IT, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *European Heart Journal*. Published online 2017. doi:10.1093/eurheartj/ehx144

53. Olson RE. Discovery of the Lipoproteins, Their Role in Fat Transport and Their Significance as Risk Factors. *Journal of Nutrition*. Published online 1998. doi:10.1093/jn/128.2.439s

54. Steinberg D, Steinberg D. Thematic review series: The Pathogenesis of Atherosclerosis. An interpretive history of the cholesterol controversy: part II: the early evidence linking hypercholesterolemia to coronary disease in humans. *Journal of Lipid Research*. Published online 2005. doi:10.1194/jlr.r400012-jlr200

55. Siri-Tarino PW, Krauss RM. The early years of lipoprotein research: from discovery to clinical application. *Journal of Lipid Research*. Published online 2016. doi:10.1194/jlr.r069575

56. Feingold KR, Feingold KR, Grunfeld C, Grunfeld C. Introduction to Lipids and Lipoproteins. *null*. Published online 2015. doi:null

57. Venugopal SK, Jialal I. Biochemistry, Low Density Lipoprotein. *null*. Published online 2019. doi:null

58. Bailey A, Bailey A, Mohiuddin SS, Mohiuddin SS. Biochemistry, High Density Lipoprotein. *null*. Published online 2019. doi:null

59. Mora S, Glynn RJ, Ridker PM. High-density lipoprotein cholesterol, size, particle number, and residual vascular risk after potent statin therapy. *Circulation*. Published online 2013. doi:10.1161/circulationaha.113.002671

60. Davidson WS. HDL-C vs HDL-P: How Changing One Letter Could Make a Difference in Understanding the Role of High-Density Lipoprotein in Disease. *Clinical Chemistry*. Published online 2014. doi:10.1373/clinchem.2014.232769

61. None &NA; EXECUTIVE SUMMARY OF THE THIRD REPORT OF THE NATIONAL CHOLESTEROL EDUCATION PROGRAM (NCEP) EXPERT PANEL ON DETECTION, EVALUATION AND TREATMENT OF HIGH BLOOD CHOLESTEROL IN ADULTS. *Infectious Diseases in Clinical Practice*. doi:10.1097/00019048-200106000-00021

62. Brown MS, Brown MS, Brown MS, Goldstein JL. Receptor-Mediated Control of Cholesterol Metabolism. *Science*. Published online 1976. doi:10.1126/science.174194

63. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation*. Published online 2002. doi:10.1172/jci15593

64. Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Human Mutation*. Published online 1992. doi:10.1002/humu.1380010602

65. Cuchel M, Bruckert E, Ginsberg HN, et al. Homozigot ailevi hiperkolesterolemi klinisyenlerin taniyi ve klinik yonetimi gelistirmelerine yonelik yeni anlayislar ve rehberlik avrupa ateroskleroz dernegi nin ailevi hiperkolesterolemi uzerine uzlasi paneli yazili gorusu. *European Heart Journal*. Published online 2015. doi:10.1093/eurheartj/ehu274

66. Nordestgaard BG, Chapman MJ, Chapman MJ, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *European Heart Journal*. Published online 2013. doi:10.1093/eurheartj/eht273

67. Abifadel M, Varret M, Varret M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature Genetics*. Published online 2003. doi:10.1038/ng1161

68. Cohen JC, Boerwinkle E, Mosley TH, Hobbs HH. Sequence Variations in PCSK9, Low LDL, and Protection against Coronary Heart Disease. *The New England Journal of Medicine*. Published online 2006. doi:10.1056/nejmoa054013

69. Vega GL, Grundy SM. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. *Journal of Clinical Investigation*. Published online 1986. doi:10.1172/jci112729

70. Innerarity TL, Mahley RW, Weisgraber KH, et al. Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. *Journal of Lipid Research*. Published online 1990. doi:null

71. Hao W, Friedman A. The LDL-HDL profile determines the risk of atherosclerosis: a mathematical model. *PLOS ONE*. Published online 2014. doi:10.1371/journal.pone.0090497

72. Whitfield AJ, Barrett PHR, Bockxmeer FM van, Burnett JR. Lipid Disorders and Mutations in the APOB Gene. *Clinical Chemistry*. Published online 2004. doi:10.1373/clinchem.2004.038026

73. Libby P. The changing landscape of atherosclerosis. *Nature*. Published online 2021. doi:10.1038/s41586-021-03392-8

74. Peacock JM, Arnett DK, Atwood LD, et al. Genome Scan for Quantitative Trait Loci Linked to High-Density Lipoprotein Cholesterol The NHLBI Family Heart Study. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2001. doi:10.1161/hq1101.097804

75. Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH. Multiple Rare Alleles Contribute to Low Plasma Levels of HDL Cholesterol. *Science*. Published online 2004. doi:10.1126/science.1099870

76. Sharrett AR, Sharrett AR, Sharrett AR, et al. Coronary Heart Disease Prediction From Lipoprotein Cholesterol Levels, Triglycerides, Lipoprotein(a), Apolipoproteins A-I and B, and HDL Density Subfractions The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation*. Published online 2001. doi:10.1161/hc3501.095214

77. Wilson PWF, Wilson PWF, Wilson PWF, et al. Prevalence of coronary heart disease in the framingham offspring study: Role of lipoprotein cholesterols. *American Journal of Cardiology*. Published online 1980. doi:10.1016/0002-9149(80)90516-0

78. Barter PJ, Caulfield MJ, Eriksson M, et al. Effects of Torcetrapib in Patients at High Risk for Coronary Events. *The New England Journal of Medicine*. Published online 2007. doi:10.1056/nejmoa0706628

79. Muntner P, Lee F, Astor BC. Association of high-density lipoprotein cholesterol with coronary heart disease risk across categories of low-density lipoprotein cholesterol:

the atherosclerosis risk in communities study. *The American Journal of the Medical Sciences*. Published online 2011. doi:10.1097/maj.0b013e3181f97e4a

80. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nature Reviews Immunology*. Published online 2015. doi:10.1038/nri3793

81. Cordero A, Muñoz-García N, Padró T, et al. HDL Function and Size in Patients with On-Target LDL Plasma Levels and a First-Onset ACS. *International Journal of Molecular Sciences*. Published online 2023. doi:10.3390/ijms24065391

82. Ouimet M, Berger JS, Barrett TJ, Fisher EA. HDL and Reverse Cholesterol Transport. *Circulation Research*. Published online 2019. doi:10.1161/circresaha.119.312617

83. Rye KA, Bursill CA, Lambert G, Tabet F, Barter PJ. The metabolism and anti-atherogenic properties of HDL. *Journal of Lipid Research*. Published online 2009. doi:10.1194/jlr.r800034-jlr200

84. Yuhanna IS, Zhu Y, Zhu Y, et al. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nature Medicine*. Published online 2001. doi:10.1038/89986

85. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High Density Lipoprotein-induced Endothelial Nitric-oxide Synthase Activation Is Mediated by Akt and MAP Kinases *. *Journal of Biological Chemistry*. Published online 2003. doi:10.1074/jbc.m211394200

86. Mineo C, Deguchi H, Griffin JH, Shaul PW. Endothelial and Antithrombotic Actions of HDL. *Circulation Research*. Published online 2006. doi:10.1161/01.res.0000225982.01988.93

87. Rader DJ, Hovingh GK. HDL and cardiovascular disease. *The Lancet*. Published online 2014. doi:10.1016/s0140-6736(14)61217-4

88. Kontush A, Chantepie S, Chapman MJ. Small, Dense HDL Particles Exert Potent Protection of Atherogenic LDL Against Oxidative Stress. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2003. doi:10.1161/01.atv.0000091338.93223.e8

89. Gordon SM, Deng J, Deng J, Lu LJ, Davidson WS. Proteomic Characterization of Human Plasma High Density Lipoprotein Fractionated by Gel Filtration Chromatography. *Journal of Proteome Research*. Published online 2010. doi:10.1021/pr100520x

90. Oram JF, Heinecke JW. ATP-Binding Cassette Transporter A1: A Cell Cholesterol Exporter That Protects Against Cardiovascular Disease. *Physiological Reviews*. Published online 2005. doi:10.1152/physrev.00005.2005

91. Tall AR. Plasma cholesteryl ester transfer protein. *Journal of Lipid Research*. Published online 1993. doi:10.1016/s0022-2275(20)36957-1

92. Tall AR, Tall AR, Rader DJ. Trials and Tribulations of CETP Inhibitors. *Circulation Research*. Published online 2018. doi:10.1161/circresaha.117.311978

93. McIntyre N. Familial LCAT Deficiency and Fish-Eye Disease. *Journal of Inherited Metabolic Disease*. Published online 1988. doi:10.1007/bf01800570

94. Rader DJ. Lecithin: Cholesterol Acyltransferase and Atherosclerosis Another High-Density Lipoprotein Story That Doesn't Quite Follow the Script. *Circulation*. Published online 2009. doi:10.1161/circulationaha.109.881979

95. Rohatgi A, Westerterp M, Eckardstein A von, Remaley AT, Rye KA. HDL in the 21st Century: A Multifunctional Roadmap for Future HDL Research. *Circulation*. Published online 2021. doi:10.1161/circulationaha.120.044221

96. Hahn C, Schwartz MA. Mechanotransduction in vascular physiology and atherogenesis. *Nature Reviews Molecular Cell Biology*. Published online 2009. doi:10.1038/nrm2596

97. Gimbrone MA, García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circulation Research*. Published online 2016. doi:10.1161/circresaha.115.306301

98. Wilson D, Wilson DP. Mechanisms of Vascular Disease: Vascular smooth muscle structure and function. *null*. Published online 2011. doi:10.1017/upo9781922064004.003

99. Stenmark KR, Yeager ME, Kasmi KCE, et al. The Adventitia: Essential Regulator of Vascular Wall Structure and Function. *Annual Review of Physiology*. Published online 2013. doi:10.1146/annurev-physiol-030212-183802

100. Miano JM, Fisher EA, Majesky MW. Fate and State of Vascular Smooth Muscle Cells in Atherosclerosis. *Circulation*. Published online 2021. doi:10.1161/circulationaha.120.049922

101. Plasschaert H, Heeneman S, Daemen MJAP. Progression in atherosclerosis: histological features and pathophysiology of atherosclerotic lesions. *Topics in Magnetic Resonance Imaging*. Published online 2009. doi:10.1097/rmr.0b013e3181ea2869

102. Virmani R, Kolodgie FD, Burke AP, et al. Atherosclerotic Plaque Progression and Vulnerability to Rupture Angiogenesis as a Source of Intraplaque Hemorrhage. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2005. doi:10.1161/01.atv.0000178991.71605.18

103. Stary HC, Stary HC, Chandler AB, et al. A Definition of Advanced Types of Atherosclerotic Lesions and a Histological Classification of Atherosclerosis A Report From the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. Published online 1995. doi:10.1161/01.cir.92.5.1355

104. Napoli C, D'Armiento FP, D'Armiento FP, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *Journal of Clinical Investigation*. Published online 1997. doi:10.1172/jci119813

105. Wissler RW. USA Multicenter Study of the pathobiology of atherosclerosis in youth. *Annals of the New York Academy of Sciences*. Published online 1991. doi:10.1111/j.1749-6632.1991.tb43716.x

106. Williams KJ, Tabas I. The Response-to-Retention Hypothesis of Early Atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 1995. doi:10.1161/01.atv.15.5.551

107. Nakashima Y, Fujii H, Sumiyoshi S, Wight TN, Sueishi K. Early human atherosclerosis : Accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2007. doi:10.1161/atvbaha.106.134080

108. Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low-density lipoprotein. *Nature*. Published online 1997. doi:10.1038/386073a0

109. Salonen JT, Salonen JT, Nyyssönen K, et al. Lipoprotein Oxidation and Progression of Carotid Atherosclerosis. *Circulation*. Published online 1997. doi:10.1161/01.cir.95.4.840

110. Moore KJ, Tabas I. Reviewmacrophages in the pathogenesis of atherosclerosis. *Cell*. Published online 2011. doi:10.1016/j.cell.2011.04.005

111. Kolodgie FD, Burke AP, Nakazawa G, Virmani R. Is Pathologic Intimal Thickening the Key to Understanding Early Plaque Progression in Human Atherosclerotic Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2007. doi:10.1161/atvbaha.0000258865.44774.41

112. Bennett MR, Sinha S, Owens GK. Vascular Smooth Muscle Cells in Atherosclerosis. *Circulation Research*. Published online 2016. doi:10.1161/circresaha.115.306361

113. Szeremeta A, Jura-Półtorak A, Zoń-Giebel A, Olczyk K, Komosińska-Vassev K. TNF-α Inhibitors in Combination with MTX Reduce Circulating Levels of Heparan Sulfate/Heparin and Endothelial Dysfunction Biomarkers (sVCAM-1, MCP-1, MMP-9 and ADMA) in Women with Rheumatoid Arthritis. *Journal of Clinical Medicine*. Published online 2022. doi:10.3390/jcm11144213

114. Hofnagel O, Luechtenborg B, Stolle K, et al. Proinflammatory Cytokines Regulate LOX-1 Expression in Vascular Smooth Muscle Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2004. doi:10.1161/01.atv.0000140061.89096.2b

115. Kattoor AJ, Goel A, Mehta JL. LOX-1: Regulation, Signaling and Its Role in Atherosclerosis. *Antioxidants*. Published online 2019. doi:10.3390/antiox8070218

116. Shankman LS, Gomez D, Cherepanova OA, et al. Klf4 dependent phenotypic modulation of smcs plays a key role inatherosclerotic plaque pathogenesis. *Nature Medicine*. Published online 2015. doi:10.1038/nm.3866

117. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons From Sudden Coronary Death A Comprehensive Morphological Classification Scheme for Atherosclerotic Lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2000. doi:10.1161/01.atv.20.5.1262

118. Hutter-Schmid B, Humpel C. Alpha-Smooth Muscle Actin mRNA and Protein Are Increased in Isolated Brain Vessel Extracts of Alzheimer Mice. *Pharmacology*. Published online 2016. doi:10.1159/000448007

119. Feil S, Fehrenbacher B, Lukowski R, et al. Transdifferentiation of Vascular Smooth Muscle Cells to Macrophage-Like Cells During Atherogenesis. *Circulation Research*. Published online 2014. doi:10.1161/circresaha.115.304634

120. Rong JX, Shapiro MS, Trogan E, Fisher EA. Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proceedings of the National Academy of Sciences of the United States of America*. Published online 2003. doi:10.1073/pnas.1735526100

121. Li F, Tian L, McDermott MM, et al. The association of lesion eccentricity with plaque morphology and components in the superficial femoral artery: a high-spatial-resolution, multi-contrast weighted CMR study. *Journal of Cardiovascular Magnetic Resonance*. Published online 2010. doi:10.1186/1532-429x-12-37

122. Bentzon JF, Otsuka F, Otsuka F, et al. Mechanisms of Plaque Formation and Rupture. *Circulation Research*. Published online 2014. doi:10.1161/circresaha.114.302721

123. Bertheloot D, Latz E, Franklin BS. Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. *Cell Mol Immunol*. 2021;18(5):1106-1121. doi:10.1038/s41423-020-00630-3

124. Zimmer S, Zimmer S, Grebe A, Latz E. Danger Signaling in Atherosclerosis. *Circulation Research*. Published online 2015. doi:10.1161/circresaha.116.301135

125. Kojima Y, Kojima Y, Weissman IL, Leeper NJ. The Role of Efferocytosis in Atherosclerosis. *Circulation*. Published online 2017. doi:10.1161/circulationaha.116.025684

126. Jaminon A, Reesink KD, Kroon AA, Schurgers LJ. The Role of Vascular Smooth Muscle Cells in Arterial Remodeling: Focus on Calcification-Related Processes. *International Journal of Molecular Sciences*. Published online 2019. doi:10.3390/ijms20225694

127. Wang X, Khalil RA. Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. *Advances in pharmacology (San Diego)*. Published online 2018. doi:10.1016/bs.apha.2017.08.002

128. Cao G, Xuan X, Hu J, Zhang R, Jin H, Dong H. How vascular smooth muscle cell phenotype switching contributes to vascular disease. *Cell Communication and Signaling*. Published online 2022. doi:10.1186/s12964-022-00993-2

129. Shah PK. Mechanisms of plaque vulnerability and rupture. *Journal of the American College of Cardiology*. Published online 2003. doi:10.1016/s0735-1097(02)02834-6

130. Demer LL, Tintut Y. Vascular Calcification Pathobiology of a Multifaceted Disease. *Circulation*. Published online 2008. doi:10.1161/circulationaha.107.743161

131. Akers EJ, Nicholls SJ, Bartolo BAD. Plaque Calcification: Do Lipoproteins Have a Role? *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2019. doi:10.1161/atvbaha.119.311574

132. Hjortnaes J, New SEP, Aikawa E. Visualizing novel concepts of cardiovascular calcification. *Trends in Cardiovascular Medicine*. Published online 2013. doi:10.1016/j.tcm.2012.09.003

133. Moulton KS, Vakili K, Zurakowski D, et al. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America*. Published online 2003. doi:10.1073/pnas.0730843100

134. Sluimer JC, Daemen MJ. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol*. 2009;218(1):7-29. doi:10.1002/path.2518

135. Herrmann J, Lerman LO, Mukhopadhyay D, Napoli C, Lerman A. Angiogenesis in Atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2006. doi:10.1161/01.atv.0000233387.90257.9b

136. Takaya N, Yuan C, Chu B, et al. Presence of Intraplaque Hemorrhage Stimulates Progression of Carotid Atherosclerotic Plaques A High-Resolution Magnetic Resonance Imaging Study. *Circulation*. Published online 2005. doi:10.1161/circulationaha.104.504167

137. Jinnouchi H, Sato Y, Sakamoto A, et al. Calcium deposition within coronary atherosclerotic lesion: Implications for plaque stability. *Atherosclerosis*. 2020;306:85-95. doi:10.1016/j.atherosclerosis.2020.05.017

138. Torii S, Tanabe K, Sato Y, et al. Eruptive Calcified Nodules as a Potential Mechanism of Acute Coronary Thrombosis and Sudden Death. *Journal of the American College of Cardiology*. Published online 2021. doi:10.1016/j.jacc.2021.02.016

139. Heusch G, Gersh BJ. The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. *European Heart Journal*. Published online 2016. doi:10.1093/eurheartj/ehw224

140. Unnithan AKA, M Das J, Mehta P. Hemorrhagic Stroke. In: *StatPearls*. StatPearls Publishing; 2023. Accessed June 9, 2023. http://www.ncbi.nlm.nih.gov/books/NBK559173/

141. Roger VL, Go AS, Go AS, et al. Heart Disease and Stroke Statistics—2011 Update A Report From the American Heart Association. *Circulation*. Published online 2011. doi:10.1161/cir.0b013e3182009701

142. Feigin VL, Stark BA, Stark BA, et al. Global, regional, and national burden of stroke and its risk factors, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet Neurology*. Published online 2021. doi:10.1016/s1474-4422(21)00252-0

143. Yaghi S, Prabhakaran S, Khatri P, Liebeskind DS. Intracranial Atherosclerotic Disease. *Stroke*. Published online 2019. doi:10.1161/strokeaha.118.024147

144. Yoon C, Bushnell C. Stroke in Women: A Review Focused on Epidemiology, Risk Factors, and Outcomes. *Journal of Stroke*. Published online 2023. doi:10.5853/jos.2022.03468

145. Burn J, Burn J, Dennis M, et al. Long-term risk of recurrent stroke after a first-ever stroke. The Oxfordshire Community Stroke Project. *Stroke*. Published online 1994. doi:10.1161/01.str.25.2.333

146. Brutto VJD, Rundek T, Sacco RL. Prognosis After Stroke. *null*. Published online 2022. doi:10.1016/b978-0-323-69424-7.00017-x

147. Kernan WN, Viera AJ, Billinger SA, et al. Primary Care of Adult Patients After Stroke: A Scientific Statement From the American Heart Association/American Stroke Association. *Stroke*. Published online 2021. doi:10.1161/str.0000000000000382

148. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. *Nature Medicine*. Published online 2013. doi:10.1038/nm.3407

149. Augustin HG, Koh GY. Organotypic vasculature: From descriptive heterogeneity to functional pathophysiology. *Science*. Published online 2017. doi:10.1126/science.aal2379

150. Zhao Z, Zhao Z, Nelson AR, Betsholtz C, Zlokovic BV. Establishment and Dysfunction of the Blood-Brain Barrier. *Cell*. Published online 2015. doi:10.1016/j.cell.2015.10.067

151. Iadecola C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron*. Published online 2017. doi:10.1016/j.neuron.2017.07.030

152. Iadecola C. The pathobiology of vascular dementia. *Neuron*. Published online 2013. doi:10.1016/j.neuron.2013.10.008

153. Kisler K, Nelson AR, Montagne A, Zlokovic BV. Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer's disease. *Nat Rev Neurosci*. 2017;18(7):419-434. doi:10.1038/nrn.2017.48

154. Silence J, Collen D, Lijnen R. Persistance of atherosclerotic plaque and reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. *Fibrinolysis and Proteolysis*. Published online 2000. doi:10.1161/hq0901.097004

155. Soumian S, Soumian S, Soumian S, et al. MRNA expression of genes involved in lipid efflux and matrix degradation in occlusive and ectatic atherosclerotic disease. *Journal of Clinical Pathology*. Published online 2005. doi:10.1136/jcp.2005.026161

156. Lakhan SE, Kirchgessner A, Kirchgessner A, Tepper D, Leonard A. Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. *Frontiers in Neurology*. Published online 2013. doi:10.3389/fneur.2013.00032

157. Deane R, Wu Z, Zlokovic BV. RAGE (Yin) Versus LRP (Yang) Balance Regulates Alzheimer Amyloid β-Peptide Clearance Through Transport Across the Blood–Brain Barrier. *Stroke*. Published online 2004. doi:10.1161/01.str.0000143452.85382.d1

158. Bell RD, Deane R, Chow N, et al. SRF and myocardin regulate LRP-mediated amyloid-beta clearance in brain vascular cells. *Nature Cell Biology*. Published online 2009. doi:10.1038/ncb1819

159. Aalling N, Jessen NA, Munk ASF, Munk ASF, Lundgaard I, Lundgaard I. The Glymphatic System: A Beginner's Guide. *Neurochemical Research*. Published online 2015. doi:10.1007/s11064-015-1581-6

160. Deane R, Yan SD, S DY, et al. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nature Medicine*. Published online 2003. doi:10.1038/nm890

161. Rivera-Rivera LA, Turski PA, Johnson KM, et al. 4D flow MRI for intracranial hemodynamics assessment in Alzheimer's disease. *Journal of Cerebral Blood Flow and Metabolism*. Published online 2016. doi:10.1177/0271678x15617171

162. Bendok BR, Sherma AK, Hage ZA, et al. Periprocedural MRI perfusion imaging to assess and monitor the hemodynamic impact of intracranial angioplasty and stenting for symptomatic atherosclerotic stenosis. *Journal of Clinical Neuroscience*. Published online 2010. doi:10.1016/j.jocn.2009.04.011

163. Tarantini S, Hertelendy P, Tucsek Z, et al. Pharmacologically-induced neurovascular uncoupling is associated with cognitive impairment in mice. *Journal of Cerebral Blood Flow and Metabolism*. Published online 2015. doi:10.1038/jcbfm.2015.162

164. Mintun MA, Lundstrom BN, Snyder AZ, Vlassenko AG, Shulman GL, Raichle ME. Blood flow and oxygen delivery to human brain during functional activity: Theoretical modeling and experimental data. *Proceedings of the National Academy of Sciences of the United States of America*. Published online 2001. doi:10.1073/pnas.111164398

165. Morizawa Y, Hirayama Y, Ohno N, et al. Reactive astrocytes function as phagocytes after brain ischemia via ABCA1-mediated pathway. *Nature Communications*. Published online 2017. doi:10.1038/s41467-017-00037-1

166. Park L, Uekawa K, Uekawa K, et al. Brain Perivascular Macrophages Initiate the Neurovascular Dysfunction of Alzheimer Aβ Peptides. *Circulation Research*. Published online 2017. doi:10.1161/circresaha.117.311054

167. Sachdev PS, Blacker D, Blazer DG, et al. Classifying neurocognitive disorders: the DSM-5 approach. *Nature Reviews Neurology*. Published online 2014. doi:10.1038/nrneurol.2014.181

168. Flier WM van der, Flier WM van der, Flier WM van der, Scheltens P. Epidemiology and risk factors of dementia. *Journal of Neurology, Neurosurgery, and Psychiatry*. Published online 2005. doi:10.1136/jnnp.2005.082867

169. Smith M, Buckwalter KC, Kang H, Kang H, Ellingrod VL, Schultz SK. Dementia Care in Assisted Living: Needs and Challenges. *Issues in Mental Health Nursing*. Published online 2008. doi:10.1080/01612840802182839

170. 2023 Alzheimer's disease facts and figures. *Alzheimer's & dementia : the journal of the Alzheimer's Association*. Published online 2023. doi:10.1002/alz.13016

171. Wimo A, Seeher K, Cataldi R, et al. The worldwide costs of dementia in 2019. *Alzheimer's & Dementia*. Published online 2023. doi:10.1002/alz.12901

172. Patnode CD, Perdue LA, Perdue LA, et al. Screening for Cognitive Impairment in Older Adults: Updated Evidence Report and Systematic Review for the US Preventive Services Task Force. *JAMA*. Published online 2020. doi:10.1001/jama.2019.22258

173. Logsdon RG, McCurry SM, McCurry SM, McCurry SM, Teri L, Teri L. Evidence-Based Interventions to Improve Quality of Life for Individuals with Dementia. *null*. Published online 2007. doi:null

174. Bloom GS. Amyloid-β and Tau: The Trigger and Bullet in Alzheimer Disease Pathogenesis. *JAMA Neurology*. Published online 2014. doi:10.1001/jamaneurol.2013.5847

175. Heneka MT, Carson MJ, Khoury JE, et al. Neuroinflammation in Alzheimer's disease. *Lancet Neurology*. Published online 2015. doi:10.1016/s1474-4422(15)70016-5

176. Moujalled D, Strasser A, Liddell JR. Molecular mechanisms of cell death in neurological diseases. *Cell Death & Differentiation*. Published online 2021. doi:10.1038/s41418-021-00814-y

177. Wolters FJ, Wolters FJ, Ikram MA. Epidemiology of Vascular Dementia. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2019. doi:10.1161/atvbaha.119.311908

178. Kalaria RN. Neuropathological diagnosis of vascular cognitive impairment and vascular dementia with implications for Alzheimer's disease. *Acta Neuropathologica*. Published online 2016. doi:10.1007/s00401-016-1571-z

179. Iadecola C, Duering M, Hachinski V, et al. Vascular Cognitive Impairment and Dementia: JACC Scientific Expert Panel. *Journal of the American College of Cardiology*. Published online 2019. doi:10.1016/j.jacc.2019.04.034

180. Arvanitakis Z, Capuano AW, Leurgans S, Bennett DA, Schneider JA. Relation of cerebral vessel disease to Alzheimer's disease dementia and cognitive function in elderly people: a cross-sectional study. *Lancet Neurology*. Published online 2016. doi:10.1016/s1474-4422(16)30029-1

181. Gorelick PB, Furie KL, Iadecola C, et al. Defining Optimal Brain Health in Adults: A Presidential Advisory From the American Heart Association/American Stroke Association. *Stroke*. Published online 2017. doi:10.1161/str.0000000000000148

182. Schneider JA, Aggarwal NT, Barnes LL, Boyle PA, Bennett DA. The Neuropathology of Older Persons with and Without Dementia from Community versus Clinic Cohorts. *Journal of Alzheimer's Disease*. Published online 2009. doi:10.3233/jad-2009-1227

183. Sweeney MD, Montagne A, Sagare AP, et al. Vascular dysfunction-The disregarded partner of Alzheimer's disease. *Alzheimers & Dementia*. Published online 2019. doi:10.1016/j.jalz.2018.07.222

184. Baigent C, Keech AC, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14

randomised trials of statins. *The Lancet*. Published online 2005. doi:10.1016/s0140-6736(05)67394-1

185. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452(7189):896-899. doi:10.1038/nature06783

186. Feinberg MW, Moore KJ. MicroRNA Regulation of Atherosclerosis. *Circulation Research*. Published online 2016. doi:10.1161/circresaha.115.306300

187. Lu Y, Thavarajah T, Gu W, Gu W, Cai J, Xu Q. Impact of miRNA in Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2018. doi:10.1161/atvbaha.118.310227

188. Ouimet M, Ediriweera H, Gundra UM, et al. MicroRNA-33–dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. *Journal of Clinical Investigation*. Published online 2015. doi:10.1172/jci81676

189. Najafi-Shoushtari SH, Kristo F, Li Y, et al. MicroRNA-33 and the SREBP Host Genes Cooperate to Control Cholesterol Homeostasis. *Science*. Published online 2010. doi:10.1126/science.1189123

190. Horie T, Ono K, Horiguchi M, et al. MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. Published online 2010. doi:10.1073/pnas.1008499107

191. Rayner KJ, Suárez Y, Dávalos A, et al. MiR-33 Contributes to the Regulation of Cholesterol Homeostasis. *Science*. Published online 2010. doi:10.1126/science.1189862

192. Holdt LM, Stahringer A, Sass K, et al. Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. *Nature Communications*. Published online 2016. doi:10.1038/ncomms12429

193. Chen W, Schilperoort M, Schilperoort M, et al. Macrophage-targeted nanomedicine for the diagnosis and treatment of atherosclerosis. *Nature Reviews Cardiology*. Published online 2021. doi:10.1038/s41569-021-00629-x

194. Prilepskii AY, Serov N, Kladko DV, Vinogradov VV. Nanoparticle-Based Approaches towards the Treatment of Atherosclerosis. *Pharmaceutics*. Published online 2020. doi:10.3390/pharmaceutics12111056

195. Getz GS, Reardon CA. Animal Models of Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 2012. doi:10.1161/atvbaha.111.237693

196. Ylä-Herttuala S, Palinski W, Rosenfeld ME, et al. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *Journal of Clinical Investigation*. Published online 1989. doi:10.1172/jci114271

197. Fan J, Kitajima S, Watanabe T, et al. Rabbit models for the study of human atherosclerosis: from pathophysiological mechanisms to translational medicine. *Pharmacology & Therapeutics*. Published online 2015. doi:10.1016/j.pharmthera.2014.09.009

198. Shiomi M, Ito T, Ito T. The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: A tribute to the late Dr. Yoshio Watanabe. *Atherosclerosis*. Published online 2009. doi:10.1016/j.atherosclerosis.2009.03.024

199. Fan J, Chen Y, Chen Y, et al. Genomic and Transcriptomic Analysis of Hypercholesterolemic Rabbits: Progress and Perspectives. *International Journal of Molecular Sciences*. Published online 2018. doi:10.3390/ijms19113512

200. Veseli BE, Perrotta P, Meyer GRAD, et al. Animal models of atherosclerosis. *European Journal of Pharmacology*. Published online 2017. doi:10.1016/j.ejphar.2017.05.010

201. Ibrahim Z, Busch J, Awwad M, Wagner R, Wells KD, Cooper DKC. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. *Xenotransplantation*. Published online 2006. doi:10.1111/j.1399-3089.2006.00346.x

202. Wernersson R, Schierup MH, Jørgensen FG, et al. Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics*. Published online 2005. doi:10.1186/1471-2164-6-70

203. Colbert CM, Shao J, Hollowed JJ, et al. 3D-Printed Coronary Implants Are Effective for Percutaneous Creation of Swine Models with Focal Coronary Stenosis. *Journal of Cardiovascular Translational Research*. Published online 2020. doi:10.1007/s12265-020-10018-3

204. Schwartz MK, Luikart G, Waples RS. Genetic monitoring as a promising tool for conservation and management. *Trends Ecol Evol*. 2007;22(1):25-33. doi:10.1016/j.tree.2006.08.009

205. Lankau EW, Turner PV, Mullan RJ, Mullan RJ, Galland GG. Use of nonhuman primates in research in North America. *Journal of The American Association for Laboratory Animal Science*. Published online 2014. doi:null

206. Shively CA, Register TC, Clarkson TB. Social stress, visceral obesity, and coronary artery atherosclerosis: product of a primate adaptation. *American Journal of Primatology*. Published online 2009. doi:10.1002/ajp.20706

207. Bremer AA, Stanhope KL, Graham JL, et al. Fructose-Fed Rhesus Monkeys: A Nonhuman Primate Model of Insulin Resistance, Metabolic Syndrome, and Type 2 Diabetes. *Clinical and Translational Science*. Published online 2011. doi:10.1111/j.1752-8062.2011.00298.x

208. Zhang W, Zhang S, Zhang S, et al. A single-cell transcriptomic landscape of primate arterial aging. *Nature Communications*. Published online 2020. doi:10.1038/s41467-020-15997-0

209. Prusty S, Kemper TL, Moss MB, Hollander W. Occurrence of stroke in a nonhuman primate model of cerebrovascular disease. *Stroke*. Published online 1988. doi:10.1161/01.str.19.1.84

210. Cefalu WT, Cefalu WT, Wagner JD. Aging and atherosclerosis in human and nonhuman primates. *Age*. Published online 1997. doi:10.1007/s11357-997-0002-4

211. Shively CA, Willard SL. Behavioral and neurobiological characteristics of social stress versus depression in nonhuman primates. *Experimental Neurology*. Published online 2012. doi:10.1016/j.expneurol.2011.09.026

212. Shelton KA, Shelton KA, Clarkson TB, Kaplan JR. Nonhuman primate models of atherosclerosis. *Laboratory Animal Science*. Published online 2012. doi:10.1016/b978-0-12-381366-4.00008-0

213. Agrawal S, Schneider JA. Vascular pathology and pathogenesis of cognitive impairment and dementia in older adults. *Cereb Circ Cogn Behav*. 2022;3:100148. doi:10.1016/j.cccb.2022.100148

214. D'Agostino RB, Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation*. 2008;117(6):743-753. doi:10.1161/CIRCULATIONAHA.107.699579

215. Alloubani A, Nimer R, Samara R. Relationship between Hyperlipidemia, Cardiovascular Disease and Stroke: A Systematic Review. *Curr Cardiol Rev*. 2021;17(6):e051121189015. doi:10.2174/1573403X16999201210200342

216. Linton MF, Yancey PG, Davies SS, et al. The Role of Lipids and Lipoproteins in Atherosclerosis. In: Feingold KR, Anawalt B, Blackman MR, et al., eds. *Endotext*. MDText.com, Inc.; 2000. Accessed June 14, 2023. http://www.ncbi.nlm.nih.gov/books/NBK343489/

217. Moore KJ, Rayner KJ, Suárez Y, Fernández-Hernando C. The role of microRNAs in cholesterol efflux and hepatic lipid metabolism. *Annu Rev Nutr*. 2011;31:49-63. doi:10.1146/annurev-nutr-081810-160756

218. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*. 2010;79:351-379. doi:10.1146/annurev-biochem-060308-103103

219. Ouimet M, Ediriweera H, Afonso MS, et al. miR-33 regulates macrophage autophagy in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2017;37(6):1058-1067. doi:10.1161/ATVBAHA.116.308916

220. Rayner KJ, Sheedy FJ, Esau CC, et al. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J Clin Invest*. 2011;121(7):2921-2931. doi:10.1172/JCI57275

221. Rayner KJ, Esau CC, Hussain FN, et al. Inhibition of miR-33a/b in non-human primates raises plasma HDL and reduces VLDL triglycerides. *Nature*. 2011;478(7369):404-407. doi:10.1038/nature10486

222. Hollander W, Prusty S, Kemper T, Rosene DL, Moss MB. The effects of hypertension on cerebral atherosclerosis in the cynomolgus monkey. *Stroke*. 1993;24(8):1218-1226. doi:10.1161/01.STR.24.8.1218

223. Tsuji K, Nakamura S, Aoki T, Nozaki K. The cerebral artery in cynomolgus monkeys (Macaca fascicularis). *Exp Anim*. 2022;71(3):391-398. doi:10.1538/expanim.22-0002

224. Mortensen MB, Caínzos-Achirica M, Steffensen FH, et al. Association of Coronary Plaque With Low-Density Lipoprotein Cholesterol Levels and Rates of Cardiovascular Disease Events Among Symptomatic Adults. *JAMA Network Open*. 2022;5(2):e2148139. doi:10.1001/jamanetworkopen.2021.48139

225. Pejic RN. Familial Hypercholesterolemia. *Ochsner J*. 2014;14(4):669-672.

226. Rallidis LS, Iordanidis D, Iliodromitis E. The value of physical signs in identifying patients with familial hypercholesterolemia in the era of genetic testing. *Journal of Cardiology*. 2020;76(6):568-572. doi:10.1016/j.jjcc.2020.07.005

227. Wu D, Chandra A, Chen J, Ding Y, Ji X. Endovascular Ischemic Stroke Models in Nonhuman Primates. *Neurotherapeutics*. 2018;15(1):146-155. doi:10.1007/s13311-017-0586-z

228. Rottiers V, Obad S, Petri A, et al. Pharmacological inhibition of a microRNA family in nonhuman primates by a seed-targeting 8-mer antimiR. *Sci Transl Med*. 2013;5(212):212ra162. doi:10.1126/scitranslmed.3006840

229. Rosner J, Reddy V, Lui F. Neuroanatomy, Circle of Willis. In: *StatPearls*. StatPearls Publishing; 2023. Accessed June 13, 2023. http://www.ncbi.nlm.nih.gov/books/NBK534861/

230. O'Connor JPB. Thomas Willis and the background to Cerebri Anatome. *J R Soc Med*. 2003;96(3):139-143.

231. Roher AE, Tyas SL, Maarouf CL, et al. Intracranial atherosclerosis as a contributing factor to Alzheimer's disease dementia. *Alzheimers Dement*. 2011;7(4):436-444. doi:10.1016/j.jalz.2010.08.228

232. Leng X, Wong KS, Liebeskind DS. Evaluating Intracranial Atherosclerosis Rather Than Intracranial Stenosis. *Stroke*. 2014;45(2):645-651. doi:10.1161/STROKEAHA.113.002491

233. Ma R, Xie Q, Li Y, et al. Animal models of cerebral ischemia: A review. *Biomedicine & Pharmacotherapy*. 2020;131:110686. doi:10.1016/j.biopha.2020.110686

234. Taha A, Bobi J, Dammers R, et al. Comparison of Large Animal Models for Acute Ischemic Stroke: Which Model to Use? *Stroke*. 2022;53(4):1411-1422. doi:10.1161/STROKEAHA.121.036050

235. Denswil NP, van der Wal AC, Ritz K, et al. Atherosclerosis in the circle of Willis: Spatial differences in composition and in distribution of plaques. *Atherosclerosis*. 2016;251:78-84. doi:10.1016/j.atherosclerosis.2016.05.047

236. Roth W, Morgello S, Goldman J, et al. Histopathological Differences Between the Anterior and Posterior Brain Arteries as a Function of Aging. *Stroke*. 2017;48(3):638-644. doi:10.1161/STROKEAHA.116.015630

237. Clarkson TB, Alexander NJ, Morgan TM. Atherosclerosis of cynomolgus monkeys hyper- and hyporesponsive to dietary cholesterol. Lack of effect of vasectomy. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Published online 1988. doi:10.1161/01.atv.8.5.488

238. Hasbani NR, Ligthart S, Brown MR, et al. American Heart Association's Life's Simple 7: Lifestyle Recommendations, Polygenic Risk, and Lifetime Risk of Coronary Heart Disease. *Circulation*. Published online 2022. doi:10.1161/circulationaha.121.053730

239. Kim SJ, Schneider DJ, Feldmann E, Liebeskind DS. Intracranial atherosclerosis: Review of imaging features and advances in diagnostics. *International Journal of Stroke*. 2022;17(6):599-607. doi:10.1177/17474930211066427

240. Kaplan, Kaplan JR, Adams MR, et al. Psychosocial factors, sex differences, and atherosclerosis: lessons from animal models. *Psychosomatic Medicine*. Published online 1996. doi:10.1097/00006842-199611000-00008

241. Aikawa M, Rabkin E, Okada Y, et al. Lipid lowering by diet reduces matrix metalloproteinase activity and increases collagen content of rabbit atheroma: a potential mechanism of lesion stabilization. *Circulation*. 1998;97(24):2433-2444. doi:10.1161/01.cir.97.24.2433

242. Johnston SC, Easton JD, Farrant M, et al. Clopidogrel and Aspirin in Acute Ischemic Stroke and High-Risk TIA. *New England Journal of Medicine*. 2018;379(3):215-225. doi:10.1056/NEJMoa1800410

243. Carnicelli AP, Hong H, Connolly SJ, et al. Direct Oral Anticoagulants Versus Warfarin in Patients With Atrial Fibrillation: Patient-Level Network Meta-Analyses

of Randomized Clinical Trials With Interaction Testing by Age and Sex. *Circulation*. 2022;145(4):242-255. doi:10.1161/CIRCULATIONAHA.121.056355

244. Hackam DG, Hegele RA. Cholesterol Lowering and Prevention of Stroke. *Stroke*. 2019;50(2):537-541. doi:10.1161/STROKEAHA.118.023167

245. Hankey GJ. Angiotensin-Converting Enzyme Inhibitors for Stroke Prevention. *Stroke*. 2003;34(2):354-356. doi:10.1161/01.STR.0000054261.97525.4B

246. Strauss MH, Hall A. Angiotensin Receptor Blockers Should Be Regarded as First-Line Drugs for Stroke Prevention in Both Primary and Secondary Prevention Settings. *Stroke*. 2009;40(9):3161-3162. doi:10.1161/STROKEAHA.109.559062

247. Sabatine MS, Giugliano RP, Keech AC, et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *The New England Journal of Medicine*. Published online 2017. doi:10.1056/nejmoa1615664

248. Soehnlein O, Libby P. Targeting inflammation in atherosclerosis — from experimental insights to the clinic. *Nat Rev Drug Discov*. 2021;20(8):589-610. doi:10.1038/s41573-021-00198-1

249. Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ. An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. *J Lipid Res*. 2005;46(5):872-884. doi:10.1194/jlr.M400492-JLR200

250. Crooke ST, Geary RS. Clinical pharmacological properties of mipomersen (Kynamro), a second generation antisense inhibitor of apolipoprotein B. *Br J Clin Pharmacol*. 2013;76(2):269-276. doi:10.1111/j.1365-2125.2012.04469.x

251. Santos RD, Duell PB, East C, et al. Long-term efficacy and safety of mipomersen in patients with familial hypercholesterolaemia: 2-year interim results of an open-label extension. *Eur Heart J*. 2015;36(9):566-575. doi:10.1093/eurheartj/eht549

252. Reeskamp LF, Kastelein JJP, Moriarty PM, et al. Safety and efficacy of mipomersen in patients with heterozygous familial hypercholesterolemia. *Atherosclerosis*. 2019;280:109-117. doi:10.1016/j.atherosclerosis.2018.11.017

253. Fogacci F, Ferri N, Toth PP, Ruscica M, Corsini A, Cicero AFG. Efficacy and Safety of Mipomersen: A Systematic Review and Meta-Analysis of Randomized Clinical Trials. *Drugs*. 2019;79(7):751-766. doi:10.1007/s40265-019-01114-z

254. Gerin I, Clerbaux LA, Haumont O, et al. Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. *J Biol Chem*. 2010;285(44):33652-33661. doi:10.1074/jbc.M110.152090

255. Singaraja RR, Van Eck M, Bissada N, et al. Both hepatic and extrahepatic ABCA1 have discrete and essential functions in the maintenance of plasma high-density

lipoprotein cholesterol levels in vivo. *Circulation*. 2006;114(12):1301-1309. doi:10.1161/CIRCULATIONAHA.106.621433

VITA

Peter I. Hecker

Doctoral Candidate
Pharmacology & Nutritional Sciences
SAHA Cardiovascular Research Center
University of Kentucky College of Medicine

Education and Training

United States Coast Guard 1999-2005
Paramedic (Flight Medicine) Training

United States Army 2005-2016
Combat Medicine Training
Medical Laboratory Medicine Training
Instructor Training
Advanced Leadership Training
Master Resiliency Training

University of Colorado, Colorado Springs, CO 2012-2015
Bachelor of Science, Dietetics and Clinical Nutrition

University of Kentucky, Lexington, KY 2015-Expected 2023
Doctoral Candidate in Philosophy, Pharmacology & Nutritional Sciences

Funding

2019-2022: **NIH R21 Targeting microRNA-33 to Reduce Intracranial Atherosclerosis and Other Hallmarks of Vascular Cognitive Impairment and Dementia (R21NS111979)**, Graduate Research Assistant, Department of Physiology, University of Kentucky, Lexington, KY

2018-2020: **NIH T32 Training in Translational Research in Alzheimer's and Related Dementias (TRIAD) (T32AG057461)**, Trainee, Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY

2017-2018: **NIH T32 Pharmacology and Nutritional Sciences: Multidisciplinary Approaches for Metabolic Disease (T32DK007778)**, Trainee, Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY

Academic and Professional Honors

Completed Nikon Advanced NIS Elements Training	2018
T32 Training Grant (TRIAD)	2018
ISTAART Student Volunteer Travel Award	2018
Bright Focus Foundation Travel Award	2018
T32 Training Grant Pharmacology & Nutritional Sciences	2017
United States Army Service Awards and Achievements (Upon Request)	2016
Graduated Salutatorian University of Colorado	2015
Outstanding Achievement Award (Biochemistry)	2014
United States Coast Guard Service Awards and Achievements (Upon Request)	2005

Professional Peer Reviewed Publications

Kukida M, Cai L, Ye D, Sawada H, Katsumata Y, Franklin MF, **Hecker PI**, Campbell KS, Jan Danser AH, Mullick AE, Daugherty A, Temel RE, Lu H. Renal Angiotensinogen Is Predominantly Liver Derived in Nonhuman Primates. *ATVB*. September 2021; 41:2851-2853. doi: https://doi.org/10.1161/ATVBAHA.121.316590

Yi S, Zhang X, Sangji H, Liu Y, Allen SD, Xiao B, Bobbala S, Braverman CL, Cai L, **Hecker PI**, DeBerge M, Thorp EB, Temel RE, Stupp SI, and Scott EA. Surface Engineered Polymersomes for Enhanced Modulation of Dendritic Cells During Cardiovascular Immunotherapy. *Adv. Funct. Mater.* August 2019; Volume 29, Issue 42. Doi: https://doi.org/10.1002/adfm.201904399

Allen S, Bobbala S, Liu YG, Cai L, **Hecker PI**, Temel RE, Scott EA. Polymersomes scalably fabricated via flash nanoprecipitation are non-toxic in non-human primates and associate with leukocytes in the spleen and kidney following intravenous administration. *Nano Research*. October 2018; Volume 11, Issue 10, pp 5689–5703. doi: https://doi.org/10.1007/s12274-018-2069-x

Professional Research Presentations (Abstract/Talk Presentations)

Intracranial Atherosclerosis in Nonhuman Primates is Associated with Neuroinflammation and Vascular Hallmarks of Cognitive Impairment and Dementia.
International Conference on Alzheimer's and Parkinson's Diseases and Related Neuropathological disorders. Lisbon, Portugal. 2019

Intracranial Atherosclerosis in Nonhuman Primates.
Gordon Research Conference. Waterville Valley, NH USA. 2018

Professional Research Presentations (Abstract/Poster Presentations)

Peter I Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Intracranial atherosclerosis promotes vascular hallmarks of cognitive.
impairment and dementia.
Cardiovascular Research Day. Lexington, KY USA. September. 2019

Peter I Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Intracranial atherosclerosis in nonhuman primates is associated with
neuroinflammation and vascular hallmarks of cognitive impairment and
dementia.
Gordon Research Conference. Sunday River, ME USA. June. 2019

Peter I Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Intracranial atherosclerosis in nonhuman primates is associated with
neuroinflammation and vascular hallmarks of cognitive impairment and
dementia.
International Conference on Alzheimer's and Parkinson's Diseases and
Related Neuropathological disorders. Lisbon, Portugal. March. 2019

Peter I Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Intracranial atherosclerosis along with other hallmarks of vascular
cognitive impairment and dementia in nonhuman primates promotes a
neuroinflammatory response.
Cardiovascular Research Day. Lexington, KY USA. September. 2018

Peter I Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Intracranial atherosclerosis in nonhuman primates promotes a
neuroinflammatory response.
Gordon Research Conference. Waterville Valley, NH USA. June. 2018

Peter I. Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel.
Atherosclerosis in the cerebrovasculature of non-human primates
promotes reactive gliosis: a potential model for vascular contributions.
to cognitive impairment and dementia.
Cardiovascular Research Day. Lexington, KY USA. November. 2017

Peter I. Hecker, Lei Cai, Donna M. Wilcock, Elizabeth Head, Ryan E. Temel
Neurovascular damage and inflammation in the brains of nonhuman
primates fed an atherogenic diet.
Dementia and Cognition: A Vascular Perspective. Montreal, Canada. May. 2017

Peter I. Hecker