PCSK9 INHIBITION AND CORONARY ARTERY DISEASE IN MICE

INVERSTIGATING THE EFFECTS OF PCSK9 INHIBITION ON CORONARY ARTERY ATHEROSCLEROSIS AND MYOCARDIAL INFARCTION IN MICE

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TITLE: Investigating the Effects of PCSK9 Inhibition on Coronary Artery Atherosclerosis and Myocardial Infarction in Mice

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Abstract

The underlying pathological process of coronary artery disease (CAD) is the development of coronary artery atherosclerotic occlusions and associated myocardial infarction. Both increased chronic inflammation and plasma low-density lipoprotein (LDL) cholesterol levels promote atherosclerosis. Inhibiting proprotein convertase subtilisin/kexin type 9 (PCSK9) is widely known for its role in enhancing LDL receptor (LDLR)-mediated cholesterol lowering when the LDLR-apolipoprotein E (APOE) axis is intact and protecting against atherosclerosis progression by reducing plasma cholesterol levels. In this thesis, we sought to test the effects of PCSK9 inhibition mediated cholesterol lowering on pre-existing CAD as well as the plasma cholesterol independent effects of PCSK9 inhibition on CAD by utilizing different mouse models.

One year old scavenger receptor class B type I (*Sr-b1*) *knockout* (*KO*) mice which have an intact LDLR-APOE axis, develop coronary artery atherosclerosis and myocardial fibrosis induced by a high fat, high cholesterol and cholate containing (HFCC) diet. Weekly anti-PCSK9 antibody treatment initiated one week before switching to an HFCC diet increased hepatic LDLR protein levels, and reduced plasma cholesterol levels and the progression of atherosclerosis in both the aortic sinus and coronary arteries in one year old *Sr-b1 KO* mice (maintained on an HFCC diet for 7 weeks). Weekly anti-PCSK9 antibody treatment initiated 7 weeks after switching to an HFCC diet also increased hepatic LDLR protein levels and reduced plasma cholesterol levels in one year old *Sr-b1 KO* mice (maintained on an HFCC diet for 12 weeks). More importantly, anti-PCSK9

antibody treatment during the last 5 weeks of the 12-week HFCC diet feeding period also slowed down the growth of pre-existing atherosclerosis in both the aortic sinus and coronary arteries and reduced myocardial fibrosis and damage.

Mice deficient in both *Sr-b1* and *ApoE* (*Sr-b1/ApoE double KO* (*dKO*) mice) spontaneously and rapidly develop features reminiscent of human CAD. Whole body *Pcsk9* genetic *KO* in both female and male *Sr-b1/ApoE dKO* mice did not affect plasma cholesterol levels despite increased hepatic LDLR protein levels, presumably due to the lack of APOE. However, genetic *Pcsk9* inactivation significantly attenuated atherosclerosis in both the aortic sinus and coronary arteries, myocardial fibrosis and damage, left ventricle (LV) dysfunction and cardiac enlargement in both female and male *Sr-b1/ApoE dKO* mice. Restoring circulating PCSK9 by a recombinant adeno associated virus 8 (AAV8)-mediated hepatic expression of a *Pcsk9* cDNA in *Pcsk9/Sr-b1/ApoE triple KO* mice reversed the plasma cholesterol independent protective effects of genetic PCSK9 KO on aortic sinus and coronary artery atherosclerosis and myocardial fibrosis and damage in both females and males. Treatment of *Sr-b1/ApoE dKO* mice with an anti-PCSK9 antibody which disrupts the interaction between the LDLR and PCSK9 protected against aortic sinus and coronary artery atherosclerosis in males but not in females and did not protect either males or females against myocardial fibrosis and damage, LV dysfunction or cardiac enlargement.

My thesis demonstrates that anti-PCSK9 antibody mediated plasma cholesterol lowering delays the continued development of pre-existing CAD. My thesis also demonstrates that liver-derived, circulating PCSK9 promotes CAD in a plasma

cholesterol independent manner in *Sr-b1/ApoE dKO* mice and these effects appear to be largely independent of the PCSK9-LDLR interaction, particularly in females.

Acknowledgement

It is a touching moment to write the acknowledgement and rememorize the scientific journey that I have travelled through during the last 7 years. Foremost, I would like to express my gratitude from the bottom of my heart to Dr. Bernardo (Dino) Trigatti for giving me a chance to learn and conduct research in his lab. Dino has always been a supportive supervisor. His knowledge, passion for science and scientific rigor have tremendous positive influence on me and my future career choice. I have also learned a lot from his exceptional scientific writing and communication skills. Thank you Dino for your constant guidance and constructive suggestions for the last 7 years. Without you, I would have not transitioned from a person who knows little about research and science to where I am now. I would like to extend my gratitude to my committee members, Dr. Richard Austin and Dr. Colin Kretz. Thank you for your constructive feedback on my projects and your encouragement. Moreover, I would also like to thank my collaborators, Dr. Richard Austin, Dr. Nabil Seidah and Dr. Annik Prat, for their contributions and inputs for the projects. This thesis would have not been possible without the support from all of you.

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To my boyfriend and life partner, Hector, thank you for making the past few years as the best time in my life. I would have not been able to reach to my current stage without your love, sacrifice and support. To our 4 parents, thank you for your unconditional love to us. Your support is the reason that we are able to pursue what we are passionate about.

To everyone who has been a part of my journey, I feel very lucky to be surrounded by you. I am looking forward to the next chapter of my life.

- Ting

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List of Abbreviations

aa	Amino acids
AAV8	Adeno associated virus 8
ABCA-1	ATP-binding cassette subfamily A member 1
ABCG-1	ATP-binding cassette subfamily G member 1
ACAT	Acyl-CoA cholesterol acyltransferase
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ANGPTL3	Angiopoietin-like protein 3
APOA-I	Apolipoprotein A-I
APOB	Apolipoprotein B
APOB48	Apolipoprotein B48
APOB100	Apolipoprotein B100
APOBEC-1	*APOB* mRNA editing catalytic polypeptide-1
APOC-II	Apolipoprotein C-II
APOE	Apolipoprotein E
ApoER2	Apolipoprotein E receptor 2

ATP	Adenosine triphosphate
BAX	Bcl2 associated X
Bcl-2	B-cell lymphoma 2
CETP	Cholesteryl ester transfer protein
CAD	Coronary artery disease
CCL2	C-C motif chemokine ligand 2
CCR7	C-C chemokine receptor 7
CD36	Cluster determinant 36
CE	Cholesterol ester
CK-MB	Creatine-kinase myocardial band
COX-1	Cyclo-oxygenase 1
CVE	Cardiovascular event
CVD	Cardiovascular disease
DC	Dendritic cell
11-dh-TxB2	11-Dehydro-thromboxane B2
dKO	*Double knockout*
EC	Endothelial cell

ECM	Extracellular matrix
EGF	Epidermal growth factor
eNOS	Endothelial NO synthase
ER	Endoplasm reticulum
ERK5	Extracellular signal-regulated kinase 5
NO	Nitric oxide
FFA	Free fatty acid
FH	Familial hypercholesterolemia
GP IIb/IIIa	Glycoprotein IIb/IIIa
HDL	High-density lipoprotein
HFCC	High fat, high cholesterol and cholate containing
HIF-1α	Hypoxia inducible factor-1 α
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
H/R	Hypoxia/reoxygenation
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1

IDL	Intermediate-density lipoprotein
IFN	Interferon
IHD	Ischemic heart disease
IL-1β	Interleukin 1 beta
IL-10	Interleukin 10
I/R	Ischemia/reperfusion
JNK	Jun N-terminal kinase
KO	*Knockout*
LCAL	Left coronary artery ligation
LCAT	Lecithin cholesterol acyl transferase
LDL	Low-density lipoprotein
LDLR	LDL receptor
LIMP-2	Lysosomal integral membrane protein 2
LOX-1	Lectin-like ox-LDL receptor-1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP-1	LDLR related protein 1

LRP-5	LDLR-related protein 5
LV	Left ventricle
LXR	Liver X receptor
Ly6C	Lymphocyte antigen 6C
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MHC-I	Major histocompatibility complex class-I
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
MTTP	Microsomal triacylglycerol transfer protein
mPTP	Mitochondrial permeability transition pore
NPC1L1	Niemann-Pick C1-like 1
Ox-LDL	Oxidized LDL
RCT	Reverse cholesterol transport

ROS	Reactive oxygen species
TGF-β	Transforming growth factor-beta
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDGF	Platelet-derived growth factor
SDS-PAGE	SDS–polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SR-A	Scavenger receptor class A
SR-B1	Scavenger receptor class B type 1
SREBP-2	Sterol regulatory element-binding protein 2
STAT6	Signal transducer and activator of transcription 6
TBS	Tris-buffered saline
TC	Total cholesterol
TG	Triglyceride
Th17	T helper-17
TNF-α	Tumor necrosis factor alpha
$T_X A_2$	Thromboxane A2

VACM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein receptor
VSMC	Vascular smooth muscle cell

PREFACE

This is a sandwich thesis. Figures in each chapter are numbered starting from figure 1. Figures within the same chapter are referred to only by their sequence number within the chapter. Figures with the same sequence number in different chapters are not distinguished by numbering different chapters, for example, both chapters 1 and 2 have “Figure 2”, however, when in chapter 1, “Figure 2” is used to indicate Chapter 1, figure 2, while when in chapter 2, “Figure 2” is used to indicated Chapter 2, figure 2.

Chapter 1: General Introduction

PREFACE

The whole section 1.8.3 of this chapter is adapted from a published review paper entitled "The local effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the inflammatory atheroma: beyond LDL cholesterol lowering". Ting Xiong contributed to the draft of the following sections of the review paper: Abstract, Introduction, VSMCs and ECs in the vascular wall, Platelets, Cardiomyocytes and Conclusions. Alex Qian contributed to the draft of the following sections of the review paper: Monocytes/Macrophages, Dendritic cells and T cells. Ting Xiong, Alex Qian and Bernardo L. Trigatti all contributed to the editing and finalizing of the review paper. The complete reference of the review paper is: Xiong, T., Qian, A. S. & Trigatti, B. L. The local effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the inflammatory atheroma: beyond LDL cholesterol lowering. Vessel Plus 5, (2021).

1.1 Cardiovascular Disease Overview

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality globally (G. A. Roth et al., 2018). Ischemic heart disease (IHD) is the primary contributor to the CVD burden, and it accounts for 9 million annual deaths worldwide (G. A. Roth et al., 2018). With the increased longevity, urbanization, lifestyle changes and expanding population in developing countries, it is projected that the burden of IHD would be even higher in the future (Yusuf et al., 2001). The major mechanism of IHD is atherosclerotic plaque development in coronary arteries. Epidemiologic studies have identified multiple modifiable risk factors for atherosclerotic coronary artery disease (CAD), including elevated low-density lipoproteins (LDL), reduced high-density lipoproteins (HDL), smoking, high blood pressure, elevated glucose, stress, physical inactivity, obesity and poor diet (Hajar, 2017; Joseph et al., 2017; Yusuf et al., 2001). Large clinical studies have also demonstrated that lowering LDL cholesterol levels by treatment with statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) reduced cardiovascular events (CVEs) (Bybee et al., 2008; Downs et al., 1998; Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, 1998; Ridker et al., 2008; Sacks et al., 1996; Scandinavian Simvastatin Survival Study Group, 1994). Despite the wide statin utilisation in developed countries, significant residual risk remains for CAD (Guadamuz et al., 2022; Wong et al., 2017), suggesting that further research and new treatments are needed for reducing the burden of CAD.

1.2 Atherosclerosis

1.2.1 Atherosclerosis Initiation

Atherosclerosis is the combinational outcome of long-lasting hypercholesterolemia and low-grade chronic inflammation. It is initiated by the retention of LDLs in the intima of the artery wall at preferred sites, such as artery curvature and bifurcations, where blood flow is non-laminar and disturbed and therefore with lower shear stress (Malek et al., 1999; Moore et al., 2013; Schwenke & Carew, 1989). The hemodynamic properties at these susceptible regions induce the activation and dysfunction of endothelial cells (ECs) that are lining the vessel wall (Malek et al., 1999). Dysfunctional ECs exhibit pro-inflammatory and pro-thrombotic phenotypes and have a disrupted barrier function (Gimbrone & García-Cardeña, 2016). Conventionally, it is thought that LDLs passively enter the subendothelial layer due to the impaired barrier function of ECs. However, recent studies have demonstrated that LDLs cross the endothelium at the lesion-prone sites by transcytosis, a transcellular process where macromolecules were transported through the cytoplasm of individual cells (Armstrong et al., 2015; Huang et al., 2019). LDL particles that have infiltrated into the intima of the artery wall undergo modifications, such as oxidation, aggregation, glycation and enzymatic cleavage (Gleissner et al., 2007). Modified LDLs, mainly oxidized LDLs (Ox-LDLs), stimulate the expression of adhesion molecules in the overlying endothelium, including P-selectin, vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Gebuhrer et al., 1995; Iiyama et al., 1999a; Ley et al.,

2007; Tabas et al., 2015). Those adhesion molecules assist the rolling and firm adhesion of monocytes to the endothelium (Gebuhrer et al., 1995; Iiyama et al., 1999a; Ley et al., 2007; Tabas et al., 2015). Ox-LDLs also stimulate the release of the monocyte chemoattractant protein 1 (MCP-1, also known as C-C motif chemokine ligand 2, CCL2) in ECs and plaque macrophages (Moore et al., 2013). MCP-1 then promotes the transmigration of monocytes from the blood across the endothelial layer and into the artery wall (Moore et al., 2013). Furthermore, the adhesion of platelets to the endothelium also facilitates the recruitment of monocytes to the lesion site through diverse mechanisms (Lievens & von Hundelshausen, 2011). Those mechanisms involve the induction of chemokine production and secretion by both platelets and ECs, the expression of immune receptors in platelets that enhance the crosstalk between monocytes and ECs, and the induction of adhesion molecule expression in ECs (Lievens & von Hundelshausen, 2011). The continued retention of LDLs, dysfunctional ECs and chemokine production collectively drive monocyte infiltration into the subendothelial layer (Moore et al., 2013). There, those monocytes proliferate and differentiate into macrophages stimulated by macrophage colony stimulating factor (M-CSF). Macrophages take up native and modified LDLs, and become cholesterol-laden foam cells, which is the hallmark of early-stage atherosclerosis (Moore et al., 2013).

1.2.2 Atherosclerosis Progression and Disruption

Plaque macrophages secrete both pro-inflammatory and anti-inflammatory cytokines. Plaque macrophages exhibit different phenotypes in vivo, spanning extremes referred to as M1 and M2. M1 macrophages secrete pro-inflammatory cytokines and

macrophages exhibiting M1 phenotypic markers dominate plaques during atherosclerosis progression, while M2 macrophages secrete anti-inflammatory cytokines and promote tissue repair and macrophages exhibiting M2 phenotypic markers are enriched in plaques during atherosclerosis regression (Moore et al., 2013). Recent studies have revealed the transcriptional and phenotypic heterogeneity of aortic macrophages in both human and murine atherosclerosis (Cochain et al., 2018; Depuydt et al., 2020; Fernandez et al., 2019; Zernecke et al., 2020). Five major macrophage subsets have been identified in murine atherosclerotic plaques, including resident macrophages, inflammatory macrophages, interferon (IFN) inducible macrophages, foamy macrophages and cavity macrophages (Zernecke et al., 2020). The exact functions of those macrophage subsets in atherosclerosis are not clear. However, it is known that the inflammatory macrophages are characterized by increased inflammatory gene expression, including CCL2, interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), and the foamy macrophages express high levels of Trem2 (triggering receptor expressed on myeloid cells-2) (Zernecke et al., 2020). Studies have demonstrated that foamy macrophages are not pro-inflammatory (K. Kim et al., 2018; Zernecke et al., 2020). Furthermore, it has been shown that foam cells in the atherosclerotic plaques were not only derived from macrophages but also vascular smooth muscle cells (VSMCs) (Shankman et al., 2015; Y. Wang et al., 2019). VSMCs in the medial layer of the artery wall are differentiated and at a mature contractile state (Gomez & Owens, 2012). However, mature contractile VSMCs remain remarkably plastic (Gomez & Owens, 2012). They can de-differentiate, proliferate, and migrate to the intimal layer of the artery wall. There, they can acquire a

macrophage-like phenotype and express macrophage markers, such as cluster of differentiation 68 (CD68) and galectin 3 (Lgals3), and therefore are referred to as macrophage-like SMCs (Allahverdian et al., 2014a; K. Kim et al., 2018; Zernecke et al., 2020). De-differentiated and macrophages-like SMCs can also take up modified LDLs and become cholesterol-laden foam cells (Pryma et al., 2019).

As the disease progresses, the overload of free cholesterol in foam cells results in endoplasm reticulum (ER) stress. The unresolved ER stress activates the prolonged unfolded protein response and ultimately triggers foam cell apoptosis (Tabas & Ron, 2011). Apoptosis is a form of programmed cell death that normally occurs without eliciting a pro-inflammatory response. Apoptosis is characterized by membrane blebbing, chromatin and nuclear condensation and cell shrinkage. At early stages of atherosclerosis, apoptotic bodies are cleared efficiently by surrounding macrophages by a process called efferocytosis. Effective efferocytosis requires the interaction of "eat me" signals on the apoptotic cells, receptors on macrophages and sometimes bridging molecules (Gonzalez et al., 2017; Hochreiter-Hufford & Ravichandran, 2013). However, in the advanced atherosclerotic plaque, more macrophages have dysregulated lipid metabolism and are unable to process the ingested lipid from the apoptotic cells, which might impair efferocytosis (Moore et al., 2013). Moreover, studies have shown that the reduced expression of "eat me" signals and the increased abundance of "don't eat me" signals on apoptotic cells and the deficiency of macrophage receptors and bridging molecules in advanced lesions may all contribute to the impaired efferocytosis (Yurdagul et al., 2018). The defective efferocytosis leads to secondary necrosis and the exposure of cellular lipids

and pro-inflammatory and thrombogenic contents. Other pro-inflammatory forms of cell death have also been demonstrated to occur in atherosclerosis plaques, including programmed necroptosis and pyroptosis (Gonzalez et al., 2017; Karunakaran et al., 2016; Kluck et al., 2023; Rayner, 2017). Ultimately, the accumulation of cell debris, cholesterol crystals, extracellular lipids and inflammatory cytokines form a necrotic core.

Stimulated by growth factors and inflammatory microenvironment, VSMCs migrate to the surface of atherosclerotic plaque and acquire the synthetic phenotype (Basatemur et al., 2019; Tabas et al., 2015). The synthetic SMCs secret collagen rich extracellular matrix (ECM) (Alexander & Owens, 2012). ECM along with SMCs form a thick fibrous cap, which stabilizes the plaque against rupture (Alexander & Owens, 2012). However, at late stages of disease, reduced SMC numbers and collagen synthesis in the fibrous cap and the degradation of ECM by matrix metalloproteinases (MMPs) derived from SMCs and macrophages, lead to the thinning of the fibrous cap (Alexander & Owens, 2012). Plaques with a large necrotic core and a thin fibrous cap are prone to rupture, exposing the contents of the plaque to the blood. This activates platelets and the coagulation cascade and results in thrombus generation. So-called atherothrombosis in coronary arteries reduces or even blocks the supply of blood to the myocardium, therefore leading to myocardial infarction (MI) (Figure 1). Alternatively, plaques with a fibrous cap containing abundant SMCs and ECM without a lipid-rich necrotic core may undergo superficial plaque erosion (Virmani et al., 2000). The eroded plaque typically has a disrupted endothelial layer (Libby, 2013). The underlying mechanism of plaque erosion is

not clear. However, plaque erosion also leads to thrombotic complications, and it accounts for approximately 20 to 25 % of acute MI cases (Arbab-Zadeh et al., 2012; Libby, 2013).

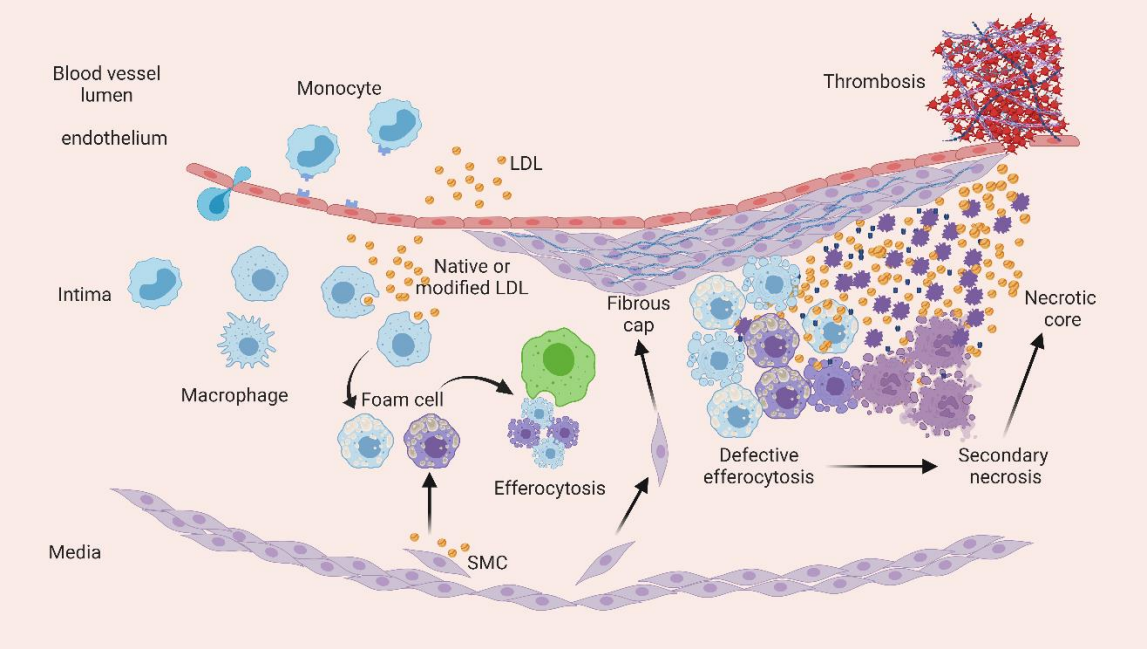

Figure 1: Atherosclerosis progression. Plasma LDL particles infiltrate into the intima of artery wall, triggering the recruitment of circulating monocytes. Monocytes in the subendothelial layer differentiate into macrophages that engulf retained LDL particles and become macrophage foam cells. SMCs de-differentiate and acquire either a synthetic or macrophage-like phenotype. Macrophage-like SMCs can also take up LDL particles and become cholesterol-laden foam cells. Both macrophage and SMCs derived foam cells undergo apoptosis and are cleared by phagocytes through efferocytosis. In advanced stages, efferocytosis is defective, leading to secondary necrosis. Cell debris, cholesterol crystals, extracellular lipids and inflammatory cytokines form a necrotic core. The

synthetic SMCs migrate to the surface of atherosclerotic plaque and secrete ECMs. ECM and SMCs form a thick fibrous cap which stabilizes the plaque. However, a large necrotic core and a thin fibrous cap ultimately result in the rupture of plaques, exposing the plaque thrombogenic contents. This activates platelets and the coagulation cascade and causes thrombus generation. So-called atherothrombosis in coronary arteries blocks the supply of blood to the myocardium, therefore leading to myocardial infarction. Figure created with BioRender.com.

1.2.3 The Stabilization and Regression of Established Atherosclerotic Plaques

Atherosclerosis develops starting from childhood (McGill et al., 2000) and nearly all people have established atherosclerotic plaques when reaching their 40s. To prevent catastrophic clinical sequela, it is critical to both limit the further growth of plaque sizes and increase plaque stability. The conventional view considers atherosclerotic plaque development beyond an early stage to be irreversible. However, recent studies have indicated that stabilization and even regression of established atherosclerotic plaques can be achieved (Barrett, 2020a). The Fisher lab has developed several mouse models of atherosclerotic plaque regression by normalizing hyperlipidemia (Feig et al., 2011; Peled et al., 2017; Reis et al., 2001). One model involves transplanting an aortic segment containing hyperlipidemia induced atherosclerosis into a normolipidemic recipient mouse (Reis et al., 2001). Reduction in plaque sizes in different regression models is accompanied by increased plaque stability indicated by reduced plaque macrophage content and increased collagen levels (Feig et al., 2011; Peled et al., 2017; Rahman et al., 2017). On the other hand, infusion of HDL or overexpression of apolipoprotein A-I

(APOA-I) which is the major component of HDL, also mediates atherosclerotic plaque regression in mouse models (Shah et al., 2001; Tangirala et al., 1999). The murine regression models suggest that extensive cholesterol lowering in atherogenic lipoproteins (VLDL + LDL) combined with raising HDL particle levels could provide beneficial outcomes on established atherosclerosis.

However, large clinical studies have shown that robust LDL cholesterol lowering led to only modest decreases in the volume of pre-existing plaques (typically less than 1.5%) (Nicholls et al., 2016; Nissen et al., 2004, 2006), while CVEs were significantly reduced. The discrepancy between the changes in plaque volume and CVEs suggests that plaque stabilization and changes in plaque composition might predominate in reducing coronary atherothrombotic events by lipid lowering interventions (Rahman & Fisher, 2018). Nevertheless, two thirds of patients on statin treatment still experienced CVEs and the residual risk remains after effective cholesterol lowering (Libby, 2005). This suggests only targeting LDL cholesterol levels does not lead to the optimal clinical benefits for patients with established atherosclerosis. The CANTOS trial which targets IL-1β has highlighted the importance of managing residual inflammatory risk in addition to cholesterol lowering in cardiovascular patients (Ridker, Everett, et al., 2017).

Importantly, the development and utilisation of murine atherosclerotic plaque regression models has provided valuable insights into the pathways involved in plaque regression (Barrett, 2020b; Rahman & Fisher, 2018). Understanding of macrophage polarization, macrophage emigration and cholesterol efflux during plaque regression might help resolve the residual risk and plaque inflammation. *In vitro*, macrophages can

be polarized toward the M2 subset by interleukin 4 (IL-4) or interleukin 13 (IL-13) via the signal transducer and activator of transcription 6 (STAT6) (Rahman & Fisher, 2018). A study using the transplanting regression model has shown that the recruitment of circulating lymphocyte antigen 6C (Ly6C)^{high} monocytes into the plaque and their conversion into the M2 macrophages via STAT6 is essential for the plaque regression (Rahman et al., 2017). This suggests promoting a healthier microenvironment that favors macrophage polarization toward M2 subset might be important for plaque regression. Macrophage emigration via the lymphatics is a characteristic of plaque regression in the transplanting regression model (Llodrá et al., 2004). C-C chemokine receptor 7 (CCR7) is important for dendritic cells (DCs) emigration from the peripheral tissues to the draining lymph nodes. During plaque regression, macrophages gain the expression of CCR7 and it is essential for macrophage emigration and plaque regression (Trogan et al., 2006).

In summary, beyond extensive plasma cholesterol lowering, directions aimed at promoting anti-inflammatory macrophage polarization, macrophage emigration, cholesterol efflux in macrophage and SMC derived foam cells and synthetic ECM-secreting SMC phenotype in the atherosclerotic plaque might help identify novel targets for therapeutic interventions against CVD.

1.3 Myocardial Infarction and Cardiac Remodeling

1.3.1 Cardiomyocyte Death during Myocardial Infarction

The occlusive atherothrombosis in coronary arteries leads to the blockage of blood supply to the nearby myocardium and causes ischemic cardiomyocyte death. This acute

event is defined as myocardial infarction. Studies have suggested that necrosis, apoptosis and autophagy are all involved in the death of cardiomyocytes in the infarcted heart (Frangogiannis, 2015).

Cardiomyocyte necrosis is characterized by cellular and organellar swelling and rupture of the plasma membrane, leading to the release of pro-inflammatory cellular contents. Cytoplasmic Ca^{2+} levels are increased in cardiomyocytes during ischemia. The entry of Ca^{2+} into the mitochondria via the mitochondrial Ca^{2+} uniporter triggers the opening of the mitochondrial permeability transition pore (mPTP) (Frangogiannis, 2015). Although mPTP has been proposed to be associated with early apoptosis, recent evidence has suggested the opening of the mPTP is central to necrosis (Kinnally et al., 2011). The opening of the mPTP results in the influx of water into the mitochondrial matrix, which causes mitochondrial swelling. Increased Ca^{2+} levels also cause the loss of mitochondrial membrane potential, reducing adenosine triphosphate (ATP) generation. The depletion of ATP and mitochondrial swelling ultimately lead to cardiomyocyte necrosis.

Apoptosis of cardiomyocytes is executed through both the intrinsic and extrinsic pathways (Chiong et al., 2011; Frangogiannis, 2015). The intrinsic pathway involves the activation of proapoptotic signaling molecules of the B-cell lymphoma 2 (Bcl-2) family in the mitochondria, which mediates the release of mitochondrial cytochrome C and the following activation of caspase 9 (Frangogiannis, 2015). The extrinsic pathway involves the activation of death receptors on the cardiomyocyte cell surface, including TNF-α and Fas receptors and the subsequent activation of caspase 8 (Chiong et al., 2011;

Frangogiannis, 2015). Both intrinsic and extrinsic pathways convergently activate the caspase 3 mediated apoptosis of cardiomyocytes.

Autophagy is a process where dysfunctional or unnecessary cell parts are degraded in the lysosome. Under normal conditions, it is an adaptive response to promote cell survival and energy hemostasis. In the normal heart, autophagy is observed at a baseline level (D. L. Li et al., 2016) and it is critical for maintaining myocardial structure and function. Autophagy levels are upregulated in cardiomyocytes by ischemia, which is dependent on the AMP-activated protein kinase (AMPK) pathway (Matsui et al., 2007a). It has been reported that autophagy during ischemia protects against cardiomyocyte death (Kanamori et al., 2011; Matsui et al., 2007a). On the other hand, autophagy levels during reperfusion are further upregulated via the beclin 1–dependent pathway, which might be maladaptive and detrimental (Matsui et al., 2007a). Whether autophagy is protective or detrimental for the infarcted heart is controversial at this stage (Gustafsson & Gottlieb, 2008; Lavandero et al., 2013).

1.3.2 Cardiac Remodeling Overview

The death of significant numbers of cardiomyocytes triggers cardiac repair with the development of myocardial fibrosis, leading to a fibrotic scar mainly containing collagen. The reparative healing process is critical for maintaining structural integrity and preventing cardiac rupture. However, the adverse remodeling which replaces the lost cardiomyocytes with excessive myocardial fibrosis, reduces the contractile function of the heart and even leads to ventricle dilation or ultimately heart failure. The infarct healing

process is divided into three phases, including the inflammatory, proliferative and maturation phases. During the inflammatory phase, monocytes and neutrophils are recruited to clear the dead bodies and ECM debris. The activation of the inflammatory cascade is necessary for a transition to the proliferative phase, which is characterized by the inflammatory resolution and myofibroblast mediated ECM deposition. A delicate balance between the inflammatory and proliferative phases is required for proper wound healing. At the maturation phase, myofibroblasts are removed or become quiescent and ECM is cross-linked to form a mature scar (Figure 2).

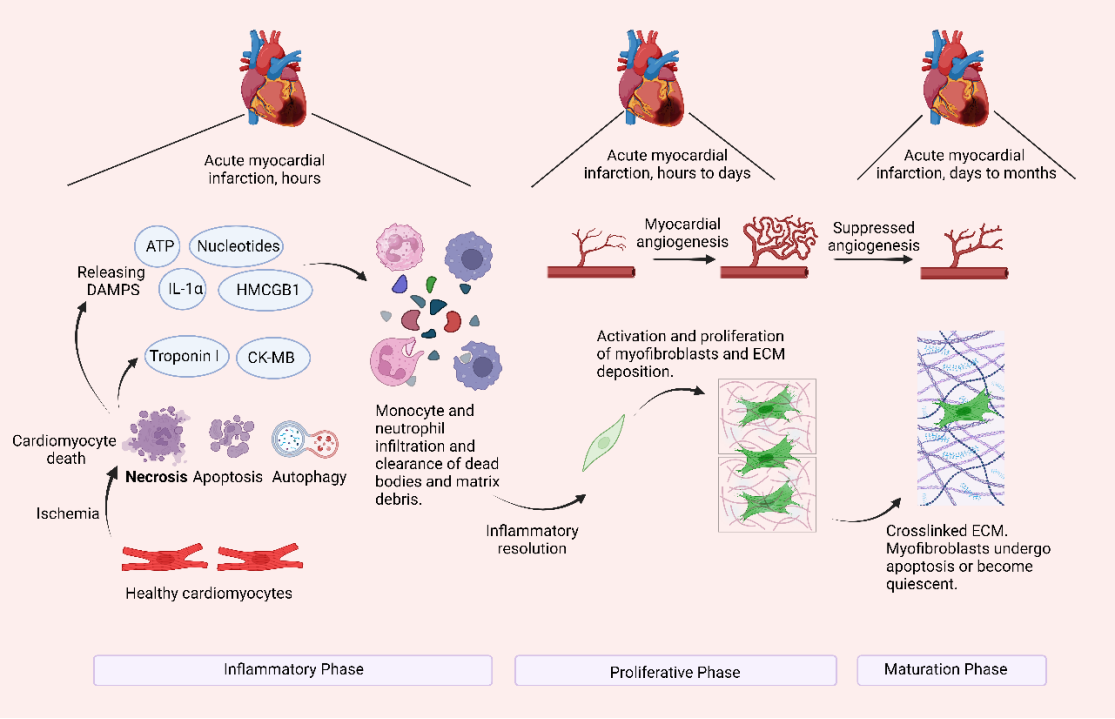

Figure 2: Cardiac remodeling post myocardial infarction. Cardiac remodeling can be divided into inflammatory, proliferative and maturation phases. The ischemia induced by

myocardial infarction results in the death of cardiomyocytes in the forms of necrosis, apoptosis and autophagy. During the inflammatory phase, cardiomyocytes undergoing necrosis release cardiomyocyte proteins, such as troponin I and CK-MB, as well as DMAPs, such as HMCGB1, nucleotides, ATP and IL-1α. DAMPs in the infarct zone attract the infiltration of circulating neutrophils and monocytes. Neutrophils and macrophages then clear the dead cardiomyocytes and matrix debris, which leads to the inflammatory resolution. During the proliferative phase, fibroblasts differentiate into myofibroblasts which secrete and deposit ECMs, mainly collagen. Collagen-based fibrotic scar prevents cardiac rupture. Myocardial angiogenesis also happens at the proliferative phase to supply the reparative infarct with nutrients and oxygen. During the maturation phase, myofibroblasts undergo apoptosis or become quiescent and ECM are cross-linked. Myocardial angiogenesis is inhibited. Figure is adapted from Wen, Z. J. et al. Emerging roles of circRNAs in the pathological process of myocardial infarction. Mol Ther Nucleic Acids 26, 828–848 (2021) with the permission pending from the publisher and created with BioRender.com.

1.3.3 Inflammatory Phase during Cardiac Remodeling

Following the myocardial ischemia, the acute inflammatory response is initiated which involves the heart itself (including cardiomyocytes, fibroblasts, ECs, and the interstitium) and the infiltrated immune cells (including neutrophils, monocytes and lymphocytes). Ischemic cardiomyocytes undergoing necrosis release cardiomyocyte proteins, such as creatine-kinase myocardial band (CK-MB) and Troponin I, as well as damage associated molecular patterns (DAMPs), such as ATP, mitochondrial (mt)DNA,

extracellular RNA, interleukin 1 alpha (IL-1α) and high mobility group box 1 (HMCGB1) (Ong et al., 2018). Those DAMPs induce leukocyte infiltration, serve as danger signals and activate the innate immune system by binding to the pattern recognition receptors (Frangogiannis, 2015).

The complement system is activated in response to DAMPs in the infarcted heart demonstrated by the C3 fragments and other complement cascade proteins in the myocardial infarcts (Hill & Ward, 1971; Yasojima et al., 1998). Complement components, DAMPs, inflammatory cytokines and chemokines all attract the infiltration of circulating neutrophils and inflammatory Ly6C^{high} monocytes (Ong et al., 2018; Prabhu & Frangogiannis, 2016). Neutrophils are the first immune cells that arrive at the infarct area, predominately at the border zone. Inflammatory monocytes are recruited to the injury site following the infiltration of neutrophils. The ligand and receptor pairs between circulating leukocytes and activated ECs, including selectin ligand-selectin, chemokine receptor-chemokine and integrin-integrin ligand are critical for neutrophils and inflammatory monocytes transmigrating through the activated endothelial layer (Frangogiannis, 2008; Prabhu & Frangogiannis, 2016). Infiltrated neutrophils and inflammatory monocytes release MMPs to mediate ECM degradation and phagocytize the dead cells and matrix debris (Frangogiannis, 2015). Neutrophils also generate reactive oxygen species (ROS) and secret factors that attract circulating monocytes (Ong et al., 2018). Inflammatory monocytes phagocytize apoptotic neutrophils (Frangogiannis, 2015). They also secret cytokines, chemokines and growth factors that may either exacerbate the inflammatory

response or assist the transition to the reparative phase depending on the microenvironment (Ong et al., 2018).

1.3.4 Proliferative Phase during Cardiac Remodeling

As dead cells and matrix debris are cleared, the major proinflammatory stimuli are eliminated. The proliferative phase is then initiated when the inflammation gets resolved and myofibroblasts deposit ECMs such as collagen and mediate the reparative response. The resolution of inflammation starts with neutrophil apoptosis and its subsequent clearance by phagocytotic macrophages, which then induces an anti-inflammatory phenotype in macrophages (Frangogiannis, 2015). Neutrophils secret pro-resolving lipid mediators that inhibit neutrophil transmigration and infiltration (Serhan et al., 2008). Neutrophils also secret factors that induce macrophage polarization towards a reparative phenotype (Horckmans et al., 2017). During the reparative phase, proinflammatory circulating Ly6C^{high} monocytes are recruited at the infarct site and switch their phenotype to anti-inflammatory and reparative Ly6C^{low} macrophages (Courties et al., 2014; Hilgendorf et al., 2014), which secret anti-inflammatory, profibrotic, and angiogenic factors, such as interleukin 10 (IL-10), transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) (Prabhu & Frangogiannis, 2016). Tissue neovascularization is required to supply the reparative infarct with nutrients and oxygen at the proliferative phase. Angiogenic pathway is activated indicated by the upregulation of VEGF in the border zone cardiomyocytes and VEGF receptors in new vessels (J. Li et al., 1996). At later stages, newly developed vessels mature by acquiring a coat of

pericytes and SMCs, which is dependent on the platelet-derived growth factor (PDGF)-PDGF receptor β signaling (Zymek et al., 2006).

As the microenvironment becomes less inflammatory, fibroblasts are activated and acquire the synthetic myofibroblast phenotype, which has increased levels of proliferation. Studies have suggested that there are a variety of origins of myofibroblasts, including resident fibroblasts, bone marrow progenitors, endothelial cells, epicardial cells, cardiac pericytes, interstitial cells and SMCs (Frangogiannis, 2015). However, the major source of myofibroblasts might be resident fibroblasts (Yano et al., 2005). The myofibroblast transdifferentiation involves TGF-β and angiotensin-II signaling pathways. TGF-β and angiotensin-II also trigger the matrix-preserving program that induces ECM synthesis in myofibroblasts (Frangogiannis, 2015; Prabhu & Frangogiannis, 2016). Fibrin and fibronectin derived from plasma form the initial provisional scaffold during the inflammatory and proliferative phases (Frangogiannis, 2021). Activated myofibroblasts at the proliferative stage also produce fibronectin initially and later collagen type I and type II which are assembled to form a fibrotic scar and maintain the structural integrity (Weber et al., 2012).

1.3.5 Maturation Phase during Cardiac Remodeling

The transition from the proliferative phase to the maturation phase is marked by the cross-linking of collagen, the reduced number of myofibroblasts and suppressed angiogenesis. Apoptosis might contribute to the significantly reduced myofibroblast numbers in the infarct. The remaining myofibroblasts become quiescent or have reduced

capacity for ECM synthesis. The signaling pathways that are involved in the quiescence and suppressed proliferation of myofibroblasts in the infarct at the maturation phase are unclear. However, the clearance of fibrogenic growth factors and matricellular proteins and the activation of STOP signals for TGF-β and angiotensin-II might all be involved.

1.4 Lipids and Lipoproteins

1.4.1 Lipids - Cholesterol and Triglycerides

Lipids are the essential structural components and energy sources of cells. There are three main categories of lipids, including cholesterol, triglycerides (TGs), and phospholipids. Cholesterol is a component of cell membranes. The structure of cholesterol contains a tetracyclic ring which is responsible for the cell membrane's fluidity. Cholesterol also plays a number of other biological roles, including as the precursor for the synthesis of steroid hormones, bile acids and vitamin D and as signaling molecules (Hegele, 2009). The majority of circulating cholesterol is produced from endogenous synthesis in the ER of hepatic cells. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol synthesis pathway, and it is the target of statins which is the most commonly prescribed class of drugs for CAD. TGs are the most energy-dense molecules. They are composed of a glycerol backbone ester-linked to three free fatty acids (FFA) molecules. TGs are synthesized in intestinal enterocytes and hepatocytes and hydrolyzed into FFAs to provide energy through β-oxidation in muscle cells or store energy as resynthesized to TG in adipocytes.

Cholesterol and TGs are insoluble in plasma, therefore, the transportation of them throughout the body is carried out by macromolecules called lipoproteins, which have a spherical shape and are compatible with the aqueous plasma. Lipoproteins have a hydrophobic core containing TGs and cholesterol esters (CEs), and a hydrophilic surface monolayer consisting of phospholipids, free cholesterol and associated protein called apolipoprotein (Figure 3). Based on the density of lipoproteins, they are broadly classified as chylomicrons, very-low density lipoproteins (VLDL), LDL and HDL. The density order is chylomicron < VLDL < LDL < HDL.

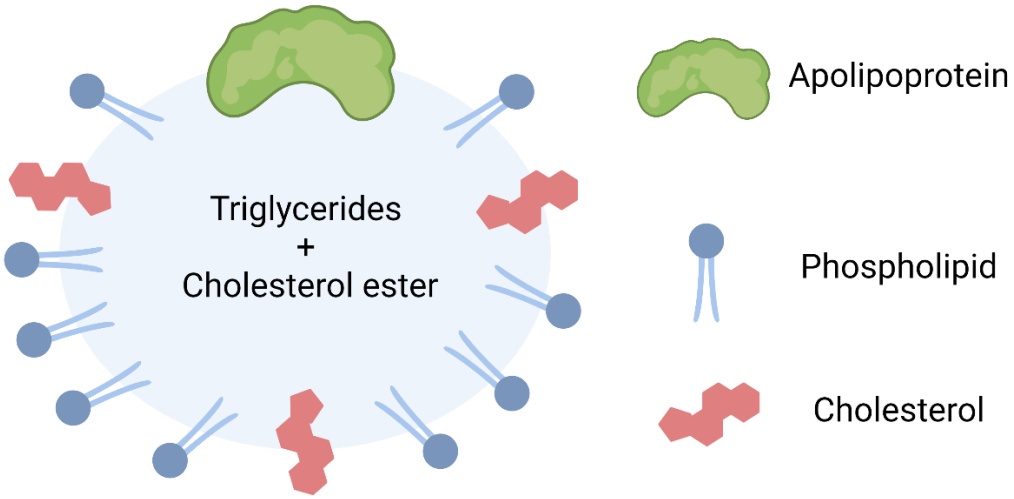

Figure 3: Lipoprotein structure. Lipoproteins are spherical particles with a hydrophobic core containing triglycerides and cholesterol esters and surrounded by a hydrophilic surface monolayer consisting of phospholipids, free cholesterol and apolipoprotein.

1.4.2 Chylomicron Formation and Metabolism

Chylomicron formation in intestine starts with the entry of FFAs and cholesterol into intestinal enterocytes from the intestinal lumen. FFAs in the intestinal lumen mainly come from the diet and enter enterocytes via passive diffusion or via a fatty acid transporter (Feingold, 2022). FFAs and monoacylglycerols in enterocytes are synthesized into TGs. Cholesterol in the intestinal lumen enters enterocytes via the Niemann-Pick C1-like 1 (NPC1L1) transporter (Betters & Yu, 2010). Ezetimibe, as a cholesterol lowering drug, inhibits NPC1L1 and the absorption of cholesterol (Betters & Yu, 2010). Cholesterol in enterocytes is converted to CE by acyl-CoA cholesterol acyltransferase (ACAT) (Hai & Smith, 2021). In the ER of enterocytes, TGs, CEs, and a single apolipoprotein B48 (APOB48) are packaged together to form one chylomicron particle. The microsomal triacylglycerol transfer protein (MTTP) plays an essential role in adding TGs to nascent APOB48 (Hussain et al., 2012). Both APOB48 and apolipoprotein B100 (APOB100) are encoded by the *APOB* gene, however, APOB48 is formed by an RNA editing enzyme - *APOB* mRNA editing catalytic polypeptide-1 (APOBEC-1) that creates an early stop codon in the *APOB100* mRNA. APOB48 protein constitutes the first 48% of the amino acid sequences for APOB100 protein and lacks the C-terminal LDLR binding region in APOB100 protein.

Once released into the circulation, chylomicrons acquire apolipoprotein E (APOE) and apolipoprotein C-II (APOC-II) from HDL, donate APOA-I to HDL and become mature chylomicrons. TGs in mature chylomicrons are hydrolyzed by lipoprotein lipase (LPL) on the capillary endothelial cell surface (Olivecrona, 2016). APOC-II is an

essential cofactor for the activity of LPL(Kumari et al., 2021; Wolska et al., 2017). The hydrolysis of chylomicron TGs results in the release of FFAs for either energy production or storage and the formation of chylomicron remnants. Chylomicron remnants return APOC-II to HDL, acquire more APOE from HDL and become CE-rich, APOE-containing lipoproteins. The APOE-containing chylomicron remnants are removed from the circulation by the liver via the LDL receptor (LDLR) or the LDLR related protein 1 (LRP-1) (Daniels et al., 2009) (Figure 4).

1.4.3 VLDL and LDL Formation and Metabolism

De novo fatty acid synthesis, breakdown of TG-containing lipoproteins and circulating fatty acids are the sources of fatty acids for TG synthesis in the liver (Feingold, 2022). Each VLDL particle is formed by the packaging of TGs, cholesterol, and a single APOB100 together in the ER of hepatocytes. MTTP is involved in the transfer of TGs to APOB100 (Hussain et al., 2012)**.** Newly synthesized VLDL is secreted into the circulation. Similar to chylomicrons, VLDL receives APOE and APOC-II from HDL and then undergoes hydrolysis of associated TGs mediated by LPL to release FFAs and form VLDL remnants (Olivecrona, 2016). VLDL remnants are also called intermediate-density lipoproteins (IDL). IDL is either removed from the circulation by the hepatic LDLR or LRP-1, or converted to LDL by hepatic lipase mediated hydrolysis which removes excess TGs and phospholipids (Daniels et al., 2009; Kobayashi et al., 2015). The resulting LDLs are enriched in CE and the only apolipoprotein in each LDL particle is one APOB100. Angiopoietin-like protein 3 (ANGPTL3) is a glycoprotein secreted from the liver and inhibits the activity of LPL and other lipases (Kersten, 2021;

Tikka & Jauhiainen, 2020). ANGPTL3 inhibition is a target for lowering plasma LDL cholesterol and TGs levels (Adam et al., 2020; Kersten, 2021; Tikka & Jauhiainen, 2020).

LDL particles deliver cholesterol to extrahepatic tissues for utilization. However, high levels of plasma LDL cause the accumulation of LDL cholesterol in the artery wall and initiate atherogenesis. LDLR in the liver is the major pathway that mediates the clearance of circulating LDL cholesterol. LDLR on the cell surface binds to APOB100 of the LDL particle and mediates the endocytosis of the LDLR:LDL particle complex via a clathrin-coated pit (Goldstein & Brown, 2009). Once the LDLR-LDL complex is being internalized, LDLR disassociates from the LDL particle in the endosome at the late stage with a low pH. The released LDL particle further travels to the lysosome and gets degraded and the LDLR recycles back to the cell surface for reuse. Mutations in the LDLR and APOB100 genes that result in the reduction of LDLR mediated endocytosis of LDL particles, are two of the causes of familial hypercholesterolemia (FH), characterized by significantly increased plasma LDL cholesterol levels (Santos et al., 2016) (Figure 4).

1.4.4 HDL Formation and Metabolism

HDL plays a central role in reverse cholesterol transport (RCT), a process where HDL particles transport cholesterol from peripheral tissues (including macrophages in the artery wall) to the liver for reuse or excretion (B. L. Trigatti et al., 2000; Von Eckardstein et al., 2001). HDL formation starts with APOA-I secretion from the liver and intestine. This monomolecular lipid-free APOA-1 acquires free cholesterol and phospholipids via ATP-binding cassette subfamily A member 1 (ABCA-1) from those tissues (Kathiresan et

al., 2008; Rye & Barter, 2004). They later are converted to discoidal HDL which has at least two molecules of APOA-I that wrap around the phospholipid bilayer (Z. Wu et al., 2007). The discoidal HDL acquires more free cholesterol and phospholipids via ABCA-1 and becomes lipid-rich HDL (Daniels et al., 2009; Fielding et al., 2000). The free cholesterol on lipid-rich HDL undergoes esterification mediated by the lecithin cholesterol acyl transferase (LCAT). LCAT is secreted from the liver, located on the surface of discoidal HDL particles and activated by APOA-I (Pavanello & Calabresi, 2020). The esterified cholesterol moves from the surface to the core of HDL particles, resulting in the HDL morphological change from a discoidal to a spherical shape (M. Wang & Briggs, 2004). The mature, spherical, CE-rich HDL can also acquire more free cholesterol from macrophage foam cells via ATP-binding cassette subfamily G member 1 (ABCG-1) or passive diffusion facilitated by the scavenger receptor class B type I (SR-B1) (Kardassis et al., 2014).

Spherical mature HDL particles exchange CE for TG with VLDL and LDL particles. This process removes cholesterol from HDL particles and is mediated by the cholesteryl ester transfer protein (CETP) (Barter et al., 2003). The other major pathway to remove HDL cholesterol is through SR-B1 mediated selective uptake of cholesterol in the liver, a key step in RCT (B. L. Trigatti et al., 2003). Hepatic SR-B1 depletes cholesterol from the bound HDL particle, which is then released back into the circulation and serves another round for extracting excess cholesterol from peripheral tissues (Krieger, 1999). Alternatively, lipid-poor HDL particles can be degraded in the kidney (Hammad et al., 1999) (Figure 4).

Epidemiological studies have shown that low HDL levels are associated with an increased risk of CVD (Gordon et al., 1989; Pekkanen et al., 1990; Wilson et al., 1988). However, elevating HDL cholesterol levels by different interventions did not reduce CVEs in large clinical trials (Barter et al., 2007; Kastelein et al., 2007; MJ et al., 2014; Nissen et al., 2007). These results have prompted the field to consider that it is the function of HDL particles instead of the HDL cholesterol level that protects against CVD. HDL cholesterol levels do not necessarily reflect the abundance of HDL particles or RCT capacity as each HDL particle may carry different amounts of cholesterol. During RCT, HDL mediates cholesterol efflux from macrophage foam cells through the transporters ABCA1 and ABCG-1 (Yvan-Charvet et al., 2010). ABCA1 and ABCG1 expressions are upregulated by the liver X receptors (LXRs) which are activated in response to increased intracellular cholesterol levels (Beyea et al., 2007). *In vivo* studies have shown that raising functional HDL particle levels or enhancing ABCA1 and LXR expression promoted plaque regression in murine models (Designed Research; J & Performed Research; J, 2011; Feig et al., 2010; Rayner et al., 2011; Shah et al., 2001). It has also been shown that ABCA1 expression was reduced in SMC-derived foam cell, contributing to the excess lipid accumulation in the plaque (Allahverdian et al., 2014b). These studies suggest that stimulating cholesterol efflux from macrophage and SMC foam cells might be a potential strategy to promote plaque regression.

Lipoprotein metabolism and regulation are slightly different between humans and mice. Humans express APOBEC-1 exclusively in the intestine and therefore, produce APOB48 in the intestine and APOB100 in the liver, respectively. However, mice express

APOBEC-1 in both the liver and intestine, and mice produce predominantly APOB48 in the intestine and produce both APOB48 and APOB100 in the liver (Nakamuta et al., 1996). Furthermore, humans express CETP whereas mice lack CETP, leading to the absence of exchange of cholesterol for TG between VLDL and HDL in mice. Therefore, humans carry plasma cholesterol mainly in LDL particles, while mice carry plasma cholesterol predominantly in HDL particles. This phenomenon may contribute to the inherent resistance of atherosclerosis development in mice. The differences in lipoprotein metabolism between humans and mice must be taken into account when using mice to model human lipoprotein metabolism and associated disease.

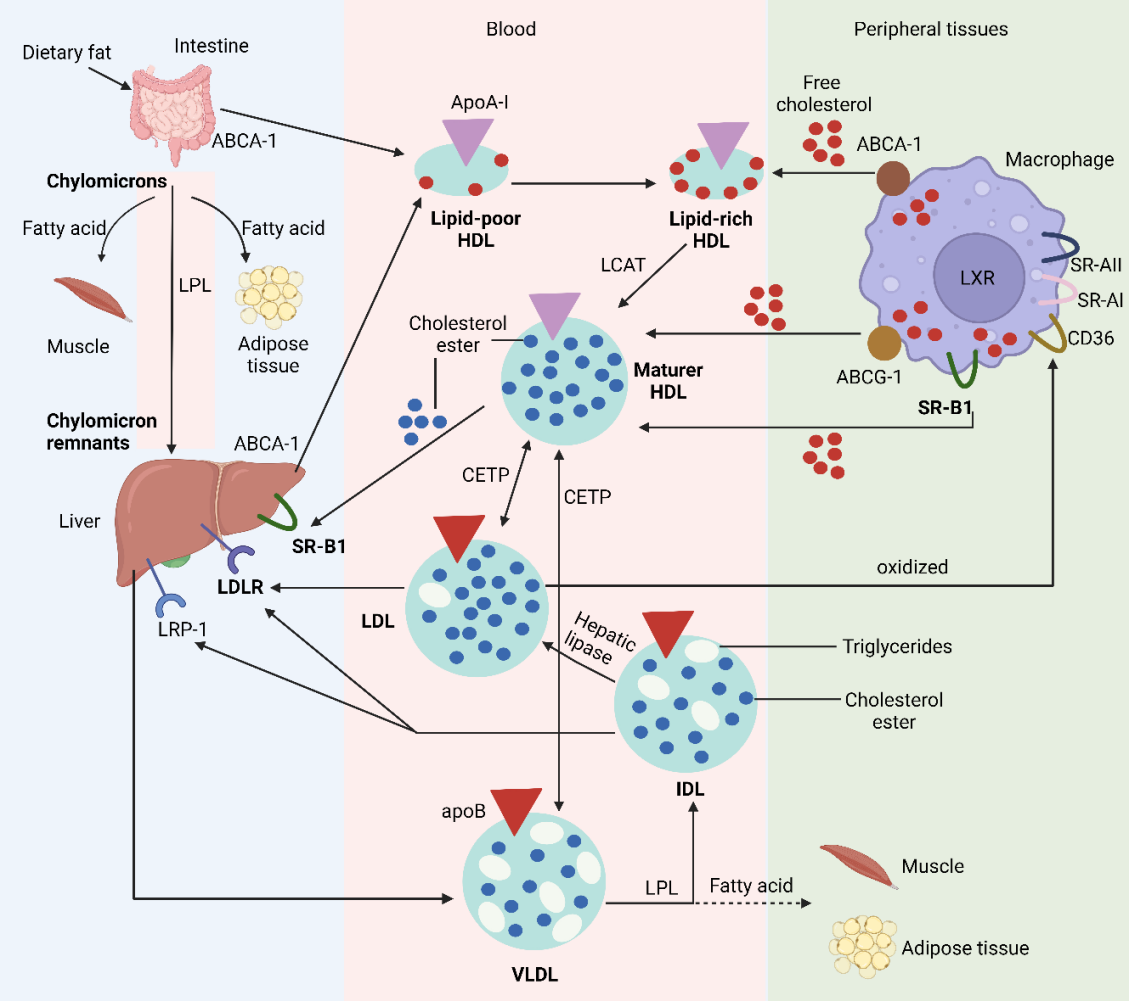

Figure 4: Lipoprotein metabolism. Chylomicrons are secreted from the intestine into the lymph and delivered to the circulation. Triglycerides in chylomicrons are hydrolyzed by the LPL to provide free fatty acids to muscle cells for energy production or adipose tissue for energy storage. The hydrolysis of chylomicrons results in the formation of chylomicron remnants which are cleared by the liver. Liver secrets VLDL particles which are hydrolyzed by the LPL, resulting in the formation of IDL particles and free fatty acids used by muscle cells and adipose tissue. IDL particles are either cleared by the liver through the LDLR and LRP-1 or are further converted into LDL particles by the hepatic

lipase. Circulating LDL is cleared by the liver through the LDLR. Circulating LDL enters the artery wall and is modified by oxidation. Oxidized LDL can be taken up by macrophages in the artery wall mediated by the cell surface CD36, SR-AI and SR-AII, leading to foam cell formation. ApoA-I obtains lipids from the intestine and liver mediated by the ABCA-1 and becomes lipid-poor HDL. Lipid-poor HDL acquires more cholesterol from peripheral tissues (e.g. macrophages in the artery wall) via ABCA-1 and becomes lipid-rich HDL. Free cholesterol on lipid-rich HDL undergoes esterification mediated by the LCAT which leads to the formation of mature HDL. Mature HDL can acquire more cholesterol from peripheral tissues via ABCG-1 or SR-B1. Mature HDL can also exchange cholesterol for triglycerides with VLDL and LDL mediated by the CETP. SR-B1 on hepatocytes mediates selective cholesterol uptake from HDL and the cholesterol-depleted HDL is released into circulation and reused to extract cholesterol from peripheral tissues. The movement of cholesterol from peripheral tissues to the liver carried by HDL and mediated by SR-B1 is called reverse cholesterol transport. Figure is adapted from Kathiresan, S. et al. Translating molecular discoveries into new therapies for atherosclerosis. Nature 451, 904–914 (2008) with the permission pending from the publisher and created with BioRender.com.

1.5 Conventional Mouse Models of Atherosclerosis

Efforts have been made to induce atherosclerosis development in mice by manipulating genes involved in lipoprotein metabolism such as the LDLR and APOE. This section will discuss genetically modified mouse models for atherosclerosis studies,

including *ApoE knockout* (*ApoE KO*) and *Ldlr KO* mice which are the two most frequently used mouse models and *APOE*3Leiden.CETP* mice that have an intact endogenous expression of both the LDLR and APOE.

1.5.1 *ApoE KO* Mice

APOE presents on the surface of chylomicrons, VLDL, and HDL particles. It mediates the clearance of plasma chylomicron remnants and VLDL remnants by binding to the LDLR and LRP-1 in the liver. In 1992, two separate groups generated *ApoE KO* mice where the *ApoE* gene was inactivated by homologous recombination in mouse embryonic stem (ES) cells (Piedrahita et al., 1992; Plump et al., 1992a). *ApoE KO* mice have markedly increased plasma cholesterol levels (~10-15 mM) compared to the wild type littermates (~2 mM) on a normal chow diet (4.5% fat and 0.02% cholesterol) (Breslow, 1996; Piedrahita et al., 1992; Plump et al., 1992a). The majority of cholesterol carried by HDL particles in wild type mice is shifted to the VLDL sized lipoproteins in *ApoE KO* mice (Plump et al., 1992a; S. H. Zhang et al., 1992a). On a normal chow diet, *ApoE KO* mice develop fatty streak consisting of foam cells in mice as young as 2 months of age (Nakashima et al., 1994). Those fatty streak lesions progress to more advanced atherosclerosis containing necrotic cores and a fibrous cap by the 8-10 months of age (Reddick et al., 1994). A Western-type diet (21% fat, 0.15% cholesterol) feeding further drastically increases plasma cholesterol levels and accelerates atherosclerosis development in *ApoE KO* mice (Nakashima et al., 1994; S. H. Zhang et al., 1994). Atherosclerotic lesions in *ApoE KO* mice first develop at the aortic sinus and later spread throughout the aorta and its principal branches (Nakashima et al., 1994). However, *ApoE*

KO mice do not frequently develop atherosclerosis in coronary arteries and the associated myocardial infarction, which are the fundamental characteristics of CAD (Braun et al., 2002; Gonzalez L et al., 2016).

1.5.2 *Ldlr KO* Mice

Mutations in the *LDLR* gene are the most common causes of FH. Human patients with homozygous loss of function mutations in the *LDLR* gene have very high levels of plasma LDL cholesterol and often die from myocardial infarction before age of 15 years old (Goldstein & Brown, 1979). LDLR is a cell membrane glycoprotein containing a cytoplasmic domain, a transmembrane anchor and extracellular domains (Go & Mani, 2012). The extracellular domains of LDLR consist of LDLR type A repeats (ligand binding domain), an epidermal growth factor (EGF)-like domain, a β-propeller domain and an O-linked sugar domain (Go & Mani, 2012). The type A repeats of the LDLR recognize and bind to APOE and APOB100 and mediates the clearance of APOE-containing and APOB100-containing lipoproteins from the circulation. Unlike *ApoE KO* mice, *Ldlr KO* mice fed a normal chow diet only develop mild hypercholesterolemia (~6 mM) associated with increased amounts of LDL sized lipoproteins in blood (Ishibashi et al., 1993) and minimal atherosclerosis (Palinski et al., 1995; Whitman, 2004). However, feeding them Western-type and other high fat/high cholesterol diets leads to severe hypercholesterolemia (>25 mM) and extensive aortic atherosclerosis in *Ldlr KO* mice (Lichtman et al., 1999; Palinski et al., 1995). Nevertheless, similar to *ApoE KO* mice, *Ldlr KO* mice rarely develop coronary artery atherosclerosis and they do not exhibit substantial myocardial fibrosis (Gonzalez L et al., 2016).

Both *ApoE KO* and *Ldlr KO* mice lack the intact APOE-LDLR axis, which is required for certain cholesterol lowering agents such as statins. One mechanism of statin mediated cholesterol lowering is the upregulation of hepatic LDLR levels (Oesterle et al., 2017). Statins reduce intracellular cholesterol levels which activates sterol regulatory element-binding protein 2 (SREBP2), therefore increasing the transcription of SREBP2 target genes, including the *LDLR*. Studies have shown that statin treatment in some cases did not reduce plasma cholesterol levels in mice lacking either *Ldlr* or *ApoE*(Z. Chen et al., 2002; Enomoto et al., 2009; Monetti et al., 2007; Schroeter et al., 2009; Zadelaar et al., 2007).

1.5.3 *APOE*3Leiden.CETP* Mice

There are three major isoforms of APOE, including APOE2, APOE3 and APOE4, encoded by three alleles (*APOE*2, APOE*3* and *APOE*4*) of the *APOE* gene (Zannis & Breslow, 1981). APOE2, APOE3 and APOE4 have different mobilities upon isoelectic focusing by one charge unit. While APOE4 is the most basic isoform, APOE2 is the most acidic isoform. The *APOE*3Leiden* mutant form of the human *APOE* gene is identical to the *APOE*4* allele but has an in-frame repeat of 21 nucleotides in exon 4 (van den Maagdenberg et al., 1989; Wardell et al., 1989). This *APOE*3Leiden* mutation leads to an insertion of 7 amino acids containing one extra negatively charged glutamyl residue when compared to the APOE4 and results in the protein focusing in the APOE3 position (Wardell et al., 1989). The *APOE*3Leiden* mutation is associated with familial dysbetalipoproteinemia, characterized by the impaired clearance of APOE-containing chylomicron and VLDL remnants (De Knijff et al., 1991). *APOE*3Leiden* transgenic

mice were generated by introducing a construct containing the human *APOE*3Leiden* gene and *APOCI* gene into C57 Bl/6 mice (Van den Maagdenberg et al., 1993). *APOE*3Leiden* transgenic mice express endogenous APOE, however, the clearance of APOE-containing lipoproteins is impaired by the dominant role of the *APOE*3Leiden* mutant (Van den Maagdenberg et al., 1993). *APOE*3Leiden* transgenic mice on a normal chow diet have increased plasma cholesterol and TG levels carried by VLDL/LDL sized lipoproteins (Van den Maagdenberg et al., 1993). *APOE*3Leiden.CETP* mice were developed by crossing *APOE*3Leiden* mice with human *CETP* transgenic mice (Westerterp et al., 2006). Human *CETP* expression in *APOE*3Leiden* mice increases plasma cholesterol levels and shifts cholesterol associated with HDL to VLDL/LDL (Westerterp et al., 2006). *APOE*3Leiden.CETP* mice develop more severe atherosclerosis in the aortic sinus compared to *APOE*3Leiden* mice when fed with a Western-type diet (15% fat and 0.25% cholesterol) (Westerterp et al., 2006). *APOE*3Leiden.CETP* mice represent a useful atherosclerosis model for testing potential therapeutics for cholesterol lowering that require the intact expression of both LDLR and APOE or target CETP. However, like *ApoE KO* and *Ldlr KO* mice, characteristics of CAD including substantial coronary artery atherosclerosis and myocardial fibrosis have not been reported in *APOE*3Leiden.CETP* mice.

1.6 Scavenger Receptor Class B Type I (SR-B1)

SR-B1, encoded by the *SCARB1* gene, is a member of the scavenger receptor class B family along with other class B members, which includes cluster determinant 36

(CD36) and lysosomal integral membrane protein 2 (LIMP-2) (Shen et al., 2018). SR-B1 is abundantly expressed in the liver and steroidogenic tissues (adrenal glands and ovaries), and expressed at low levels in other cell types, such as macrophages, ECs and SMCs (Acton et al., 1996; Krieger, 1999; Shen et al., 2018). SR-B1 protein concentrates in cholesterol- and glycolipid-rich microdomains on the plasma membrane called caveolae (Babitt et al., 1997). As a multiligand membrane-spanning receptor, SR-B1 contains a large extracellular domain, two transmembrane domains and short N and C terminal cytoplasmic domains (B. L. Trigatti, 2017). Based on the LIMP-2 structure and the homology remodeling of SR-B1, it is proposed that the extracellular domain of SR-B1 comprises an antiparallel β-barrel core with many short α-helical segments (Neculai et al., 2013).

1.6.1 The Role of SR-B1 in Reverse Cholesterol Transport

SR-B1 is a high-affinity receptor for HDL and plays an important role in RCT (Acton et al., 1996). SR-B1 facilitates free cholesterol efflux from macrophage foam cells to mature HDL (Phillips, 2014). HDL particles transport cholesterol in the form of CE through the circulation to the liver. Hepatic SR-B1 then mediates selective CE uptake from HDL (Rigotti et al., 1997a) (Figure 4). HDL-derived cholesterol taken up by the liver is secreted into the bile or used for the repackaging of new lipoproteins. Selective cholesterol uptake has been described as a two-step process, which involves the binding of HDL to SR-B1 followed by cholesterol movement from HDL to the plasma membrane (Gu et al., 1998). The α-helix bundle at the apex of the SR-B1 extracellular domain creates a cluster of basic residues, which facilitates the binding of SR-B1 to the

amphipathic α-helix domain of apoA-I on the surface of HDL (Neculai et al., 2013; Phillips, 2014). The extracellular domain of SR-B1 is predicted to contain a hydrophobic channel that allows for the movement of CE down a concentration gradient from HDL to the plasma membrane (Neculai et al., 2013; Phillips, 2014; B. Trigatti et al., 2000). The lipid-depleted HDL particle is then thought to dissociate from SR-B1 and re-enters the circulation. Consistent with the critical role of SR-B1 in RCT, *Sr-b1 knockout* in mice results in increased plasma cholesterol levels associated with enlarged HDL particles due to impaired selective cholesterol uptake (Rigotti et al., 1997a). The enlarged HDL particles have abnormally high free cholesterol to total cholesterol ratios accompanied by reduced LCAT activities (Braun et al., 2003; Ma et al., 2005). SR-B1 also regulates biliary cholesterol levels (the last step of RCT) where deficiency of *Sr-b1* reduces and overexpression of hepatic *Sr-b1* increases biliary cholesterol concentrations (Kozarsky et al., 1997; B. Trigatti et al., 1999).

1.6.2 The Role of SR-B1 beyond Reverse Cholesterol Transport

SR-B1 expression in the steroidogenic tissues also mediates selective cholesterol uptake from HDL for storage or steroid hormone production. Deficiency of *Sr-b1* leads to the reduction of cholesterol in adrenal glands and ovaries and infertility in female mice (Rigotti et al., 1997a; B. Trigatti et al., 1999). *Sr-b1 KO* mice develop thrombocytopenia characterized by reduced platelet counts and active circulating platelets, and their platelets are abnormally large (Dole et al., 2008; Korporaal et al., 2011). The abnormalities of platelets in *Sr-b1 KO* mice were proposed to be induced by the abnormal lipid

environment with a high free cholesterol to total cholesterol ratio (Dole et al., 2008; Korporaal et al., 2011).

SR-B1 is expressed at low levels in macrophages and ECs, however, it plays an important role in these cells in atherosclerosis development. SR-B1 in macrophages mediates HDL stimulated cell migration which has been hypothesized to play a potential role in atherosclerotic plaque regression (Al-Jarallah et al., 2014a). It also plays an anti-inflammatory role in macrophages. *Sr-b1* deficiency in bone marrow derived macrophages increased IL-6 and TNF-α production stimulated by lipopolysaccharides (LPS) (Cai et al., 2012). Furthermore, the absence of *Sr-b1* expression in bone marrow derived cells promotes atherosclerosis development in multiple mouse models without altering plasma cholesterol levels (Covey et al., 2003; Pei et al., 2013; Tao et al., 2015; W. Zhang et al., 2003). SR-B1 in ECs plays a dual role in atherosclerosis development. As mentioned in atherosclerosis initiation, SR-B1 on ECs mediates LDL transcytosis through the endothelium layer and promotes atherosclerosis (Armstrong et al., 2015; Huang et al., 2019). However, SR-B1 is also required for HDL signaling mediated atheroprotective effects in ECs, such as activation of endothelial nitric oxide (NO) synthase (eNOS) and promoting migration and proliferation and suppressing activation of ECs (B. L. Trigatti & Fuller, 2016a).

1.7 Mouse Models of Coronary Artery Disease

SR-B1 is expressed in a variety of tissues and cells and its roles in RCT and beyond RCT are crucial to atherosclerosis development. Traditional genetically modified

mouse models of atherosclerosis that do not develop substantial coronary artery occlusions obtain characteristics of human CAD when in combination with *Sr-b1* deficiency, such as *Sr-b1/ApoE double knockout (dKO)* and *Sr-b1/Ldlr dKO* mice. Furthermore, it has been recently demonstrated that inactivation of *Sr-b1* alone in mice promotes an atherogenic diet induced coronary artery atherosclerosis (M. T. Fuller et al., 2023). Here, I describe two mouse models that develop coronary artery atherosclerosis and myocardial fibrosis used in my thesis, including *Sr-b1/ApoE dKO* and *Sr-b1 KO* mice.

1.7.1 *Sr-b1/ApoE Double KO* Mice

In 2002, Monty Krieger's lab described the CAD phenotype of *Sr-b1/ApoE dKO* mice(Braun et al., 2002). *Sr-b1/ApoE dKO* mice have significantly increased plasma cholesterol levels associated with VLDL sized lipoproteins and abnormally high free cholesterol to total cholesterol ratios compared to *ApoE KO* mice (Braun et al., 2002, 2003). On a normal chow diet, these *Sr-b1/ApoE dKO* mice develop accelerated atherosclerosis in the aortic sinus by 5 weeks of age (Braun et al., 2002). Importantly, they also rapidly develop substantial coronary artery atherosclerotic occlusions and myocardial fibrosis (Braun et al., 2002). Fibrin and platelet accumulation were detected in coronary artery atherosclerotic plaques, suggesting atherothrombosis in coronary arteries (Braun et al., 2002; Yu et al., 2018). Moreover, they exhibit cardiac enlargement and dysfunction and abnormalities of cardiac conduction system associated with a significantly reduced lifespan (50% mortality around 6 weeks of age) (Braun et al., 2002). Myocardial lipid and macrophage accumulation was also reported in these *dKO* mice

(Braun et al., 2002). Together, *Sr-b1/ApoE dKO* mice exhibit multiple characteristics of human CAD, making it an attractive mouse model to test potential therapeutics or study mechanisms of CAD. Various interventions have been tested in *Sr-b1/ApoE dKO* mice to date(Al-Jarallah et al., 2013a; Braun et al., 2003, 2008; Yu et al., 2018). In 2018, we reported that rosuvastatin treatment protected *Sr-b1/ApoE dKO* mice against atherosclerosis in the aortic sinus and coronary arteries, myocardial fibrosis and cardiac enlargement despite in the absence of cholesterol lowering (Yu et al., 2018). Interestingly, these protective effects of rosuvastatin were accompanied by increased plasma levels of proprotein convertase subtilism/kexin type (PCSK9) (Yu et al., 2018), which plays an important role in cholesterol metabolism and atherosclerosis development and will be discussed in more detail in Chapter 1.8.

1.7.2 *Sr-b1 Single KO* Mice

Sr-b1 single KO mice only develop minimal atherosclerosis in the aortic sinus induced by a high fat (15% fat and 0.25% cholesterol) or a high fat/high cholesterol (7.5% fat and 1.25% cholesterol) diet despite more than 20 weeks of feeding with those atherogenic diets (Harder et al., 2007; Hildebrand et al., 2010; Van Eck et al., 2003). On the other hand, when *Sr-b1 KO* mice were fed a high fat, high cholesterol and cholate containing (20% fat, 1.25% cholesterol and 0.5% cholate) diet for 11 weeks, extensive aortic sinus atherosclerosis was observed (Huby et al., 2006). Nevertheless, those studies did not examine atherosclerosis in coronary arteries of *Sr-b1 KO* mice. Recently, our lab reported that female *Sr-b1 KO* mice fed a high fat, high cholesterol and cholate containing (15.8% fat, 1.25% cholesterol and 0.5% cholate, HFCC) diet for 20 weeks developed

widespread atherosclerosis in multiple artery sites, including the aortic sinus, aorta and importantly coronary arteries (M. T. Fuller et al., 2023). Coronary artery atherosclerosis development in HFCC diet fed *Sr-b1 KO* mice was associated with increased leukocytosis and VCAM-1 expression in the endothelium of coronary arteries (M. T. Fuller et al., 2023). Furthermore, unpublished data from our lab (S. Lee *at al., manuscript in preparation*) found that aging (one year old) exacerbated an HFCC diet (12 weeks) induced coronary artery atherosclerosis and myocardial fibrosis in female *Sr-b1 KO* mice accompanied by a reduced survival. Platelet accumulation was also observed in coronary artery atherosclerotic plaques in an HFCC diet (12 weeks) fed *Sr-b1 KO* mice, suggesting the existence of coronary artery atherothrombosis. Therefore, *Sr-b1 KO* mice fed an HFCC diet represents a mouse model of age-induced coronary artery atherosclerosis and myocardial fibrosis. This mouse model allows for testing the effects of potential therapeutics on CAD that requires an intact LDLR-APOE axis.

1.8 Proprotein Convertase Subtilisn/Kexin Type 9 (PCSK9)

1.8.1 PCSK9 in Lipid Metabolism

PCSK9 is a secreted serine protease and the ninth member of the kexin-like proprotein convertase subtilisin family (Seidah et al., 2003a). *PCSK9* was initially discovered as a mRNA that is upregulated during apoptosis in neuronal cells (Seidah et al., 2003a). Soon after, *PCSK9* was identified as the third gene linked to autosomal dominant FH (Abifadel et al., 2003a). Gain of function mutations in the *PCSK9* gene are associated with hypercholesterolemia, while loss of function mutations in the *PCSK9*

gene are associated with hypocholesterolemia (Abifadel et al., 2003a; Cohen et al., 2005). *PCSK9* gene is located on the short arm of chromosome 1p32.3. PCSK9 is mainly expressed in the liver, intestine and kidney (Seidah et al., 2003b). However, it is also expressed in a variety of other tissues, such as VSMCs, ECs, macrophages and cardiomyocytes (although to a much lesser extent) (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015; Ding, Wang, et al., 2018; Ferri et al., 2012a; J. Li et al., 2017; L.-S. Liu et al., 2009a; TANG et al., 2012). PCSK9 protein is synthesized as an inactive zymogen consisting of a signal sequence (amino acids (aa): 1-30), a prodomain (aa: 31-152), a catalytic domain (aa: 153-451) and a C-terminal region (aa: 452-692) that are rich in cysteines and histidines. The precursor of PCSK9 undergoes autocatalytic cleavage between the prodomain and the catalytic domain in the ER and then becomes mature PCSK9 (Seidah et al., 2003a). This autocatalytic processing is the only protease activity of PCSK9 and no other substrate beyond itself has been reported (Seidah & Prat, 2022). Different from other proprotein convertase members, the prodomain of PCSK9 always remains noncovalently attached to its catalytic domain, presumably inhibiting its catalytic activity (Seidah et al., 2003a; Seidah & Prat, 2022). It was suggested that the prodomain attachment is required for the secretion of mature PCSK9 through the trans-Golgi network. The catalytic domain of secreted circulating PCSK9 binds to the EGF-A domain of LDLR on the cell surface (D. W. Zhang et al., 2007). The PCSK9-LDLR complex undergoes autosomal recessive hypercholesterolemia (ARH) adaptor protein-dependent, clathrin-mediated endocytosis (Lagace et al., 2006; Qian et al., 2007). After the PCSK9-LDLR complex is internalized, PCSK9 diverts LDLR to the lysosome for degradation,

preventing LDLR recycling back to the cell surface. Besides the extracellular pathway, PCSK9 also mediates LDLR degradation through an intracellular pathway where intracellular PCSK9 binds to LDLR and directs it from the trans-Golgi network to lysosome for degradation (Poirier et al., 2009). However, the extracellular/circulating PCSK9 plays a dominant role in LDLR degradation in the liver (Lagace et al., 2006) and the majority of circulating PCSK9 derives from the liver (Zaid et al., 2008a). The consequence of circulating PCSK9 mediated hepatic LDLR degradation is the substantially reduced clearance of plasma LDL, resulting in the accumulation of LDL cholesterol in the bloodstream (Figure 5). Studies have suggested that PCSK9 also regulates plasma TG levels, however, this effect is relatively modest when compared to its effects on cholesterol metabolism (Dijk et al., 2018). Beyond LDLR, PCSK9 has also been implicated in mediating the degradation of other receptors, including LRP1, CD36, the very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor-2 (apoER2) and major histocompatibility complex class-I (MHC-I) in different tissues (Byun, Lebeau, Platko, Carlisle, Faiyaz, Chen, Macdonald, et al., 2022; Canuel et al., 2013; Da Dalt et al., 2021; Demers, Samami, Lauzier, Des Rosiers, Sock, et al., 2015; X. Liu et al., 2020; Poirier et al., 2008; Roubtsova et al., 2011a), which will be discussed in more detail in 1.8.3.

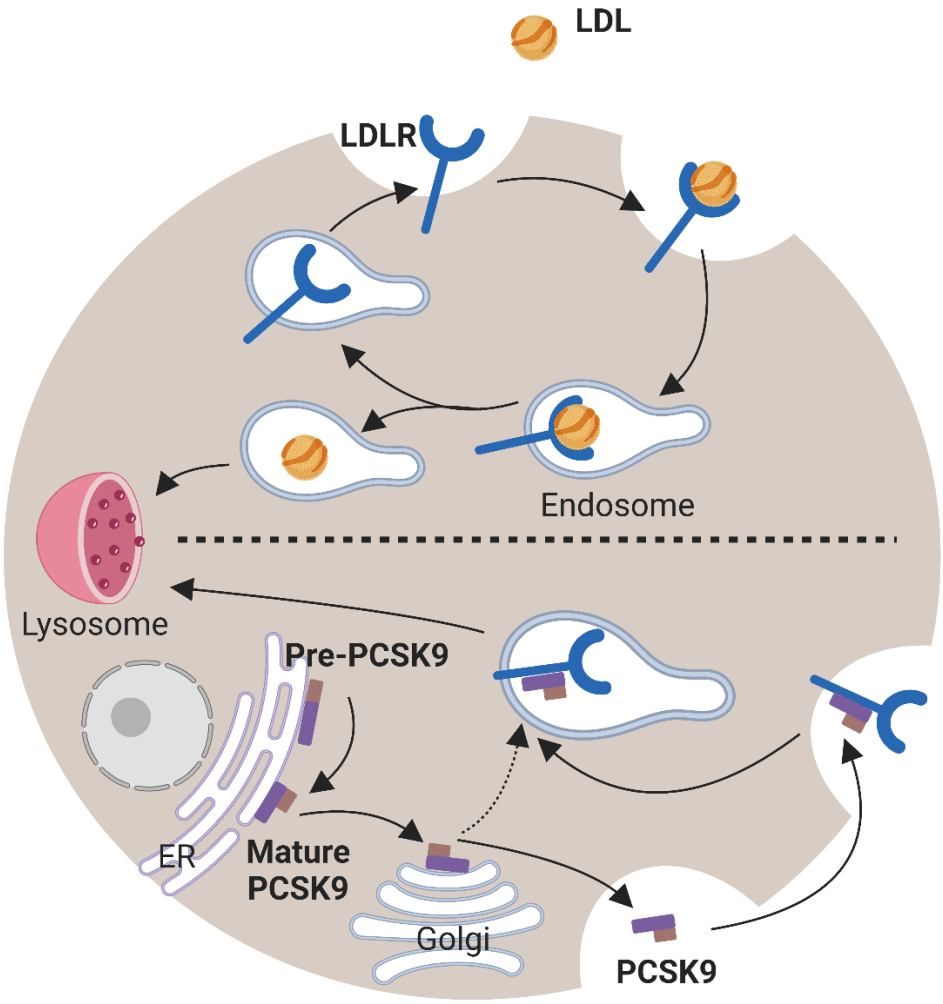

Figure 5: PCSK9 mediates LDLR degradation in hepatocytes. LDLR on the cell surface of hepatocytes binds to the LDL particle and mediates the endocytosis of the LDLR:LDL particle complex via a clathrin-coated pit. LDLR disassociates from the LDL particle in the endosome. LDL particle further travels to the lysosome and gets degraded and the LDLR recycles back to the cell surface for reuse. Pre-PCSK9 undergoes autocleavage between the prodomain and the catalytic domain in the ER and becomes mature PCSK9. Mature PCSK9 travels through the trans-Golgi network and is secreted

from hepatocytes into circulation. Circulating PCSK9 binds to the LDLR on the cell surface of hepatocytes and the LDLR-PCSK9 complex is internalized and diverted to the lysosome for degradation. Therefore, PCSK9 reduces the levels of cell surface LDLR in the liver. Figure is adapted from Horton, J. D. et al. Molecular biology of PCSK9: its role in LDL metabolism. Trends Biochem Sci 32, 71–77 (2007) with the permission pending from the publisher and created with BioRender.com.

1.8.2 PCSK9 Inhibition in Plasma Cholesterol Lowering and Atherosclerosis

PCSK9 diverts the LDLR from the cell surface to the lysosome for degradation in liver cells (Benjannet et al., 2004; Sahng et al., 2004). It is an attractive target for lowering LDL cholesterol in the blood (Sabatine et al., 2017; Schwartz et al., 2018). Inhibiting PCSK9 provides a chance to decrease cholesterol levels for people who are intolerant to or have major side effects from statin treatment. Indeed, two monoclonal antibodies (mAbs) against PCSK9 (evolocumab and alirocumab), have shown potent cholesterol lowering effects and reduced CVEs in large clinical studies (Sabatine et al., 2017; Schwartz et al., 2018). The LDL cholesterol lowering response to statin therapy has considerable variability (Taylor & Thompson, 2016). One potential reason might be that statin also increases PCSK9 levels by activating SREBP-2, which is a transcription factor and induces the expression of its downstream target genes, including the *LDLR* and *PCSK9* (Taylor & Thompson, 2016). Therefore, the combination of PCSK9 inhibition and statin treatment provides greater beneficial outcomes on reducing CVEs compared to the statin treatment alone (Sabatine et al., 2017; Schwartz et al., 2018).

Studies in model organisms have suggested that similar to statins, PCSK9 inhibition mediated cholesterol lowering also requires both LDLR and APOE (Ason, van der Hoorn, et al., 2014; Denis et al., 2012; Z.-H. Tang et al., 2017). By using Western-type diet fed *APOE*3Leiden.CETP* mice that have the intact expression of both LDLR and APOE, PCSK9 inhibition mediated by mAbs or a nanoliposome-based vaccine, successfully reduced plasma cholesterol levels and significantly reduced aortic sinus atherosclerosis (Ason, van der Hoorn, et al., 2014; Kühnast, Van Der Hoorn, et al., 2014; Landlinger et al., 2017; Schuster et al., 2019). While all studies reported PCSK9 inhibition did not affect plasma cholesterol levels in *ApoE KO* mice, conflicting results have been reported about atherosclerosis development (Ason, van der Hoorn, et al., 2014; Z.-H. Tang et al., 2017). One study reported that neither genetic *KO* nor mAb mediated PCSK9 inhibition affected aortic sinus atherosclerosis levels in *ApoE KO* mice (Ason, van der Hoorn, et al., 2014). Other studies, however, reported that PCSK9 inhibition reduced aortic atherosclerosis despite the absence of cholesterol lowering (Denis et al., 2012; Z.-H. Tang et al., 2017), suggesting PCSK9 might affect atherosclerosis development independent of plasma cholesterol levels.

1.8.3 The Effects of PCSK9 on CVD: beyond LDL Cholesterol Lowering

PCSK9 has gained extensive attention since the discovery of its role in mediating hepatic LDLR degradation, and therefore regulating plasma LDL cholesterol clearance. However, emerging studies have indicated that PCSK9 may have pleiotropic effects on the development of atherosclerosis and eventually cardiac dysfunction by mechanisms independent of the regulation of LDL cholesterol. A meta-analysis revealed that by

achieving the same levels of LDL cholesterol lowering, the relative risk reduction for CVEs tended to be greater with PCSK9 inhibitors compared with statins, although the apparent lower risk was not statistically significant (Silverman et al., 2016). More importantly, it has been shown that circulating PCSK9 levels predicted future CVEs, independently of established risk factors, including LDL cholesterol (Leander et al., 2016). This suggests that in addition to exerting cardioprotective effects through lowering LDL cholesterol, inhibition of PCSK9 may have cardioprotective effects that are independent of LDL cholesterol lowering. Accumulating research has suggested that PCSK9 may exert effects on atherogenesis within the artery wall beyond its influences on LDL cholesterol levels (Denis et al., 2012; Ferri et al., 2016; Giunzioni et al., 2016a; Karagiannis et al., 2018; Z.-H. Tang et al., 2017; Tavori et al., 2016). Studies in model organisms have shown that overexpressing *PCSK9* elevated the plaque size at the aortic sinus and root in *ApoE KO* mice, without altering plasma cholesterol levels (Denis et al., 2012; Tavori et al., 2016). On the other hand, silencing *Pcsk9* through lentivirus-mediated shRNA mediated *Pcsk9* knockdown, decreased atherosclerosis development in the aortic sinus in *ApoE KO* mice, independently of plasma cholesterol levels (Z.-H. Tang et al., 2017). Atherothrombosis development in coronary arteries involves a variety of different cell types, including but not limited to VSMCs, ECs, macrophages, T cells, dendritic cells (DCs) (Zernecke et al., 2020), and platelets. In this section (adapted from a published review paper: Xiong *et al*. 2021), I will discuss evidence related to how PCSK9 modulates cellular responses and the pro-inflammatory microenvironment in the artery wall, and how those processes may affect atherothrombosis progression locally (Figure

6). I will also discuss the potential direct role of PCSK9 on the myocardium, which is critical for heart function after ischemia and post-myocardial infarction remodeling (Figure 6). These pathways may be critical for understanding the mechanisms of PCSK9's cardioprotective effects beyond LDL cholesterol lowering.

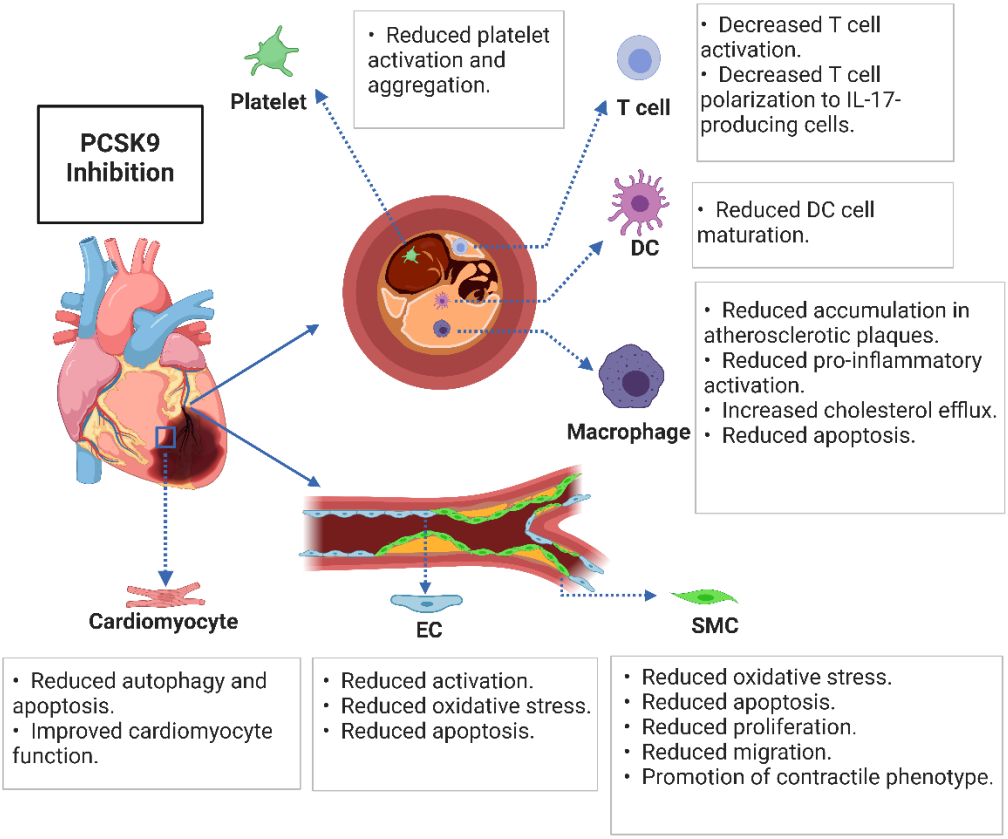

Figure 6: Potential mechanisms by which PCSK9 inhibition protects against CVD independently of plasma LDL cholesterol lowering. Inhibition of PCSK9 affects

cellular responses in bone marrow derived cells (monocytes/macrophages, DCs, T cells and platelets), ECs and VSMCs in the artery wall, and cardiomyocytes, which might all contribute to cardioprotective effects mediated by inhibition of PCSK9. Figure is previously published in Xiong, T. et al. The local effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the inflammatory atheroma: beyond LDL cholesterol lowering. Vessel Plus 5, (2021) and reproduced with permission.

1.8.3.1 VSMCs and ECs in the vascular wall

Although circulating PCSK9 is mainly derived from hepatocytes, PCSK9 is also found to be expressed in a variety of tissues (Zaid et al., 2008b). Interestingly, it was reported to be expressed in VSMCs, and, to a lesser extent, in ECs and macrophages, and PCSK9 protein has been detected in human atherosclerotic plaques (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015; Ferri et al., 2012b). This raises the possibility that PCSK9 derived locally from the artery wall might have an involvement in plaque development. In vitro studies have shown that PCSK9 secreted by cultured VSMCs can decrease LDLR protein levels in macrophages (Ferri et al., 2012b; Grune et al., 2017) (Figure 7). Several studies suggested that different factors regulate PCSK9 expression levels in VSMCs and ECs, including shear stress, ROS, and pro-inflammatory stimuli, such as Ox-LDL, TLR4 ligands such as LPS, and pro-inflammatory cytokines (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a; Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015; Grune et al., 2017; S. Liu et al., 2020). Ding *et al*. found that in mouse aortas, at arterial branching points and at the bifurcation of the iliac arteries, expression levels of PCSK9 were elevated along with expression levels of VCAM-1, known to be elevated at sites of EC

activation (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015; Iiyama et al., 1999b) (Figure 6). Inhibition of PCSK9 by alirocumab treatment reduced levels of EC expression of ICAM-1, another marker of EC activation (Iiyama et al., 1999b; Kühnast, Van Der Hoorn, et al., 2014). The transcriptional regulator, NF-κB, is known to be a key mediator of EC activation in regions of the mouse aorta exposed to hemodynamic factors that predispose to atherosclerotic plaque development (Hajra et al., 2000). Hemodynamic forces appear to regulate PCSK9 expression along the mouse aorta partly through a TLR4-myeloid differentiation primary response 88 (MyD88)-NF-κB pathway (S. Liu et al., 2020). Moreover, VSMCs and ECs exposed to low shear stress in culture exhibit increased PCSK9 expression compared to that in cells exposed to high shear stress (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). This pattern of PCSK9 expression parallels patterns of oxidative stress and EC activation (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015) (Figure 6). There appears to be a positive feedback relationship between PCSK9 expression and ROS levels (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). ROS at moderate levels generated by normal physiological processes appear to serve as signaling molecules (Mittler et al., 2011); however, excessive ROS generation in the vascular wall is pathological and promotes atherosclerosis, by contributing to lipoprotein oxidation, endothelial dysfunction, leukocyte migration and activation, VSMC proliferation, DNA damage, and MMPs-induced collagen degradation (Goncharov et al., 2015; H. Li et al., 2014). Ox-LDLs, generated by ROS mediated oxidation of LDLs, lead to further ROS production via a pathway mediated by the lectin-like Ox-LDL receptor-1 (LOX-1) (Cominacini et al., 2000; Xu et al., 2013). Ding *et al*. also found that there is a

bidirectional interplay between PCSK9 and LOX-1 expression in VSMCs and ECs (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a; Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). Small interfering (si)RNA mediated *LOX-1* knockdown reduced LPS-induced PCSK9 expression in VSMCs and ECs (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a; Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). On the other hand, recombinant human PCSK9 (hPCSK9) was reported to increase LOX-1 protein levels, and siRNA mediated *PCSK9* knockdown decreased LPS-induced LOX-1 levels in VSMCs and ECs (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a; Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). Conversely, Sun *et al.* reported that PCSK9 associated with LDL particles, but not either PCSK9 or LDL alone, increased gene expression of LOX-1 and ICAM-1 along with CCL2, C-C motif chemokine ligand 6 (CCL6), IL-1β and IL-6 in cultured ECs, suggesting that PCSK9 affects EC gene expression by modulating the properties of LDL particles (Sun et al., 2018). The reciprocal regulation between PCSK9 and LOX-1 expression may be mediated through mitochondrial ROS (mtROS), as it affected both PCSK9 and LOX-1 expression levels (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a). Excessive mtROS production leads to mtDNA damage, which can induce autophagy, inflammation, or apoptosis (Ding et al., 2013; Quan et al., 2020; Ricci et al., 2008). It is reported that induced mtROS production by antimycin A, rotenone and thenoyltrifluoroacetone, increased PCSK9 expression and mtDNA damage in VSMCs (Ding et al., 2016). It has also been shown that there is a positive feedback interaction between PCSK9 expression levels and mtDNA damage in VSMCs, partially mediated by mtROS (Ding et al., 2016). Enhancement of mtDNA damage induced by

autophagy inhibitors increased and inhibition of mtDNA damage via an autophagy inducer (rapamycin) decreased PCSK9 expression in VSMCs (Ding et al., 2016). The effect of antimycin induced mtDNA damage on PCSK9 protein levels was mediated by the activation of p38 mitogen-activated protein kinase (MAPK) (Ding et al., 2016), which has been shown to respond to environmental stress, cytokines and DNA damage (Wood et al., 2009; Zarubin & Han, 2005). Conversely, recombinant hPCSK9 increased and siRNA mediated *PCSK9* knockdown decreased phosphorylation of mammalian target of rapamycin (mTOR) and LPS-induced mtDNA damage in VSMCs (Ding et al., 2016). This result suggests that PCSK9 might regulate mtDNA damage via mTOR activation, as mTOR inhibition by rapamycin decreased mtDNA damage (Ding et al., 2016). However, the importance of PCSK9, ROS, and LOX-1 interaction in VSMCs and ECs on atherosclerosis development in vivo has not been elucidated yet.

Apoptosis is a significant feature of atherosclerotic plaque development. EC apoptosis compromises the barrier function of the EC layer, contributing to increased lipid deposition and plaque vulnerability (Y. Zhang, Qin, et al., 2015). Apoptosis in SMCs may result in reduced collagen production and decreased plaque stability (Clarke et al., 2006). In vitro studies have shown that PCSK9 inhibition reduced apoptosis levels in both in human umbilical vein endothelial cells (HUVECs) and VSMCs (Ding et al., 2016; J. Li et al., 2017; C.-Y. Wu et al., 2012) (Figure 6). The Bcl2/ Bcl2 associated X (Bax)–caspase9–caspase3 pathway is involved in the protective effects of PCSK9 inhibition on EC and VSMC apoptosis (Ding et al., 2016; J. Li et al., 2017; C.-Y. Wu et al., 2012). However, the link between PCSK9 and the Bcl2/Bax pathway is unclear. Studies have

shown that c-Jun N-terminal kinases (JNK) and p38 MAPK mediated the phosphorylation and mitochondrial translocation of Bax, led to apoptosis in human hepatoma HepG2 cells (B. J. Kim et al., 2006). It was also shown that JNK and p38 MAPK mediated the phosphorylation of Bcl-2, which can enhance Bcl-2 degradation (Breitschopf et al., 2000; De Chiara et al., 2006). Interestingly, knockdown of *PCSK9* by shRNA decreased phosphorylation of JNK and p38 MAPK in HUVECs (J. Li et al., 2017), suggesting PCSK9 might trigger apoptosis through the JNK/p38 MAPK – Bcl-2/Bax signaling pathway. Yet interventional studies should be conducted to test if PCSK9 mediated apoptosis is affected by inhibiting JNK/p38 MAPK activation. Whether PCSK9 induces apoptosis of ECs and VSMCs during atherosclerotic plaque development also needs to be investigated in vivo.

The dramatically increased proliferation capability of neo-intimal compared to medial VSMCs contributes to both neo-intimal hyperplasia and atherosclerosis progression. Importantly, Ferri *et al*. have shown that knocking out the *Pcsk9* gene mitigated neo-intimal formation and VSMC accumulation within the neo-intima in mice subjected to a non-occlusive collar placement around carotid arteries (Ferri et al., 2016). Furthermore, aortic VSMCs isolated from *Pcsk9* deficient mice have been shown to exhibit reduced proliferation rates and migration levels when compared to those from *Pcsk9* expressing mice (Ferri et al., 2016) (Figure 6). *Pcsk9* deficient VSMCs reportedly exhibited a more spindle and elongated shape and increased expression of smooth muscle (SM) α-actin and major histocompatibility complex class-II (MHC-II) compared to VSMCs from *Pcsk9* expressing mice, suggesting that inactivation of the *Pcsk9* gene

might modulate VSMC phenotypes (Ferri et al., 2016) (Figure 6). Knock out of the *Pcsk9* gene in mice has been reported to increase CD36 protein levels in VSMC (Ferri et al., 2016) and CD36 is considered as an important Ox-LDL receptor involved in macrophage foam cell formation and activation in atherosclerotic plaques (Febbraio et al., 2000, 2004; Mäkinen et al., 2010; Podrez et al., 2000) (Figure 7). VSMCs in the neo-intima are thought to adopt a macrophage-like phenotype including the ability to take up Ox-LDL and accumulate lipid droplets and a foam cell-like morphology in atherosclerotic plaques (Allahverdian et al., 2014a). However, whether PCSK9 inhibition affects the ability of VSMC to adopt a macrophage-like phenotype has not yet been reported.

1.8.3.2 Monocytes/Macrophages

Monocytes are recruited to the vessel wall at sites of atherosclerotic lesion development and infiltrate through the EC layer into the tunica intima layer of the artery where they mature into macrophages that can take up modified lipoproteins (Moore et al., 2013). PCSK9 may promote monocyte recruitment into atherosclerotic plaques at multiple steps including, as indicated above, through activation of ECs (VCAM-1 and ICAM-1 expression) (Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015; Kühnast, Van Der Hoorn, et al., 2014; Schuster et al., 2019). Once in the artery wall, macrophages become foam cells as they accumulate lipids through scavenger receptor-mediated uptake of Ox-LDL particles. Recent studies suggest that PCSK9 may play an important role in foam cell formation by increasing lipid accumulation and limiting lipid efflux in macrophages (Adorni et al., 2017; Ding, Liu, et al., 2018). Macrophage CD36 has been reported to play important roles in atherosclerosis development by virtue of its involvement in Ox-LDL

uptake, pro-inflammatory responses, engulfment of apoptotic cells, and macrophage emigration (Silverstein & Febbraio, 2009). In culture, the exposure of TNF-α primed mouse peritoneal macrophages to exogenous PCSK9 resulted in increased Ox-LDL uptake and this correlated with an upregulation of gene expression and protein levels of scavenger receptors CD36, scavenger receptor class A (SR-A) and LOX-1 (Ding, Liu, et al., 2018) (Figure 7). Furthermore, *Pcsk9* gene deletion or siRNA-mediated silencing in macrophages has been reported to reduce SR-A, LOX-1, and CD36 expression (Ding, Liu, et al., 2018). However, another study has shown that conditioned media containing PCSK9 did not change CD36 protein levels in phorbol 12-myristate 13-acetate (PMA)-induced THP-1 macrophages compared with control media which is deficient in PCSK9 (Demers, Samami, Lauzier, Des Rosiers, Sock, et al., 2015). On the other hand, PCSK9 plays a well-known role in receptor internalization and degradation. In addition to PCSK9 directed degradation of LDLR and its close family members VLDLR and ApoER2 (Poirier et al., 2008), PCSK9 has also been reported to mediate internalization and degradation of CD36 in cultured adipocytes and hepatocytes, limiting fatty acid uptake and triglyceride accumulation (Demers, Samami, Lauzier, Des Rosiers, Sock, et al., 2015). Therefore, the reports that *Pcsk9* inactivation in macrophages reduces CD36 expression is unexpected. However, the reported effects of PCSK9 on macrophage CD36 levels await confirmation from in vivo pre-clinical or clinical studies.

In human macrophages derived from monocytes isolated from healthy blood donors, PCSK9 and LDLR-related protein 5 (LRP5) have been reported to interact to control lipid uptake from aggregated LDL (Badimon et al., 2020) (Figure 7). LRP5

appears to mediate macrophage uptake of aggregated LDL and PCSK9 has been reported to form an intracellular complex with LRP5 and assist in its trafficking to the cell surface (Badimon et al., 2020). Silencing of either *LRP5* or *PCSK9* results in reduced uptake of aggregated LDL by macrophages (Badimon et al., 2020). On the other hand, the treatment of mouse peritoneal macrophages with purified hPCSK9 has been reported to result in reduced gene expression and protein levels of ABCA1 (Adorni et al., 2017). Consequently, PCSK9 treatment of macrophages reduced ABCA1 dependent cholesterol efflux to APOA-I (Adorni et al., 2017) (Figure 6). ABCA1 mediated cholesterol efflux from macrophages to APOA-I is believed to play a critical atheroprotective role in the removal of excess cholesterol from macrophages via a process called reverse cholesterol transport (Mireille et al., 2019). Taken together, it appears that PCSK9 derived from both macrophages themselves or extracellular PCSK9 encountered by macrophages influence macrophage cholesterol homeostasis to promote macrophage foam cell formation, a process generally considered to contribute to atherosclerotic plaque formation.

Studies have reported that LDL levels appear to regulate the level of CCR2 expression on monocytes (Bernelot Moens et al., 2017; Grune et al., 2017). CCR2 on monocytes appears to play a critical role in regulating circulating monocyte counts and monocyte migration across the intact EC barrier of arteries in response to its ligand CCL2, a key step in monocyte accumulation in the atherosclerotic artery wall (Maik et al., 2015). One study reported that monocyte CCR2 expression correlated with LDL-cholesterol levels in patients with familial hypercholesterolemia, who display elevated LDL cholesterol due to impaired LDLR mediated LDL clearance (Bernelot Moens et al.,

2017). Administration of anti-PCSK9 mAb treatment to familial hypercholesterolemia patients, which reduced LDL cholesterol, coordinately reduced lipid accumulation and CCR2 expression in circulating monocytes, as well as the capacity of those monocytes to migrate through cultured EC monolayers in response to added CCL2 (Bernelot Moens et al., 2017). In contrast, another study reported that LDL dependent upregulation of monocyte CCR2 expression was dependent on LDLR expression in monocytes (Grune et al., 2017). In that study, PCSK9 secreted from VSMCs reduced CCR2 expression on monocytes by virtue of reducing monocyte LDLR levels and LDLR mediated LDL uptake by monocytes (Grune et al., 2017). This study suggests that PCSK9, secreted locally in the environment of the atherosclerotic plaque, may actually limit monocyte or macrophage chemotaxis within the plaque itself. Whether this also impacts monocyte recruitment to the plaque has not been tested. It is also unclear whether the effects of PCSK9 on LDL-cholesterol levels or on monocyte LDL- cholesterol uptake predominates in regulating monocyte CCR2 levels.

Emerging research reveals the potential role of PCSK9 in modulating monocyte and macrophage inflammation in the progression of atherosclerosis. The global overexpression of mouse or human PCSK9 in *ApoE KO* or *Ldlr KO* mice did not alter plasma cholesterol levels due to the absence of the key components of the APOE-LDLR axis for LDL clearance targeted by PCSK9 (Denis et al., 2012; Tavori et al., 2016). However, despite the lack of changes in plasma lipids, *PCSK9* overexpression increased aortic lesion size and the abundance of pro-inflammatory Ly6C^{high} monocytes in atherosclerotic plaques (Denis et al., 2012; Tavori et al., 2016) (Figure 6). Human *PCSK9*

overexpression in *ApoE KO* mice was also accompanied by increased levels of PCSK9 protein in atherosclerotic plaques (Tavori et al., 2016). Interestingly, the increase in $Ly6C^{high}$ monocytes and PCSK9 expression in plaque were not evident in *Ldlr KO* mice, suggesting that PCSK9 exerts pro-inflammatory effects in an LDLR dependent manner. The silencing of *Pcsk9* in *ApoE KO* mice by lentivirus-mediated shRNA reduced atherosclerosis development and reduced the abundance of macrophages and expression of inflammatory markers within atherosclerotic plaques (Z.-H. Tang et al., 2017). Exogenous PCSK9 can induce a pro-inflammatory response in human macrophages by upregulating the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (CCL2, C-X-C motif ligand 2 (CXCL2)) (Ricci et al., 2018a) (Figure 6). The release of pro-inflammatory cytokines such as TNF-α and IL-1β by PCSK9 seems to involve TLR4-NF-κB signaling in a manner similar to that described for EC activation (Badimon et al., 2020; Ding et al., 2020; Giunzioni et al., 2016a; S. Liu et al., 2020; Schuster et al., 2019; Z. Tang et al., 2012; Z.-H. Tang et al., 2017).

Human and murine primary monocytes and macrophages express intracellular and secreted PCSK9 in culture (Badimon et al., 2020; Z. Tang et al., 2012). Although a majority of circulating PCSK9 is derived from the liver, endogenous expression of PCSK9 in monocytes and macrophages may affect local environments such as within the atherosclerotic lesion. When human *PCSK9* was overexpressed selectively in bone marrow-derived cells transplanted into *ApoE KO* mice, atherosclerotic lesion sizes were not increased compared to *ApoE KO* mice transplanted with bone marrow from non-transgenic controls (Giunzioni et al., 2016b). However, selective *PCSK9* overexpression

in bone marrow-derived cells was sufficient to result in increased levels of Ly6C^{high} monocytes in atherosclerotic plaques of *ApoE KO* mice but not in those of *Ldlr KO* mice (Giunzioni et al., 2016b). This suggests that PCSK9 expression in bone marrow-derived cells, such as monocytes, may play a role in altering monocyte inflammatory profiles and circulating monocyte counts independently of PCSK9 expression from other sources, such as the liver. In support of this, Giunzioni *et al* reported that overexpression of human *PCSK9* in macrophages increased LPS stimulated induction of mRNA levels of the pro-inflammatory markers TNF-α and IL-1β and suppression of the anti-inflammatory markers IL-10 and arginase (Arg1) (Giunzioni et al., 2016b) (Figure 6). Conversely, others have reported that PCSK9 expression is upregulated in cultured mouse peritoneal macrophages exposed to LPS, TNF-α, or Ox-LDL (Ding, Liu, et al., 2018). Similar findings have been reported in the liver in vivo and in cultured VSMCs (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015a; Feingold et al., 2008). It has also been recently demonstrated that activation of the NLRP3 inflammasome upregulated intracellular PCSK9 expression and PCSK9 secretion from murine peritoneal macrophages, suggesting the possibility of a positive feedback loop of PCSK9 expression and macrophage activation (Ding et al., 2020). The secretion of PCSK9 was dependent on IL-1β and MAPK pathway signalling and IL-1β deficient mice fed a high-fat diet had reduced serum PCSK9 levels (Ding et al., 2020). Determining the mechanistic link between PCSK9, TLR4/NF-κB signalling, and NLRP3 inflammasome activation represents an interesting area of future research.

Macrophage cell death is a key driver in atherosclerosis progression and the formation of necrotic cores within lesions. As described earlier, PCSK9 seems to influence apoptosis of ECs and VSMCs (Ding et al., 2016; J. Li et al., 2017; C.-Y. Wu et al., 2012). Similarly, several lines of evidence suggest it may also influence apoptosis of macrophages. Partial knockdown of *PCSK9* by siRNA in THP-1 macrophages seems to reduce cell death induced by Ox-LDL (L.-S. Liu et al., 2009b) (Figure 6). The mechanism of this effect will need to be explored further, however some studies suggest PCSK9 may influence cell death by mediating the degradation of ApoER2, a LDLR family member which binds to APOE and exerts anti-inflammatory and anti-apoptotic effects (Bai et al., 2018). In neurons, PCSK9 was shown to increase apoptosis by the degradation of ApoER2 (Kysenius et al., 2012). ApoER2 is also expressed in macrophages(Waltmann et al., 2014a). The knockdown of *ApoER2* in RAW 264.7 macrophages increased Ox-LDL induced cell death, inhibited pro-survival Akt signalling, and increased peroxisome proliferator-activated receptor γ (PPARγ) expression (Waltmann et al., 2014a). This deficiency of *ApoER2* in *Ldlr KO* mice translated to larger and more advanced atherosclerotic lesions with large necrotic cores and plaque apoptosis (Waltmann et al., 2014b). Of course, whether PCSK9 controls macrophage apoptosis in a manner similar to neurons, ECs, or VSMCs remains to be determined.

1.8.3.3 Dendritic Cells and T Cells

Along with monocytes and macrophages, activated T cells (Saigusa et al., 2020a) and antigen-presenting DCs (Gil-Pulido & Zernecke, 2017) are commonly found in atherosclerotic lesions. Kuhnast *et al*. observed that inhibition of PCSK9 with alirocumab

reduced T cell abundance within aortic lesions (Kühnast, Van Der Hoorn, et al., 2014). However, it is not clear if this was a consequence of the inactivation of circulating PCSK9, derived largely from the liver, or of PCSK9 locally in the atherosclerotic plaque. More recently, it has been discovered that PCSK9 is involved in DC maturation and T cell activation and polarization to T helper-1 (Th1) and T helper-17 (Th17) subsets (A. Liu & Frostegård, 2018) (Figure 6). PCSK9 inhibition in DCs prevented T-cell activation induced by Ox-LDL and promoted T regulatory cell populations through increased DC secretion of TGF-β and IL-10 (A. Liu & Frostegård, 2018) (Figure 6). Furthermore, *Pcsk9* gene inactivation in mice deficient in both *Ldlr* and *Apobec1* resulted in a reduction of levels of IL-17 in the plasma and Th17 cells in atherosclerotic plaques (Y. U. Kim et al., 2019) (Figure 6). These results may suggest that PCSK9 is associated with T cell programming within atherosclerotic lesions. However, there remains debate over whether IL-17 and Th17 cells protect against or promote atherosclerosis development (Soraya et al., 2015).

1.8.3.4 Platelets

Platelets contribute to atherothrombosis by accelerating both atherosclerosis progression (Huo et al., 2003) and thrombus generation. Activated platelets facilitate the binding of leukocytes to ECs on the atherosclerotic lesion sites and release pro-inflammatory cytokines, therefore promoting atherosclerosis (Huo et al., 2003; Lievens & von Hundelshausen, 2011). Exposure of subendothelial collagen to circulating platelets when the plaque ruptures initiates primary hemostasis, which leads to thrombus generation. Platelet adhesion, activation and aggregation are important steps during the

primary hemostasis. Multiple factors can activate platelets, including collagen, adenosine diphosphate (ADP), thrombin and thromboxane A2 ($T_X A_2$). $T_X A_2$, generated from arachidonic acid through cyclo-oxygenase -1 (COX-1)/thromboxane synthase pathway in activated platelets, is unstable and rapidly hydrolyzed into thromboxane B2 ($T_X B_2$) (Gurbel et al., 2017). $T_X B_2$ is further converted in the liver into 11-dehydro-thromboxane B2 (11-dh-TxB2), which is excreted in the urine (Gurbel et al., 2017). An observational study has shown plasma PCSK9 levels are correlated with urinary levels of 11-dh-TxB2 in patients with atrial fibrillation (Pastori et al., 2017). However, $T_X A_2$ derives not only from the COX-1/thromboxane synthase pathway in activated platelets but also from the COX-2/thromboxane synthase pathway in leukocytes and endothelial cells exposed to inflammatory stimuli (Gurbel et al., 2017; Tantry et al., 2009). Therefore, the association between plasma PCSK9 levels and urinary 11-dh-TxB2 levels may alternatively reflect effects on inflammatory pathways. Plasma PCSK9 levels have also been observed to be correlated with levels of ADP-induced ex vivo platelet aggregation in patients with acute coronary syndrome (Navarese et al., 2017), directly suggesting an association between plasma PCSK9 levels and platelet activation. Interestingly, anti-PCSK9 mAb treatment in patients with hypercholesterolemia was associated with reduced levels of ex vivo ADP-induced platelet aggregation and reduced plasma markers of platelet activation, including soluble CD40 ligand, platelet factor-4, and soluble P-selectin (Barale et al., 2020). In mice subjected to $FeCl_3$-induced carotid artery injury, *Pcsk9* deficiency was associated with reduced levels of activated glycoprotein IIb/IIIa (GP IIb/IIIa), P-selectin and platelet–leukocyte aggregates, and thrombus formation at the injury site (Camera et al.,

2018). A factor which complicates the interpretation of the effects of PCSK9 inhibition on platelet activation and thrombus formation is the reduced LDL cholesterol levels resulting from anti-PCSK9 antibody treatment or *Pcsk9* gene inactivation, since LDL cholesterol levels have been reported to affect platelet activation (N. Wang & Tall, 2016). Therefore, direct measurements of the effect of PCSK9 on platelet activation including studies in model organisms should be conducted. LDL cholesterol levels are not altered by plasma PCSK9 inhibition or overexpression in *Ldlr KO* mice (Ason, van der Hoorn, et al., 2014; Denis et al., 2012; Tavori et al., 2016), which becomes an advantage for investigating the direct role of PCSK9 on platelets in vivo. In one such study in *Ldlr KO* mice, evolocumab treatment reduced $FeCl_3$-induced thrombus formation in mesenteric arterioles (Qi et al., 2021a) (Figure 6). Moreover, evolocumab treatment also reduced ex vivo ADP-induced aggregation of platelets from LDLR KO mice (Qi et al., 2021a) (Figure 6). These results suggest that PCSK9 plays a direct role in platelet activation and thrombus generation.

CD36 on platelets can bind to Ox-LDL, which has been shown to promote platelet activation (Magwenzi et al., 2015; Podrez et al., 2007; Yang et al., 2017). CD36 is also a major platelet receptor for thrombospondin-1, a platelet α-granule protein that is released upon platelet activation and promotes platelet mediated thrombus formation via interactions with CD36 (Kuijpers et al., 2014). Multiple downstream signaling pathways initiated by CD36, including Src kinase, extracellular signal-regulated kinase 5 (ERK5), JNK, and p38MAPK pathways have been shown to be involved in platelet activation (K. Chen et al., 2008; Hackeng et al., 1999; Magwenzi et al., 2015; Yang et al., 2017). In vitro studies have shown that co-incubating platelets with PCSK9 increased collagen, thrombin

and ADP stimulated platelet aggregation and oxidative stress (Cammisotto et al., 2020; Qi et al., 2021a). Such an effect on collagen-induced platelet aggregation was further amplified by the presence of LDL, which could be oxidized by PCSK9-induced oxidative stress (Cammisotto et al., 2020). Interestingly, inhibition of CD36 significantly decreased PCSK9 induced platelet aggregation with or without the presence of LDL (Cammisotto et al., 2020; Qi et al., 2021a), suggesting PCSK9 induced platelet aggregation was dependent on CD36 signaling. PCSK9 appeared able to activate several signaling pathways via CD36, including Src kinase, ERK5, JNK, and p38MAPK pathways (Qi et al., 2021a). As indicated earlier, in some cell types (e.g. hepatocytes and adipocytes) but not others (such as macrophages), PCSK9 has been shown to target CD36 for degradation (Demers, Samami, Lauzier, Des Rosiers, Sock, et al., 2015; Ding, Liu, et al., 2018). It was reported that PCSK9 did not affect CD36 protein levels in human megakaryocytic cells (Qi et al., 2021a), suggesting that PCSK9 affects platelet activation via CD36 signaling, rather than by affecting CD36 protein levels. To examine the in vivo consequences of PCSK9 mediated platelet activation via CD36, Qi *et al*. carried out studies in which mice were depleted of endogenous platelets and then reconstituted with platelets pretreated with PCSK9 before left coronary artery ligation (LCAL) surgery to induce experimental MI (Qi et al., 2021a). They reported that reconstituting mice with PCSK9-pretreated platelets significantly increased levels of microthrombi and development of MI compared with platelets that were not pretreated. They also demonstrated that this effect was reduced when platelets were from CD36 deficient donor mice (Qi et al., 2021a). These

results suggest that PCSK9 mediated platelet activation via CD36 has an influence on the expansion of MI.

1.8.3.5 Cardiomyocytes

Acute cardiac ischemia resulting from occlusive coronary artery atherothrombosis triggers MI. Early responses in the myocardium after MI include cardiomyocyte death and inflammation in the infarct border zone (Van Der Laan et al., 2014). Macrophages and neutrophils are recruited to and accumulate in the infarct zone where they play major roles in the clearance of apoptotic cells (Prabhu & Frangogiannis, 2016). However, the resolution of the inflammatory response is a prerequisite for the transition from the inflammatory to the reparative phase (Prabhu & Frangogiannis, 2016). Persistent accumulation of macrophages and pro-inflammatory cytokines, however, ultimately acts to impair cardiac remodelling and left ventricle (LV) function (Prabhu & Frangogiannis, 2016). An observational study has reported that circulating PCSK9 levels were associated with reduced LV ejection fraction 6 months after a first ST-elevation myocardial infarction (STEMI) (Miñana et al., 2020), and that this association was independent of serum LDL cholesterol levels and statin use. This study suggested that PCSK9 might contribute to adverse outcomes of cardiac remodelling post-MI.

Schulz *et al*. first reported that PCSK9 is expressed by terminally differentiated cardiomyocytes (Schlüter et al., 2017). Ox-LDL not only impaired the load-free cell shortening (an indicator of cardiomyocyte contractile function) but also increased PCSK9 expression in cardiomyocytes (Schlüter et al., 2017). Inhibiting *PCSK9* by siRNA

reversed the effects of Ox-LDL on cell shortening of cardiomyocytes (Schlüter et al., 2017) (Figure 6). Exposure of cultured cardiomyocytes to hypoxia, followed by reoxygenation (H/R), culture conditions that are meant to mimic ischemia/reperfusion (I/R) in vivo, also induced PCSK9 expression in murine primary cardiomyocytes (CL et al., 2020). Inhibiting hypoxia inducible factor-1 α (HIF-1α) attenuated hypoxia induced PCSK9 expression and autophagy in primary cardiomyocytes (Ding, Wang, et al., 2018). AMP-activated protein kinase (AMPK) is an energy sensor and maintains energy homeostasis. It promotes autophagy when phosphorylated/activated under many conditions, including ischemia, hypoxia, and glucose starvation (Gui et al., 2017; J. Kim et al., 2011; Matsui et al., 2007b). Ding *et al*. have reported that PCSK9 is involved in hypoxia induced autophagy in cardiomyocytes by bridging ROS generation and activating AMPK signalling (Ding, Wang, et al., 2018) (Figure 6). Whether PCSK9 induced autophagy of cardiomyocytes is cardio-protective or deleterious during I/R remains unclear. Nevertheless, PCSK9 expression enhanced inflammatory cytokine release in a co-culture system of cardiomyocytes and macrophages under H/R conditions (CL et al., 2020). Furthermore, the media from the co-culture system reduced the viability and increased the apoptosis of cardiomyocytes in a manner that was reversed by siRNA mediated knockdown of *PCSK9* (Figure 6) or chemical inhibition of NF-κB (CL et al., 2020). These in vitro studies suggest a direct role for PCSK9 in the viability and function of cardiomyocytes. However, the effects of PCSK9 mediated inflammatory cytokine release on cardiac remodelling still needs to be elucidated in vivo.

Ding *et al*. extended their findings in the in vivo preclinical mouse LCAL surgical model of MI (Ding, Wang, et al., 2018). They reported a dramatic increase in PCSK9 protein levels and autophagy makers in the infarct border zone (Ding, Wang, et al., 2018). Importantly, they reported that inhibiting *Pcsk9* gene expression (*Pcsk9 knockout*) or function, using peptide based inhibitors, reduced cardiomyocyte autophagy and infarct sizes, and improved LV fractional shortening in mice that had undergone LCAL, without altering plasma LDL cholesterol levels (Ding, Wang, et al., 2018). These results suggest that PCSK9 may exert direct effects on cardiomyocyte survival and cardiac function, although the contribution of altered PCSK9 mediated platelet activation described above, cannot be ruled out. One potential mechanism of the PCSK9 inhibition mediated protection on cardiac dysfunction is via modulating CD36 levels. CD36 mediates fatty acid uptake in cardiomyocytes (Silverstein & Febbraio, 2009). It has been suggested that inhibition of CD36 in cardiomyocytes reduced lipid accumulation and contractile dysfunction (Angin et al., 2012). However, in vivo studies have reported controversial results regarding the role of cardiomyocyte CD36 in cardiac function. On the one hand, cardiomyocyte-specific inhibition of CD36 improved functional recovery after I/R (Nagendran et al., 2013) and high fat diet induced cardiac hypertrophy and dysfunction (Y. Zhang, Bao, et al., 2015). On the other hand, inactivation of CD36 selectively in cardiomyocytes accelerated the progression from cardiac hypertrophy to heart failure, which was associated with energetic stress, as indicated by AMPK activation (Y. Zhang, Bao, et al., 2015). As described earlier, PCSK9 has been shown to exert different effects on CD36 levels in different cell types (Demers, Samami, Lauzier, Des Rosiers, Sock, et

al., 2015; Ding, Liu, et al., 2018). It would be interesting to investigate if PCSK9 mediates CD36 degradation or alters CD36 gene expression in cardiomyocytes. One study, however, has actually found that PCSK9 did not change CD36 protein levels in both HL-1 cardiomyocytes and heart tissues (Demers, Samami, Lauzier, Des Rosiers, Sock, et al., 2015), suggesting the protection against cardiac dysfunction in LCAL mouse model by inhibition of PCSK9 may occur independently of alterations in CD36 levels in cardiomyocytes.

1.8.3.6 Conclusions

The PCSK9 era started in 2003 when Seidah and Boileau *et al*. discovered that mutations in the *PCSK9* gene are associated with autosomal dominant hypercholesterolemia (Abifadel et al., 2003b). Since then, this field has moved forward rapidly. Two FDA approved mAbs against PCSK9 are available for clinical use (Robinson et al., 2015; Sabatine et al., 2015, 2017; Schwartz et al., 2018). While most of the focus on the role of PCSK9 inhibition in CVD has been on LDL cholesterol lowering, emerging basic studies utilizing either mouse models or cell lines have increasingly suggested that PCSK9 may also act via mechanisms that are independent of LDL cholesterol lowering. These mechanisms include influences on a variety of cell types and cellular pathways relevant to CVD. Whether these effects are the result of actions of PCSK9 derived locally in the cell types/tissues affected or due to circulating PCSK9 derived from the liver are not entirely clear, and further research will be needed to clarify this. Nevertheless, they raise the possibility that targeting PCSK9 may be beneficial even in individuals with low

LDL cholesterol or who have already achieved LDL cholesterol lowering targets by other means.

LDLR	Hepatocytes • PCSK9 mediates cell surface LDLR degradation (Zhang *et al.*, 2007; Lagace *et al.*, 2006; Qian *et al.*, 2007). Monocyte/Macrophages • PCSK9 reduces LDLR protein levels and LDLR mediated LDL uptake, affecting expression of CCR2 (Grune *et al.*, 2017).
LRP-1	B16F1 melanoma cells • PCSK9 mediates LRP-1 degradation (Canuel *et al.*, 2013).
VLDLR	Adipose tissue • PCSK9 mediates VLDLR degradation (Roubtsova *et al.*, 2011). Multiple cell lines • PCSK9 mediates VLDLR degradation in HEK293, NIH 3T3, CHO-A7, CHO-K1, Neuro2A, and HuH7 cells (Poirier *et al.*, 2008).
ApoER2	Neurons • PCSK9 mediates ApoER2 degradation (Kysenius *et al.*, 2012). Multiple cell lines • PCSK9 mediates ApoER2 degradation in HEK293, NIH 3T3, CHO-A7, CHO-K1, Neuro2A, and HuH7 cells (Poirier *et al.*, 2008).
MHC-I	Tumor cells • PCSK9 mediates MHC-I degradation and enhances intratumoral $CD8^+$ T cells infiltration (Liu *et al.*, 2020).
LOX-1	VSMCs, ECs, and Activated Macrophages • PCSK9 promotes LOX-1 expression (Ding *et al.*, 2015; Ding *et al.*, 2018).
LRP-5	Monocyte/Macrophages • PCSK9 and LRP-5 interact to facilitate lipid uptake (Badimon *et al.*, 2020).
CD36	VSMCs •PCSK9 reduces CD36 protein levels (Ferri *et al.*, 2016). Monocytes/Macrophages •PCSK9 promotes CD36 mRNA and protein levels (Ding *et al.*, 2018). Platelets •PCSK9 binds CD36 triggering platelet activation (Qi *et al.*, 2021). Cardiomyocytes •PCSK9 either does not affect (Demers *et al.*, 2015) or reduce (Da Dalt *et al.*, 2021) CD36 protein levels in the heart. Hepatocytes and Adipocytes •PCSK9 mediates cell surface CD36 degradation (Demers *et al.*, 2015). Renal cells •PCSK9 reduces cell surface CD36 levels (Byun *et al.*, 2022).

Figure 7: PCSK9-dependent effects on receptors. PCSK9's interactions with or effects on the LDLR, LRP-1, VLDLR, ApoER2, MHC-I, LOX-1, LRP-5 and CD36 in a variety of tissues, cells and cultured cell lines are summarized.

1.9 Overall Context and Objective

PCSK9 inhibition has been extensively studied for its role in lowering plasma LDL cholesterol levels and therefore protecting against atherosclerosis and CVD. However, the effects of PCSK9 inhibition have not been examined in a mouse model of pre-existing coronary artery atherosclerosis leading to myocardial infarction. Furthermore, the effects of PCSK9 that are independent of plasma cholesterol lowering, on coronary artery atherosclerosis and myocardial infarction have not been examined.

One year old *Sr-b1 KO* mice develop substantial coronary artery atherosclerosis induced by an HFCC diet. They also develop myocardial fibrosis and cardiac damage. Since *Sr-b1 KO* mice have an intact LDLR-APOE axis, they may be useful models to study the cholesterol lowering effects of therapeutic interventions on pre-existing CAD, such as PCSK9 inhibitors.

Sr-b1/ApoE dKO mice develop multiple characteristics of human CAD, including extensive coronary artery atherosclerosis and myocardial fibrosis, cardiac enlargement and dysfunction, cardiac conduction abnormalities, myocardial lipid and macrophage accumulation and early death (Braun et al., 2002). Since *Sr-b1/ApoE dKO* mice lack APOE, a ligand of LDLR, we hypothesize that PCSK9 inhibition does not change plasma

cholesterol levels in *Sr-b1/ApoE dKO* mice, making it an ideal mouse model to study the role of PCSK9 inhibition on CAD in a cholesterol independent manner.

Overall, the objective of this thesis is to study the effects of PCSK9 inhibition mediated cholesterol lowering on pre-existing CAD as well as the role of PCSK9 inhibition on CAD in a plasma cholesterol independent manner in different mouse models.

1.10 Hypothesis

We hypothesize that anti-PCSK9 antibody treatment will lower plasma cholesterol levels and reduce the continued growth of pre-existing coronary artery atherosclerosis and myocardial fibrosis induced by an HFCC diet in *Sr-b1 KO* mice and that genetic *Pcsk9 knockout* will protect against CAD in *Sr-b1/ApoE dKO* mice in a plasma cholesterol lowering independent manner.

1.11 Specific Aims

Aim of Chapter 2

To evaluate the effects of anti-PCSK9 antibody treatment on plasma cholesterol levels, the newly developing aortic sinus and coronary artery atherosclerosis and pre-existing atherosclerosis in the aortic sinus and coronary arteries and myocardial fibrosis in *Sr-b1 KO* mice fed an HFCC diet, a model of age induced coronary atherosclerosis.

Aim of Chapter 3

To evaluate whether genetic *Pcsk9 KO* protects against CAD in *Sr-b1/ApoE dKO* mice in a plasma cholesterol independent manner and whether the plasma cholesterol independent protective effects of *Pcsk9 KO* on CAD (if there are) are coming from the absence of liver-derived, circulating PCSK9 and dependent on the LDLR-PCSK9 interaction.

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Chapter 2: Anti-PCSK9 Antibody Intervention Reduces Plasma Cholesterol Levels and Protects against Established Coronary Artery Disease in *Sr-b1 KO* Mice

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Preface

TX and BLT designed the study, interpreted data, wrote and edited the paper. TX carried out the majority of experiments, data collection, all statistical analysis and lead the writing of the paper. LW carried out some experiments/data analyses (western blot in Figure 1) under the guidance of TX and/or BLT. BLT provided supervisory oversight. All authors contributed to the editing of the manuscript.

2.1 Abstract

Background: PCSK9 inhibition does not lower plasma cholesterol levels in commonly used experimental mouse atherosclerosis models - *ApoE KO* and *Ldlr KO* mice. This is due to the lack of an intact APOE-LDLR axis in these models. To understand the role of PCSK9 inhibition mediated plasma cholesterol lowering on coronary artery atherosclerosis, we tested anti-PCSK9 antibody treatment on HFCC diet (15% fat, 1.25% cholesterol, and 0.5% sodium cholate) induced atherosclerosis in one year old *Sr-b1 KO* mice that develop age and diet dependent coronary artery atherosclerosis and myocardial fibrosis.

Hypothesis: Anti-PCSK9 antibody treatment will reduce plasma cholesterol levels and decrease both new and established HFCC diet induced atherosclerotic plaque development in the aortic sinus and coronary arteries of one year old *Sr-b1 KO* mice.

Methods and results: One year old male *Sr-b1 KO* mice were fed the HFCC diet to induce atherosclerosis development. Weekly control or anti-PCSK9 antibody (10 mg/kg body weight in 0.9% saline, SQ) treatment was initiated one week before the diet switch to examine effects on new plaque development over 7 weeks of HFCC diet feeding. Alternatively, weekly control or anti-PCSK9 antibody treatment was initiated 7 weeks after the switch to HFCC diet to examine the impacts on the progression of established atherosclerotic plaques over the course of a further 5 weeks of HFCC diet feeding. Anti-PCSK9 antibody treatment significantly reduced plasma cholesterol and IL-6 levels in both cases. Anti-PCSK9 antibody treatment over the first 7 weeks of HFCC diet feeding

significantly reduced the sizes of atherosclerotic plaques in the aortic sinus and the numbers of coronary artery cross-sections exhibiting atherosclerotic plaque development. Feeding the HFCC diet for a total of 12 weeks resulted in significantly increased sizes of atherosclerotic plaques in the aortic sinus and increased proportions of coronary artery cross-sections exhibiting atherosclerotic plaque development. Treatment with the anti-PCSK9 antibody beginning at 7 weeks of HFCC diet feeding significantly attenuated the continued growth of established atherosclerotic plaques in the aortic sinus and attenuated the increase in coronary artery cross-sections with evidence of atherosclerotic plaque development. One-year-old male *Sr-b1 KO* mice fed the HFCC diet for 12 weeks also developed significant myocardial fibrosis and elevated plasma CK-MB, a marker of myocardial damage, both of which were reduced by anti-PCSK9 antibody treatment over the last 5 weeks of HFCC diet feeding.

Conclusion: Anti-PCSK9 antibody decreases plasma cholesterol levels and protects against continued growth of pre-established diet-induced atherosclerosis in the aortic sinus and coronary arteries and myocardial damage and fibrosis in one year old male *Sr-b1 KO* mice.

2.2 Introduction:

Coronary artery disease (CAD) is one of the leading causes of death worldwide. The underlying mechanism of CAD is the cholesterol-rich and highly inflammatory atherosclerotic plaques that build up in coronary artery walls. High plasma levels of cholesterol associated with apolipoprotein (apo) B-containing low-density lipoproteins (LDLs) initiate and drive atherosclerosis development. LDLs infiltrate into the intima and elicit proinflammatory responses in the susceptible regions of artery walls. Advanced atherosclerotic plaques with large necrotic cores are prone to undergo rupture and/or endothelial erosion triggering atherothrombotic occlusions in the artery lumen, blocking the blood supply to the regions of the myocardium and leading to myocardial infarction (MI), recognized by the release of cardiomyocyte proteins (such as creatine-kinase myocardial band, CK-MB or cardiac Troponin I or T) into the blood (serum or plasma) and myocardial fibrosis.

Proprotein convertase subtilisin like kexin type 9 (PCSK9) is highly expressed in the liver (Seidah et al., 2003b) from which the majority of circulating PCSK9 is derived (Zaid et al., 2008a). PCSK9 is well-known for its role in binding to LDL receptor (LDLR) and mediating LDLR lysosomal degradation in the liver (Benjannet et al., 2004; Maxwell et al., 2005; Maxwell & Breslow, 2004). Therefore, inhibition of PCSK9 reduces LDLR degradation, enhancing cell surface levels of LDLR on hepatocytes and thereby enhancing clearance of LDL from blood. Large clinical trials demonstrated that monoclonal antibodies (mAbs) and a small interfering RNA against PCSK9 substantially

lowered plasma LDL cholesterol levels (Raal et al., 2020; Ray et al., 2020; Robinson et al., 2015; Sabatine et al., 2015) and the PCSK9 mAbs reduced cardiovascular events (CV) (Sabatine et al., 2017; Schwartz et al., 2018). The most commonly used murine models of atherosclerosis (*Ldlr* or *ApoE knockout* (*KO*) mice) develop atherosclerosis beginning in their aortic sinuses and extending into the aortic arch and arteries that branch from the arch (e.g. brachiocephalic artery) and the descending aorta, but do not commonly develop atherosclerosis in coronary arteries and do not develop ischemic coronary artery disease characterized by myocardial fibrosis (Gonzalez et al., 2016; Lichtman et al., 1999; Nakashima et al., 1994). Furthermore, these mice do not respond to interventions aimed at increasing LDLR mediated cholesterol lowering because they lack either the LDLR itself or a key ligand of the LDLR, namely apoE. Therefore, they exhibit limitations to study the impacts of PCSK9 inhibition on cholesterol lowering (Ason, van der Hoorn, et al., 2014) and coronary artery atherosclerosis development. On the other hand, PCSK9-inhibition was reported to reduce plasma cholesterol levels in *ApoE*3Leiden/CETP* transgenic mice which do have an intact LDLR-ApoE axis (Ason, van der Hoorn, et al., 2014; Kühnast, van der Hoorn, et al., 2014; Landlinger et al., 2017). These studies reported that PCSK9 inhibition also reduced atherosclerotic plaque development in these mice (Ason, van der Hoorn, et al., 2014; Kühnast, van der Hoorn, et al., 2014; Landlinger et al., 2017); however, like *ApoE KO* and *Ldlr KO* mice, atherosclerosis in *ApoE*3Leiden CETP* mice does not generally involve the coronary arteries. Therefore, *ApoE*3Leiden CETP* mice are not well-suited to investigate impacts on ischemic coronary artery disease.

Scavenger receptor class B type 1 (SR-B1) is a high affinity receptor for high-density lipoproteins (HDLs). SR-B1 mediates selective HDL cholesterol uptake in the liver driving reverse cholesterol transport (Rigotti et al., 1997a). It also mediates cholesterol efflux from macrophages to HDL and HDL signaling in macrophages, endothelial cells, platelets, and cardiomyocytes (Durham, Chathely, & Trigatti, 2018; Durham, Chathely, Mak, et al., 2018; B. L. Trigatti & Fuller, 2016b). Rare loss of function mutations in *SCARB1*, the gene encoding SR-B1, have been associated with early-onset, severe CAD in people (Koenig et al., 2021). Similarly, knockout of *Scarb1* (*Sr-b1 KO*) in mice that also lack either *ApoE* or *Ldlr* expression leads to, respectively, either spontaneous or high fat/high cholesterol diet induced occlusive coronary artery atherosclerosis associated with extensive myocardial fibrosis and reduced survival (Braun et al., 2002; M. Fuller et al., 2014). Furthermore, *Sr-b1 single KO* mice develop high fat/high cholesterol, cholate containing (HFCC) diet-induced atherosclerosis in coronary arteries as well as in the aortic sinus (M. T. Fuller et al., 2023). The extent of the diet-induced coronary artery atherosclerosis is increased in older (1 year of age) *Sr-b1* KO mice and is associated with myocardial fibrosis (Lee, S. K. *et al*, manuscript in preparation). Therefore, *Sr-b1 KO* mice may be a useful model of age and diet dependent coronary artery atherosclerosis which is sensitive to interventions targeting LDLR mediated cholesterol lowering, such as anti-PCSK9 antibodies.

In this study, we examined the role of anti-PCSK9 antibody treatment on both the initiation of new atherosclerotic plaque development in the aortic sinus and coronary arteries and on the continued growth of pre-existing atherosclerotic plaques in the aortic

sinuses and coronary arteries of one-year-old male *Sr-b1 KO* mice. We show that weekly anti-PCSK9 antibody treatment reduces plasma total cholesterol and IL-6 levels and that when initiated before diet induction of atherosclerosis development, reduces atherosclerosis in both the aortic sinus and coronary arteries of 1 year old *Sr-b1 KO* mice, whereas when initiated 7 weeks into a 12 week course of atherogenic diet feeding, it attenuates the continued growth of atherosclerotic plaques in the aortic sinus and coronary arteries and attenuates the levels of the myocardial damage marker CK-MB and the degree of myocardial fibrosis that develops. This represents the first demonstration of the effects of PCSK9-inhibition mediated cholesterol lowering on coronary artery atherosclerosis and myocardial fibrosis in mice and demonstrates the utility of *Sr-b1 single KO* mice as a model of age and diet induced coronary artery disease.

2.3 Materials and Methods:

***2.3.1 Mice*:** All procedures involving mice were approved by the Animal Research Ethics Board of McMaster University and followed the guidelines of the Canadian Council on Animal Care. Mice were housed in the David Braley Research Institute animal facility and had free access to water and a normal diet containing 18% protein, 5% fat and 5% fiber (Teklad, TD2018, Envigo, Madison, WI, USA) or a high fat (15.8%), high cholesterol (1.25%) and sodium cholate containing (0.5%) diet (HFCC diet, Teklad, TD.88051, Envigo, Madison, WI, USA). *Sr-b1 KO* mice were originally provided by Monty Krieger, Massachusetts Institute of Technology, Cambridge, USA. Those mice were backcrossed >10 times onto a C57BL/6/J background in house.

2.3.2 Antibody treatment: The antibody against PCSK9 (anti-PCSK9 antibody) (Ason, van der Hoorn, et al., 2014) was kindly provided by Amgen Inc. This anti-PCSK9 antibody is a chimeric monoclonal antibody containing the variable domains of human IgG against human PCSK9 (Chan et al., 2009) and the constant domains of mouse IgG (Ason, van der Hoorn, et al., 2014). Anti-AGP3 (4D2, kindly provided by Amgen Inc.) or human IgG (027102, Invitrogen Life Technologies Inc., Burlington, ON, Canada) were used as control antibodies. There were no differences observed between anti-AGP3 and human IgG on all parameters we measured in the study and anti-AGP3 and human IgG treated mice were grouped as control antibody treated mice. All experiments used male *Sr-b1 KO* mice. *Sr-b1 KO* mice were fed with a normal diet from weaning at three weeks of age until one year old of age. *Sr-b1 KO* mice were switched to an HFCC diet at one year old of age until the experimental endpoints to induce atherosclerosis development. To investigate the role of anti-PCSK9 antibody on the newly developing atherosclerotic plaques, control antibody or anti-PCSK9 antibody (10 mg/kg body weight in 0.9% saline, SQ) treatment was initiated one week before the diet switch and administrated weekly until the experimental endpoint. This cohort of *Sr-b1 KO* mice was maintained on the HFCC diet for 7 weeks and was humanely euthanized and analyzed 7 weeks after initiation of HFCC diet feeding. To test the role of anti-PCSK9 antibody on the further development of established atherosclerotic plaques, control antibody or anti-PCSK9 antibody (10 mg/kg body weight in 0.9% saline, SQ) treatment was initiated 7 weeks after the switch to HFCC diet. The antibody treatment was continued weekly until the experimental endpoint. This cohort of *Sr-b1 KO* mice was maintained on the HFCC diet

for a total of 12 weeks and was humanely euthanized and analyzed after 12 weeks of HFCC diet feeding period. Another cohort of *Sr-b1 KO* mice was fed the HFCC diet for seven weeks and humanely euthanized and analyzed at one year and seven weeks of age. The last cohort of *Sr-b1 KO* mice was fed a normal diet until they were humanely euthanized and analyzed at one year and twelve weeks old of age.

2.3.3 ECG: Mice were anesthetized with isoflurane (1.8% O_2, induction with 5% isoflurane, maintenance with 2.5% isoflurane). ECG tracings were collected from needle electrodes inserted into the skin with a green needle electrode in the left hind limb, a red needle electrode in the left lower chest and a black needle electrode in the right upper chest. ECG tracings were recorded for 3 mins using a PowerLab 8/35 ADINSTRUMENTS and LabChart 8 software (ADInstruments, Inc., CO, USA).

2.3.4 Euthanasia and tissue and blood collection: All mice were fasted for 4 hrs and anesthetized with isoflurane/O_2. Blood for plasma analysis of CK-MB and troponin-I was collected from the submandibular vessel into a heparinized tube. For all other analyses, blood was collected by cardiac puncture into a heparinized syringe. Mice were euthanized by thoracotomy. Heparinized blood was centrifuged at 5,500 xg for 10 min and the plasma was recovered and stored at -80 °C prior to analysis. Tissues were perfused in situ with phosphate buffered saline containing 10 U of heparin/ml. Hearts were frozen in Shandon Cryomatrix (Fisher Scientific, Ottawa, ON, Canada) and then stored at -80 °C prior to further analysis. Livers were snap frozen in liquid nitrogen and stored at -80 °C for protein analysis. Aortas were harvested and fixed in 10% formalin for further analysis.

2.3.5 Liver total membrane preparation: Liver tissue (0.1 g) in 200 ul of ice-cold homogenization buffer (20mM Tris-HCl pH 7.5, 2mM $MgCl_2$, 0.25 M sucrose with the following protease inhibitors added just before use: 50uM PMSF, 2ug/ml pepstatin A, 5ug/ml leupeptin, and 10 ug/ml aprotinin) was homogenized using an Ultra-Turrax tissue homogenizer. The total liver homogenates were centrifuged at 3000 xg for 10 min at 4 °C to pellet nuclei and cell debris. Then, the supernatants were recovered and subjected to ultracentrifugation at 100,000 x g for 60 min at 4 °C using a Beckman Optima MAX-XP ultracentrifuge with an MLA-130 rotor to pellet total membranes. Membrane pellets were re-suspended in 200 µl of 10 mM sodium phosphate pH 7, containing the protease inhibitors listed above at the indicated concentrations and 0.1% SDS. Samples were then snap frozen in liquid nitrogen and stored at -80°C.

2.3.6 SDS – PAGE and immunoblotting: Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Hepatic total membrane protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide separating gels. Proteins were then transferred onto PVDF membranes in transfer buffer (150 mM glycine, 18 mM Tris-Cl, and 20% methanol). Membranes with proteins were blocked in 5% skim milk (Carnation) in Tris-buffered saline (TBS: 137mM NaCl, 2.7 mM KCl, and 24.8 mM Tris-Cl, pH 7.4) for 1 hr at room temperature. They were then incubated with goat anti-LDLR (R&D Systems, AF2255, Minneapolis, MN, USA), or rabbit anti-ε-COP (Novus Biologicals, NBP2-38512, Colorado, CO, USA) primary antibodies diluted 1:1000 in 3% BSA in TBS at 4°C overnight. Membranes were then washed three times with TBST (TBS plus 0.1%

Tween-20) and incubated with horseradish peroxidase (HRP) conjugated rabbit anti-goat (305-035-003), or donkey anti-rabbit (711-005-152) IgG (Jackson Immunoresearch Laboratory, West Grove, PA, USA) secondary antibodies diluted 1:5000 in 5% skim milk in TBS for 1 hr at room temperature. Membranes were washed with TBST three times again. HRP was then detected using the Pierce ECL Western Blotting Substrate kit (Thermo Fisher Scientific, Rockford, IL, USA) using a Gel Doc instrument (Bio-Rad Laboratories, Hercules, CA, USA) and of the protein bands were quantified using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

2.3.7 Plasma protein analysis: Plasma PCSK9, CK-MB, troponin-I, and IL-6 were measured by the mouse proprotein convertase 9 Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA), the mouse creatine kinase MB ELISA Kit (Novus Biologicals, Colorado, CO, USA), the mouse cardiac troponin-I ELISA kit (Life Diagnostics Inc., Pennsylvania, PA, USA), or the mouse IL-6 ELISA MAX™ Deluxe Set (BioLegend, San Diego, CA, USA) respectively according to manufacturer's instructions.

2.3.8 Plasma lipid Analysis: Total cholesterol levels were measured by using the Infinity^{TM} Cholesterol kit (Thermo Fisher Scientific, Ottawa, ON, Canada). Plasma triglyceride levels were measured by using the L-Type Triglyceride M Enzyme Color A and B kit (Wako Diagnosis, Richmond, VA, USA). Plasma unesterified cholesterol was measured by using the free cholesterol E (Wako Diagnosis, Richmond, VA, USA).

2.3.9 Atherosclerosis and myocardial fibrosis: Transverse cryosections (10 μm) of hearts were stained for lipids with oil red O. The aortic sinus atherosclerotic plaque volume was

calculated as the area under the curve of the plaque area versus the distance in the aortic sinus. Coronary artery atherosclerotic burdens were measured in 7 sections (300 μm apart) in the upper portion of the heart, starting from the aortic annulus (0 μm) and moving towards the apex direction (covering 1800 μm). The coronary artery occlusion levels were averaged from those 7 sections. Transverse cryosections (10 μm) of hearts were also stained for myocardial fibrosis with Masson's Trichrome (Thermo Fisher Scientific, Ottawa, ON, Canada), which stains healthy myocardium red and collagen blue. An Olympus BX 41 microscope with an automated XY stage was used to collect a gallery of images at 4X magnification for each Trichome-stained transverse section. Slidebook 6 software was used to create a composite image of each transverse section. Five sections (300 μm apart) starting from the aortic annulus (0 μm) towards the apical direction (covering 1200 μm) were analyzed to determine the average myocardial fibrosis levels from each mouse.

2.3.10 Statistical Analysis: GraphPad Prism 9.4.1 software was used for statistical analysis. For the comparison of two groups, data were first subjected to the Shapiro-Wilk normality test. Those that did not pass the normality test were analyzed by the Mann-Whitney Rank Sum test. Those which passed the normality test were then subjected to the F-test for comparing variances. Data that had significantly different variances were analyzed by the Student's t test with Welch's correction and data that had equal variances were analyzed by the Student's t test. For comparison of multiple groups, data were also first subjected to the Shapiro-Wilk normality test. Data that did not pass the normality test were analyzed by the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test.

Data that passed the normality test were then subjected to the Brown-Forsythe test for comparing standard deviations. Those that had significantly different standard deviations were analyzed by Welch's ANOVA test with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli post hoc test. Those that had equal standard deviations were analyzed by the ordinary one-way ANOVA with Tukey's post hoc test. Data are presented as mean ± standard deviation. NS: not statistically significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Results were considered statistically significant when $P < 0.05$.

2.4 Results:

2.4.1 Anti-PCSK9 antibody treatment increases hepatic LDLR protein levels and lowers plasma cholesterol levels in *Sr-b1 KO* mice fed the HFCC diet for 7 weeks.

To examine the effects of anti-PCSK9 antibody treatment on the newly developing atherosclerosis, male *Sr-b1 KO* mice were treated with either an anti-PCSK9 or a control antibody (weekly, 10 mg/kg body weight in 0.9% saline, SQ) beginning at age 51 weeks of age. Mice were switched from a normal chow diet to an HFCC diet one week after initiation of antibody treatment (52 weeks of age) until they were humanely euthanized and samples were collected 7 weeks after initiation of HFCC diet feeding (Figure 1A). Anti-PCSK9 antibody treatment did not affect body weights (Supplementary Figure 1A) compared to the control antibody treatment. Anti-PCSK9 antibody treatment significantly increased plasma PCSK9 levels compared to control antibody treatment (Figure 1B), consistent with previous reports (Byun et al., 2022; Koren et al., 2015; Oleaga et al.,

2021; Ridker et al., 2017; E. M. Roth et al., 2012; Shapiro et al., 2018; L. Zhang et al., 2012). As expected, anti-PCSK9 antibody treatment increased hepatic LDLR protein levels (Figure 1C and D) and significantly reduced plasma total cholesterol levels (Figure 1E) compared to control antibody treatment in *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. Anti-PCSK9 antibody treatment had no effects on the free to total cholesterol ratios (known to be abnormally high in these mice (M. T. Fuller et al., 2023)) or triglycerides (TG) levels (Figure 1F and G).

2.4.2 Anti-PCSK9 antibody treatment reduces new atherosclerotic plaque development in both the aortic sinus and coronary arteries of *Sr-b1 KO* mice fed the HFCC diet for 7 weeks.

One year old male *Sr-b1 KO* mice treated with the control antibody and fed the HFCC diet for 7 weeks developed substantial atherosclerosis in the aortic sinus (Figure 2A, C and D). Treatment with the anti-PCSK9 antibody significantly decreased the atherosclerotic plaque area and the volume of atherosclerotic plaques over the distance of the aortic sinus analyzed (Figure 2A-D). Those control antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks also developed atherosclerosis in coronary arteries: 43 ± 18% of coronary artery cross sections analyzed contained atherosclerotic plaques whereas 57 ± 18% of coronary artery cross sections were devoid of plaques in the control antibody-treated mice (Figure 2E and F). The extent of coronary artery atherosclerosis was reduced in mice treated with the anti-PCSK9 antibody: 24 ± 15% of coronary artery cross sections analyzed contained atherosclerotic plaques and 76 ± 15% of coronary

artery cross sections contained no plaque (Figure 2E and F). Plasma IL-6 levels were also significantly lower in the anti-PCSK9 antibody treated compared to control antibody-treated mice (Figure 2G). These findings suggest that anti-PCSK9 antibody mediated inhibition of PCSK9 in *Sr-b1 KO* mice increases hepatic LDLR protein levels and reduces HFCC-diet induced hypercholesterolemia, systemic inflammation and atherosclerosis development in the aortic sinus and coronary arteries.

2.4.3 Anti-PCSK9 antibody treatment reduces the growth of pre-existing atherosclerotic plaques in the aortic sinus and coronary arteries and myocardial fibrosis in 1 year old *Sr-b1 KO* mice fed the HFCC diet for 12 weeks.

To determine if anti-PCSK9 antibody treatment of *Sr-b1 KO* mice attenuates the continued development of pre-existing atherosclerotic plaques, 1 year old male *Sr-b1 KO* mice were fed the HFCC diet for 7 weeks, at which point they started to receive 5 weekly treatments of either the control antibody or anti-PCSK9 antibody while the HFCC diet was maintained. Mice were euthanized and analyzed after a total of 12 weeks of HFCC diet feeding (Figure 3A). As a control, an additional cohort of mice was analyzed after 7 weeks of HFCC diet feeding, corresponding to the time at which anti-PCSK9 or control antibody treatment was initiated (Figure 3A). This provided a baseline measure of the extent of pre-existing atherosclerosis corresponding to the beginning of anti-PCSK9 treatment. Consistent with the results of anti-PCSK9 antibody treatment during the first 7 weeks of HFCC diet feeding (Figure 1A-G), anti-PCSK9 antibody during the last 5 weeks of a total of 12 weeks of HFCC diet feeding did not affect body weights (Supplementary

Figure 1B) but resulted in significantly increased plasma PCSK9 and hepatic LDLR protein levels (Figure 3B-D), and reduced plasma total cholesterol (Figure 3E) but not the abnormally high ratio of free/total cholesterol or TG levels (Figure 3F and G). Very little atherosclerosis was detected in either the aortic sinus or coronary artery cross sections of mice maintained on the normal diet and analyzed at a similar age (Figure 4A, E-G), whereas, as in Figure 2A-F, atherosclerosis was detected in the aortic sinuses and coronary artery cross sections of mice fed the HFCC diet for 7 weeks (Figure 4B, E-G). An additional 5 weeks of HFCC diet feeding (in the presence of the control, non-immune antibody) further promoted the growth of atherosclerotic plaques in the aortic sinus (Figure 4C, E and F) and the proportion of coronary artery cross-sections that contained atherosclerotic plaques (Figure 4G) by approximately 2-fold. Anti-PCSK9 antibody treatment during the last 5 weeks of the 12 week HFCC diet feeding period, however resulted in an approximately 50% attenuation of the level of plaque growth in the aortic sinus (Figure 4C-F) and attenuated the increase in the proportions of coronary artery cross sections that contained atherosclerotic plaques (Figure 4G).

Significant fibrosis was detected in the myocardium of the 1 year old *Sr-b1 KO* mice fed the HFCC diet for 12 weeks which were treated with the control, non-immune antibody, reaching 1.3 ± 0.7% of the cross-sectional area of the myocardium (Figure 4I and K). The extent of myocardial fibrosis was reduced by approximately 40 % in 1 year old *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with the anti-PCSK9 antibody for the last 5 weeks of the feeding period (Figure 4I-K). Consistent with the

levels of myocardial fibrosis, control antibody treated mice fed the HFCC diet for 12 weeks exhibited elevated plasma levels of the myocardial damage marker CK-MB and these levels were attenuated in 1 year old *Sr-b1 KO* mice treated with the anti-PCSK9 antibody during the last 5 weeks of the 12 week HFCC diet feeding period (Figure 4L). Levels of plasma troponin-I, another marker of cardiac damage, were undetectable in all but 2 of the control antibody treated mice, and in all of the anti-PCSK9 antibody treated mice, but differences did not reach statistical significance among the two groups (data not shown). Neither HFCC-diet feeding for 7 weeks or 12 weeks, nor anti-PCSK9 antibody treatment affected cardiac size (heart weight/tibia length; Supplementary Figure 2A,B), suggesting that, unlike the *Sr-b1/ApoE double KO* spontaneous or the *Sr-b1/Ldlr double KO* diet-dependent models of coronary artery atherosclerosis, the extent of myocardial damage and fibrosis was not sufficient to result in cardiomegaly in the one year old *Sr-b1 single KO* mice fed the HFCC diet for 12 weeks. Anti-PCSK9 antibody treatment over the last 5 weeks of the 12 week HFCC diet feeding period was also associated with a reduction in plasma IL-6 levels (Figure 4H), similar to the effects of anti-PCSK9 antibody treatment over the first 7 weeks of HFCC diet feeding (Figure 2G). These findings suggest that anti-PCSK9 antibody mediated PCSK9 inhibition over the last 5 weeks of the 12 weeks HFCC diet feeding period increased hepatic LDLR protein levels and attenuated HFCC-diet induced hypercholesterolemia, systemic inflammation, and the continued growth of pre-existing atherosclerotic plaques in the aortic sinus and coronary arteries as well as the induction of myocardial damage and fibrosis.

2.5 Discussion:

PCSK9 inhibition in experimental mice has been reported to reduce aortic sinus atherosclerosis when cholesterol lowering was achieved (Ason, van der Hoorn, et al., 2014; Kühnast, van der Hoorn, et al., 2014; Landlinger et al., 2017). This has typically been in mice with intact expression of the LDLR and APOE (specifically, *ApoE*3Leiden/CETP* transgenic mice). In contrast, cholesterol lowering is not typically seen with PCSK9 inhibition in mice lacking expression of either the *Ldlr* or the *ApoE* genes and atherosclerosis reduction is not consistently seen in *ApoE KO* mice in which PCSK9 is inactivated or inhibited (Ason, van der Hoorn, et al., 2014; Denis et al., 2012; Z. H. Tang et al., 2017). Unlike *ApoE* or *Ldlr KO* mice, *Sr-b1 KO* mice are sensitive to PCSK9-inhibition mediated cholesterol lowering (Figures 1E and 3E). Most atherosclerosis susceptible strains of mice, including the *Ldlr KO*, *ApoE KO* and *ApoE*3Leiden/CETP* transgenic mice develop atherosclerosis in their aortic sinuses but only little atherosclerosis in their coronary arteries and do not exhibit significant myocardial fibrosis (Gonzalez et al., 2016). In contrast, one year old *Sr-b1 single KO* mice (on a C57BL/6J background) fed an HFCC diet for 12 weeks develop extensive coronary artery atherosclerosis accompanied by significant myocardial fibrosis and myocardial damage as evidenced by the levels of CK-MB detected in plasma (Lee, S. K. *et al*, manuscript in preparation; Figure 4G and I-L). It is important to note that in this study very little atherosclerotic plaque development occurred in either the aortic sinus or in the coronary arteries of 1 year old male *Sr-b1* single KO mice unless they were fed the HFCC diet (Figure 4E-G), consistent with our previous findings in both younger *Sr-b1*

KO mice (M. Fuller et al., 2014) and 1 year old female *Sr-b1* KO mice (Lee, S. K. *et al*, manuscript in preparation). This demonstrates that there is very little baseline atherosclerosis development in these mice fed a normal chow diet and that atherosclerosis development in both the aortic sinus and coronary arteries is mainly HFCC-diet dependent. This allowed us to examine the effects of PCSK9-antibody treatment on the initiation of atherosclerotic plaque development in the aortic sinus and coronary arteries by initiating treatment with either the anti-PCSK9 or control antibodies one week before initiating feeding with the HFCC diet for 7 weeks. This demonstrated that PCKS9-inhibition reduced the extent of newly developing atherosclerotic plaques in both the aortic sinus and in coronary arteries of these mice (Figure 2A-F). To examine the effects of PCSK9-inhibition on the continued growth of previously initiated atherosclerosis, one year old male *Sr-b1 KO* mice were fed the HFCC diet for 12 weeks and anti-PCSK9 or control antibody treatment was initiated at week 7. This demonstrated that anti-PCSK9 antibody treatment dramatically attenuated the continued growth of pre-existing atherosclerotic plaques in the aortic sinus and the extent of atherosclerosis development in coronary arteries (Figure 4A-G), and attenuated the degree of myocardial fibrosis and myocardial damage that largely appeared to develop between weeks 7-12 of HFCC diet feeding (Figure 4I-L). This appears to more closely resemble a clinical situation in which patients, presenting with pre-existing coronary atherosclerotic disease, are put on cholesterol lowering therapy, including PCSK9-inhibition.

We noted that while the continued development of atherosclerosis was reduced by anti-PCSK9 treatment, atherosclerosis was not reversed, suggesting that PCSK9-antibody treatment alone, was not sufficient to trigger atherosclerotic plaque regression, at least in this mouse model. Atherosclerotic plaque regression has been reported in a number of pre-clinical mouse atherosclerosis models generally involving transient impairment of *Lldr* or *ApoE* gene expression/activity and high fat/high cholesterol diet feeding, followed by restoration of *Ldlr/Apo*E gene expression/activity and switching back to normal fat/normal cholesterol diets (Feig et al., 2011; Harris et al., 2002; Patel et al., 2022; Reis et al., 2001; Van Craeyveld et al., 2011). Other studies have demonstrated that atherosclerotic plaque regression can be induced pre-clinically in mouse models by enhancing apoA1 levels in addition to reducing total plasma cholesterol levels, suggesting that HDL mediated reverse cholesterol transport may be required (Shah et al., 2001; Tangirala et al., 1999). Whether atherosclerotic plaque regression can occur in *Sr-b1 KO* mice which lack hepatic SR-B1 mediated HDL cholesterol clearance and exhibit impaired reverse cholesterol transport, remains to be determined.

Nevertheless, anti-PCSK9 antibody treatment for the last 5 weeks of the 12-week HFCC diet feeding period dramatically reduced the plasma levels of the myocardial damage marker CK-MB and the extent of myocardial fibrosis (Figure 4I-L). This suggests that anti-PCSK9 antibody treatment reduced the level of myocardial infarction resulting from coronary artery atherosclerosis. This is consistent with and may be the result of the reduction in the extent of coronary artery atherosclerosis development observed in the

mice treated with the anti-PCSK9 antibody for the last 5 weeks of HFCC diet feeding (Figure 4G). Whether this is the result of the plasma cholesterol lowering triggered by anti-PCSK9 antibody treatment, or due to inhibition of some other property of PCSK9 remains to be determined. Anti-PCSK9 antibody treatment prevents PCSK9 directed degradation of the LDLR (Chan et al., 2009). In the liver, this leads to elevated LDLR protein levels (Figure 1C-D and 3C-D) and LDLR mediated clearance of plasma cholesterol (Figure 1E and 3E). However, it is possible that anti-PCSK9 antibody treatment prevents PCSK9 directed degradation of LDLR in one or more other tissues that may impact myocardial infarction. Alternatively, it is possible that anti-PCSK9 antibody treatment affects PCSK9-mediated regulation of the protein levels of one or more other targets, such as the very low-density lipoprotein receptor (VLDLR), apoE receptor 2 (ApoER2), cluster of differentiation 36 (CD36) or major histocompatibility complex I (MHC-I), all of which have been shown to be targets for PCSK9 regulation (Demers, Samami, Lauzier, Des Rosiers, Ngo Sock, et al., 2015; X. Liu et al., 2020; Poirier et al., 2008; Roubtsova et al., 2011b). Furthermore, *Pcsk9* gene inactivation has been shown to attenuate myocardial infarction resulting from coronary artery ligation (Ding, Wang, et al., 2018), raising the possibility that PCSK9 inhibition may have a direct impact on myocardial remodeling after ischemic coronary artery disease. Further studies are required to explore these possibilities.

We also observed that anti-PCSK9 antibody treatment reduced plasma IL-6 levels in *Sr-b1 KO* mice fed the HFCC diet for either 7 or 12 weeks (Figure 2G and 4H).

Whether the reduced plasma IL-6 levels were a consequence or independent of the reduced plasma cholesterol levels resulting from anti-PCSK9 antibody treatment (Figure 1E and 3E) remains to be determined. The reduction in this systemic marker of inflammation suggests that PCSK9 inhibition may have either direct or indirect anti-inflammatory effects that may contribute to the reductions in development of atherosclerotic plaques and/or reduced levels of myocardial damage and fibrosis (Figure 2 and 4). Further research is required to determine the mechanisms by which anti-PCSK9 antibody treatment leads to reduced circulating IL-6 levels and the contribution of this to the reduced coronary artery atherosclerosis, myocardial damage and myocardial fibrosis.

In summary, our studies demonstrate that anti-PCSK9 antibody effectively reduced plasma cholesterol and IL-6 levels in one year old HFCC-diet fed male *Sr-b1 KO* mice. This protected against atherosclerosis development in both the aortic sinus and coronary arteries when treatment was initiated either at the beginning of atherosclerosis induction with the HFCC diet or after 7 weeks of HFCC diet feeding when atherosclerotic plaques had become established. In the latter model anti-PCSK9 antibody treatment attenuated myocardial damage and the development of myocardial fibrosis, likely due to the reduced coronary artery atherosclerosis resulting from the observed cholesterol lowering and anti-inflammatory effects.

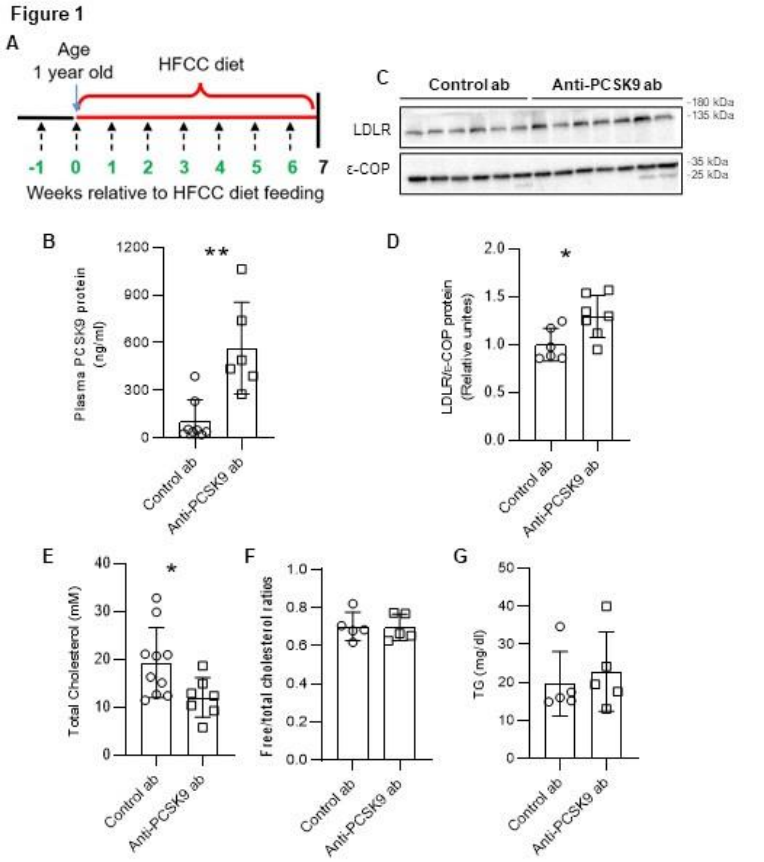


Figure 1
A
Age
1 year old
HFCC diet
-1 0 1 2 3 4 5 6 7
Weeks relative to HFCC diet feeding
C
Control ab
Anti-PCSK9 ab
LDLR
ε-COP
180 kDa
135 kDa
35 kDa
25 kDa
B
Plasma PCSK9 protein (ng/ml)
Control ab
Anti-PCSK9 ab
D
LDLR/ε-COP protein (Relative unites)
E
Total Cholesterol (mM)
F
Free/total cholesterol ratios
G
TG (mg/dl)

Figure 2

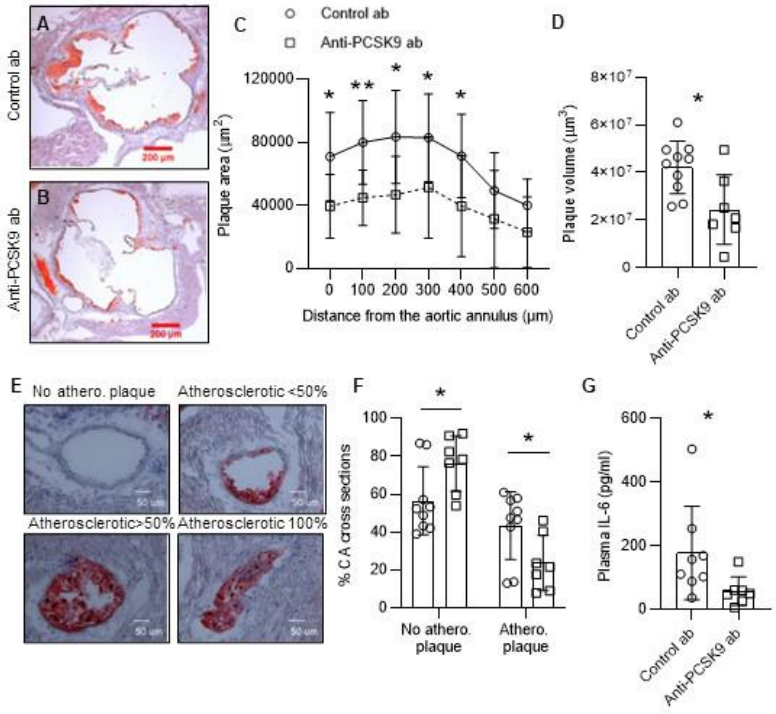
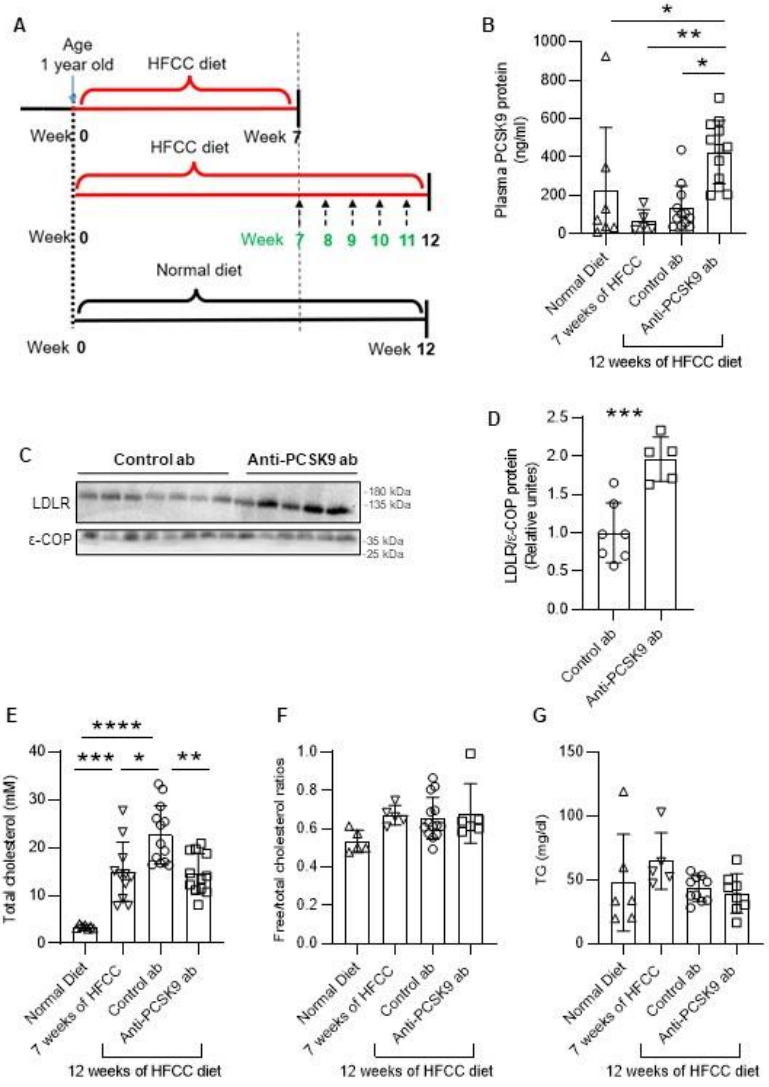

A
Control ab
B
Anti-PCSK9 ab
200 µm
C
Control ab
Anti-PCSK9 ab
Plaque area (µm²)
120000
80000
40000
0
0 100 200 300 400 500 600
Distance from the aortic annulus (µm)
D
Plaque volume (µm³)
8×10⁷
6×10⁷
4×10⁷
2×10⁷
0
Control ab
Anti-PCSK9 ab
E
No athero. plaque
Atherosclerotic <50%
Atherosclerotic>50%
Atherosclerotic 100%
50 µm
F
% C.A cross sections
100
80
60
40
20
0
No athero. plaque
Athero. plaque
G
Plasma IL-6 (pg/ml)
600
400
200
0
Control ab
Anti-PCSK9 ab

Figure 3


A
Age
1 year old
HFCC diet
Week 0
Week 7
HFCC diet
Week 0
Week 7 8 9 10 11 12
Normal diet
Week 0
Week 12
B
Plasma PCSK9 protein (ng/ml)
Normal Diet
7 weeks of HFCC
Control ab
Anti-PCSK9 ab
12 weeks of HFCC diet
C
Control ab
Anti-PCSK9 ab
LDLR
ε-COP
180 kDa
135 kDa
35 kDa
25 kDa
D
LDLR/ε-COP protein (Relative unites)
Control ab
Anti-PCSK9 ab
E
Total cholesterol (mM)
F
Free/total cholesterol ratios
G
TG (mg/dl)

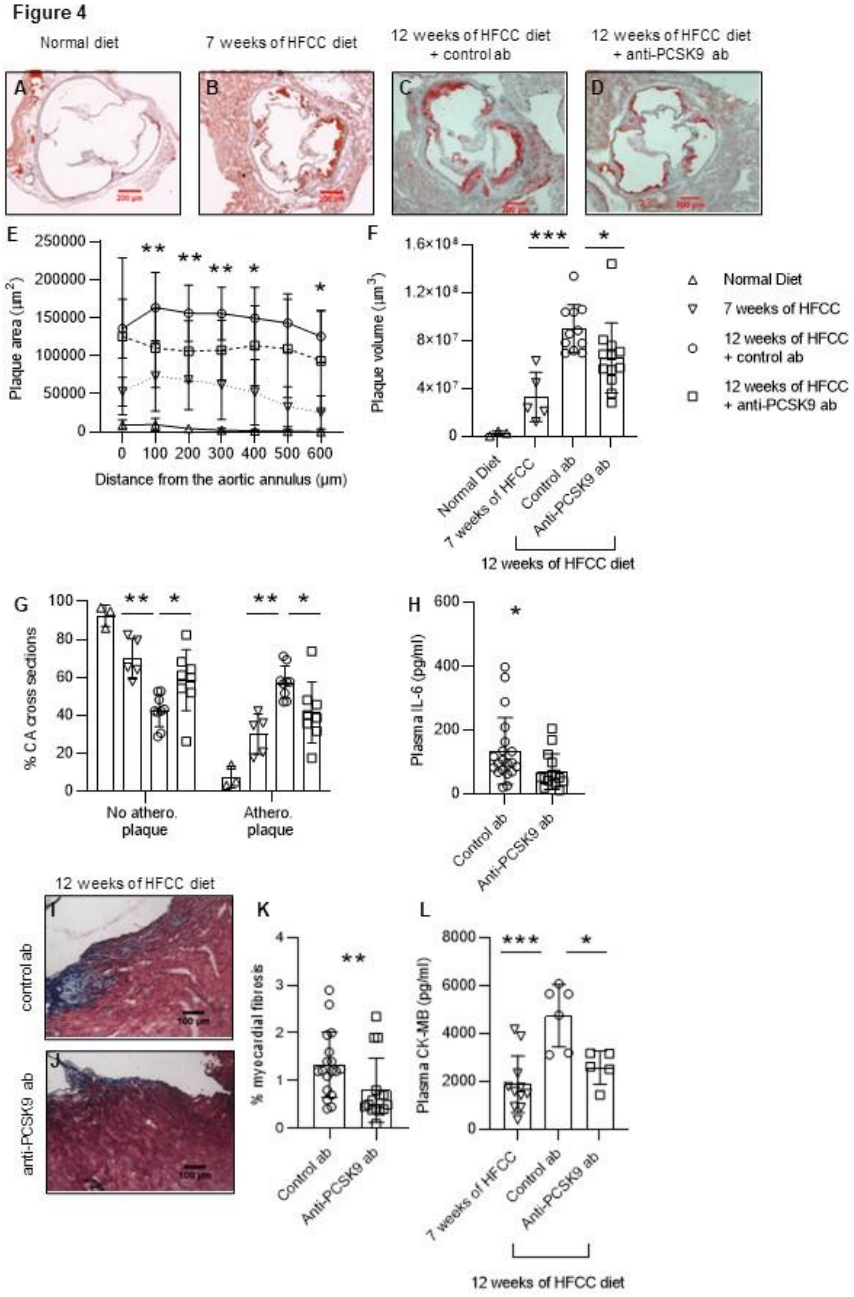


Figure 4
Normal diet
7 weeks of HFCC diet
12 weeks of HFCC diet + control ab
12 weeks of HFCC diet + anti-PCSK9 ab
A
B
C
D
E
Plaque area (μm²)
Distance from the aortic annulus (μm)
F
Plaque volume (μm³)
Normal Diet
7 weeks of HFCC
12 weeks of HFCC + control ab
12 weeks of HFCC + anti-PCSK9 ab
Normal Diet
7 weeks of HFCC
Control ab
Anti-PCSK9 ab
12 weeks of HFCC diet
G
% CA cross sections
No athero. plaque
Athero. plaque
H
Plasma IL-6 (pg/ml)
Control ab
Anti-PCSK9 ab
12 weeks of HFCC diet
I
J
control ab
anti-PCSK9 ab
K
% myocardial fibrosis
Control ab
Anti-PCSK9 ab
L
Plasma CK-MB (pg/ml)
7 weeks of HFCC
Control ab
Anti-PCSK9 ab
12 weeks of HFCC diet

2.6 Figure legends:

Figure 1: Anti-PCSK9 antibody treatment increases hepatic LDLR protein levels and lowers plasma cholesterol levels in *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (A) The schematic of experimental design. (B) Plasma PCSK9 protein levels in control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (C) Representative immunoblots and (D) quantification of LDLR protein levels (normalized to ε-COP) in liver total membranes from control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. Plasma (E) total cholesterol, (F) free cholesterol to total cholesterol ratios and (G) triglyceride levels from control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Figure 2: Anti-PCSK9 antibody treatment decreases the newly developing atherosclerosis in both the aortic sinus and coronary arteries of *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (A-B) Representative images of cross sections of aortic sinus stained with oil red O/hematoxylin, and (C) quantification of atherosclerotic plaque cross sectional areas at different positions along the aortic sinus from (A) control antibody or (B) anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (D)

Quantification of atherosclerotic plaque volumes (areas under the curves for individual mice corresponding to panel C) along 600 μm of the aortic sinus from control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (E) Representative images of transverse sections of coronary arteries stained with oil red O/hematoxylin showing either no atherosclerotic plaque or different extents of atherosclerosis (occluding <50%, >50% or 100% of the artery lumen; together considered as coronary artery cross sections with atherosclerotic plaque). (F) Quantification of the percentage of coronary artery cross sections with no atherosclerotic plaque and with atherosclerotic plaques in control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. (G) Plasma IL-6 levels in control or anti-PCSK9 antibody treated *Sr-b1 KO* mice fed the HFCC diet for 7 weeks. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

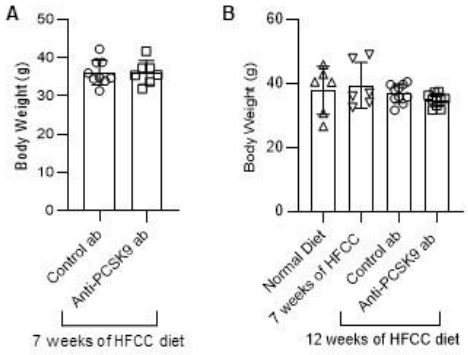
Figure 3: Anti-PCSK9 antibody treatment over the last 5 weeks of the 12 week HFCC diet feeding period increases hepatic LDLR protein levels and reduces plasma cholesterol levels in *Sr-b1 KO* mice. (A) The schematic of experimental design. (B) Plasma PCSK9 protein levels in *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, *Sr-b1 KO* mice fed the HFCC diet for 7 weeks, and *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with control antibody or anti-PCSK9 antibody from week 7 of HFCC diet feeding. (C) Representative immunoblots and (D) quantification of LDLR

protein levels (normalized to ε-COP) in liver total membranes from *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated for the last 5 weeks with control or anti-PCSK9 antibody. Plasma (E) total cholesterol, (F) free cholesterol to total cholesterol ratios and (G) triglyceride levels from *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, *Sr-b1 KO* mice fed the HFCC diet for 7 weeks, and *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with control or anti-PCSK9 antibodies for the last 5 weeks. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

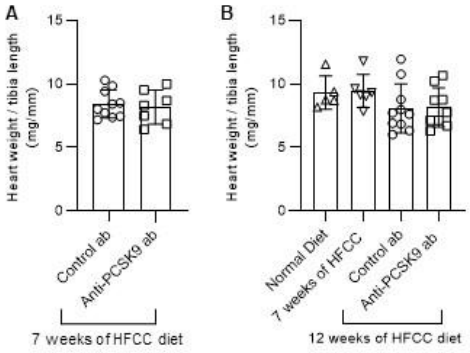
Figure 4: Anti-PCSK9 antibody treatment reduces the growth of pre-existing atherosclerotic plaques in the aortic sinus and coronary arteries and myocardial fibrosis in 1 year old *Sr-b1 KO* mice fed the HFCC diet for 12 weeks. (A-D) Representative images of cross sections of aortic sinus stained with oil red O/hematoxylin, (E) quantification of atherosclerotic plaque cross sectional areas at different positions along the aortic sinus and (F) quantification of atherosclerotic plaque volumes (areas under the curves for individual mice corresponding to panel E) along 600 μm of the aortic sinus for (A) *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, (B) *Sr-b1 KO* mice fed the HFCC diet for 7 weeks, and *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with (C) control antibody or (D) anti-PCSK9 antibody for the last 5 weeks of HFCC diet feeding. (G) Quantification of the percentage

of coronary artery cross sections with no atherosclerotic plaque and with atherosclerotic plaques in *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, *Sr-b1 KO* mice fed the HFCC diet for 7 weeks, and *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with the control or anti-PCSK9 antibodies for the last 5 weeks of HFCC diet feeding. (H) Plasma IL-6 levels in *Sr-b1 KO* mice fed the HFCC diet for 12 weeks treated with the control or anti-PCSK9 antibodies for the last 5 weeks of HFCC diet feeding. (I, J) Representative images and (K) quantification of fibrosis in trichrome-stained transverse sections of the upper portions of hearts from *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with (I) the control antibody or (J) the anti-PCSK9 antibody for the last 5 weeks of HFCC diet feeding. Healthy myocardium stains red and collagen containing myocardial fibrotic area stains blue. (L) Plasma CK-MB levels for *Sr-b1 KO* mice fed the HFCC diet for 7 weeks, and *Sr-b1 KO* mice fed the HFCC diet for 12 weeks and treated with the control antibody or anti-PCSK9 antibody for the last 5 weeks of HFCC diet feeding. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Supplementary Figure 1



Supplementary Figure 2



2.7 Supplementary figure legends:

Supplementary figure 1: Anti-PCSK9 antibody treatment does not affect body weights in *Sr-b1 KO* mice fed the HFCC diet. (A) One year old male *Sr-b1 KO* mice fed the HFCC diet and treated with either control antibody or anti-PCSK9 antibody for 7 weeks. (B) One year old male *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, or one year old male *Sr-b1 KO* mice fed the HFCC diet for 7 weeks or fed the HFCC diet for 12 weeks and treated with the control antibody or anti-PCSK9 antibody for the last 5 weeks of the HFCC diet feeding period. Refer to the schematic of the experiment in Figure 1A and 3A. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. No statistically significant differences were detected.

Supplementary figure 2: Neither HFCC diet feeding for 7 or 12 weeks, nor anti-PCSK9 antibody treatment affects heart weights in 1 year old male *Sr-b1 KO* mice. (A) One year old male *Sr-b1 KO* mice fed the HFCC diet and treated with either control antibody or anti-PCSK9 antibody for 7 weeks. (B) One year old male *Sr-b1 KO* mice fed a normal diet for an additional 12 weeks, or one year old male *Sr-b1 KO* mice fed the HFCC diet for 7 weeks or fed the HFCC diet for 12 weeks and treated with the control antibody or anti-PCSK9 antibody for the last 5 weeks of the HFCC diet feeding period. Refer to the schematic of the experiment in Figure 1A and 3A. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. No statistically significant differences were detected.

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Chapter 3: Circulating PCSK9 promotes coronary artery atherosclerosis and myocardial fibrosis independently of plasma cholesterol levels in a mouse model of coronary artery disease.

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Preface

TX and BLT designed the study, interpreted data, wrote and edited the paper. TX carried out the majority of experiments, data collection, all statistical analysis and lead the writing of the paper. LW (lipid accumulation in the heart and CD36 staining), GEGK (initial PV loop surgery) and LRC (sectioning of 9 hearts from Figure 2 and 3) carried out some experiments/data analyses under the guidance of TX and/or BLT. BLT provided supervisory oversight. AP, NGS and RCA provided expert advice. All authors contributed to the editing of the manuscript.

3.1 Abstract

Background: *Sr-b1/ApoE dKO* mice develop substantial occlusive coronary artery atherosclerosis and myocardial fibrosis, associated with cardiomegaly, cardiac dysfunction, and premature death, characteristics reminiscent of human coronary artery disease (CAD). PCSK9 has been widely investigated for its role in antagonizing LDLR-mediated plasma cholesterol-lowering. However, the effects of inhibiting PCSK9 on coronary artery atherosclerosis and myocardial infarction that are independent of plasma cholesterol-lowering, remain unknown.

Hypothesis: Inactivating *Pcsk9* by whole-body genetic deletion will protect against CAD in *Sr-b1/ApoE dKO* mice independently of plasma cholesterol-lowering.

Methods and results: Whole-body genetic deficiency of *Pcsk9* in female and male *Sr-b1/ApoE dKO* mice did not affect plasma cholesterol levels, although hepatic LDLR protein levels were increased. However, *Pcsk9* deficiency attenuated atherosclerosis in the aortic sinus and coronary arteries in both females and males. Furthermore, it reduced cardiomegaly and cardiac fibrosis and damage, decreased lipid and macrophage accumulation in the heart, and attenuated cardiac dysfunction in both females and males. Restoring circulating PCSK9 by recombinant AAV8 harboring *Pcsk9* driven by a liver-specific promoter did not affect plasma cholesterol levels, but it reversed the protective effects of *Pcsk9* genetic deficiency against aortic sinus and coronary artery atherosclerosis, myocardial fibrosis and damage, and lipid accumulation in the heart in both female and male *Pcsk9/Sr-b1/ApoE tKO* mice. Disrupting the interaction between

the LDLR and PCSK9 by anti-PCSK9 antibody treatment significantly increased circulating PCSK9 levels in both female and male *Sr-b1/ApoE dKO* mice. Anti-PCSK9 antibody treatment did not affect plasma cholesterol levels despite increased hepatic LDLR protein levels in both female and male *Sr-b1/ApoE dKO* mice. However, it protected males but not female mice against atherosclerosis in the aortic sinus and coronary arteries. Despite this, it had no effects on cardiac enlargement or myocardial fibrosis and damage in either females or males.

Conclusions: The absence of circulating PCSK9 protects *Sr-b1/ApoE dKO* mice against coronary artery atherosclerosis and myocardial fibrosis in a plasma cholesterol independent manner. While the plasma cholesterol independent effects of circulating PCSK9 on atherosclerosis appear to act partially through the LDLR (in males but not females), the effects of circulating PCSK9 on myocardial fibrosis development appear to be independent of the LDLR.

3.2 Introduction:

Atherosclerosis is the underlying cause of coronary artery disease (CAD). Both lipid retention and chronic inflammatory responses contribute to the growth of atherosclerotic plaque within the intima of arteries. Atherosclerosis development involves many cell types and processes, including endothelial cell (EC) activation, the recruitment of monocytes and smooth muscle cells (SMCs) into the intima, and the uptake of lipids by macrophages and SMCs (resulting in foam cell formation). Cellular apoptosis within atherosclerotic plaques and the inefficient clearance of apoptotic bodies (defective efferocytosis) trigger secondary necrosis and the formation of a necrotic core. The rupture of large necrotic cores in atherosclerotic plaques in coronary arteries leads to athero-thrombosis, which eventually causes myocardial infarction (MI) and sudden cardiac death.

Familial hypercholesterolemia (FH), characterized by increased circulating LDL cholesterol levels, is a risk factor for CAD. The proprotein convertase subtilisin like kexin type 9 (*PCSK9*) gene is one of three causative genes in which mutations are associated with FH (Abifadel et al., 2003b). The two other FH target genes encode the apolipoprotein B-100 and the LDL receptor (LDLR). *Pcsk9* is most abundantly expressed in hepatocytes, pancreatic β-cells, intestine and kidney (Peyot et al., 2021; Seidah et al., 2003b). Although *Pcsk9* is expressed at marginal levels in SMCs, ECs, macrophages and cardiomyocytes, their expression of PCSK9 has been reported to be involved in processes that are associated with atherosclerosis and MI development (Ding, Liu, Wang, Deng,

Fan, Sun, et al., 2015; Ding, Wang, et al., 2018; Ferri et al., 2012a; J. Li et al., 2017; Lu-Shan et al., 2008; TANG et al., 2012; Xiong et al., 2021). The majority of circulating PCSK9 is derived from the liver (Zaid et al., 2008a). Circulating PCSK9 binds to hepatic LDLR on the cell surface. This process promotes LDLR internalization, directs LDLR to the lysosome for degradation, and reduces hepatic LDLR levels on the cell surface, which ultimately increases plasma LDL cholesterol levels (Benjannet et al., 2004; Maxwell et al., 2005; Maxwell & Breslow, 2004). Therefore, gain of function mutations in *PCSK9* cause increased degradation of LDLR in the liver, increasing plasma LDL cholesterol levels (Abifadel et al., 2003b), while loss of function mutations reduce LDLR degradation in the liver, lowering plasma LDL cholesterol levels (Cohen et al., 2005). PCSK9 has also been implicated in targeting other receptors, thereby promoting their degradation (Canuel et al., 2013; Demers, Samami, Lauzier, Des Rosiers, Ngo Sock, et al., 2015; Poirier et al., 2008). These include the LDL receptor-related protein 1 (LRP1), cluster of differentiation 36 (CD36), the very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor-2 (apoER2) and major histocompatibility complex class-I (MHC-I) (Canuel et al., 2013; Demers, Samami, Lauzier, Des Rosiers, Ngo Sock, et al., 2015; X. Liu et al., 2020; Poirier et al., 2008; Roubtsova et al., 2011b).

Because PCSK9 mediates hepatic LDLR degradation, it is an appealing target for reducing cholesterol levels. Large clinical trials demonstrated that PCSK9 inhibitors, including two fully human monoclonal antibodies (mAbs) and one small interfering RNA (siRNA) are potent drugs for LDL cholesterol lowering (Raal et al., 2020; Ray et al., 2020; Robinson et al., 2015; Sabatine et al., 2015) and the PCSK9 mAbs significantly

reduced cardiovascular events (Sabatine et al., 2017; Schwartz et al., 2018). Interestingly, recent studies have indicated that PCSK9 inhibition might exhibit pleiotropic anti-atherogenic effects independent of LDL cholesterol lowering (Ferri et al., 2016; Giunzioni et al., 2016c; Karagiannis et al., 2018; Z.-H. Tang et al., 2017; Tavori et al., 2016; Xiong et al., 2021). *In vitro* studies have shown that PCSK9 short interfering (si) RNA mediated *Pcsk9* knockdown inhibited the apoptosis of human umbilical vein endothelial cells (HUVECs), SMCs and human THP-1 derived macrophages (Ding et al., 2016; J. Li et al., 2017; Lu-Shan et al., 2008; C.-Y. Wu et al., 2012). *Pcsk9*-targeting siRNA also suppressed the inflammatory response induced by oxidized (ox) LDL in human THP-1 derived macrophages (TANG et al., 2012). Furthermore, *Pcsk9* knockdown suppressed lectin-like oxLDL receptor 1 expression and reactive oxygen species (ROS) production in HUVECs and SMCs (Ding, Liu, Wang, Deng, Fan, Shahanawaz, et al., 2015b; Ding, Liu, Wang, Deng, Fan, Sun, et al., 2015). In addition, PCSK9 inhibition by the alirocumab mAb reduced intercellular adhesion molecule-1 (ICAM-1) levels, a marker for EC activation (Kühnast, van der Hoorn, et al., 2014), while the evolocumab anti-PCSK9 mAb reduced platelet activation in a cholesterol independent manner (Qi et al., 2021b). Together, these findings suggest that PCSK9 may exert a variety of atherogenic effects that are independent of its regulation of LDLR mediated cholesterol clearance.

Apolipoprotein E knockout (*ApoE KO*) mice accumulate large amounts of cholesterol associated with VLDL- sized lipoproteins and develop spontaneous atherosclerosis in the aortic sinus, aortic arch and brachiocephalic arteries (Nakashima et

al., 1994; Plump et al., 1992b; S. H. Zhang et al., 1992b). It has been shown that PCSK9-mediated regulation of plasma cholesterol levels is not only dependent on LDLR, but also on APOE (a ligand for LDLR) (Ason, Van Der Hoorn, et al., 2014; Tavori et al., 2016). Consistent with this, it has been reported in one study that inhibition of PCSK9 reduces atherosclerosis in mice in a manner dependent on the expression of APOE (Ason, Van Der Hoorn, et al., 2014). However other studies have reported that *Pcsk9 KO* or silencing can result in reduced aortic atherosclerosis in *ApoE KO* mice (Denis et al., 2012; Z.-H. Tang et al., 2017), although the extent of reduction appears to be less than when APOE is present (Ason, Van Der Hoorn, et al., 2014). Similarly, it has been reported that overexpression of either mouse or human *PCSK9*, increases atherosclerosis in *ApoE KO* mice (Denis et al., 2012; Tavori et al., 2016), even in the absence of effects on plasma cholesterol levels. These studies further raise the possibility that PCSK9 may exert direct effects on atherogenesis in the aorta that may be independent of its effects on LDL cholesterol levels. Whether such effects might be mediated by PCSK9 expressed locally in the atherosclerotic plaque or by PCSK9 expressed in the liver and secreted into the blood, is not clear.

Although *ApoE KO* mice are commonly used for atherosclerosis studies, they do not frequently develop occlusive atherosclerosis in coronary arteries or MI (Gonzalez et al., 2016). On the other hand, inactivation of the *Scarb1* gene which encodes the scavenger receptor class B type I (SR-B1) in *ApoE KO* mice (*Sr-b1/ApoE dKO* mice), results in the rapid and spontaneous development of atherosclerosis in both the aortic sinus and a significant proportion of coronary arteries (Al-Jarallah et al., 2013b; Braun et

al., 2002, 2003, 2008; Karackattu et al., 2006). *Sr-b1/ApoE dKO* mice also develop substantial myocardial fibrosis, cardiac enlargement, cardiac lipid accumulation, cardiac conductance abnormalities, left ventricular dysfunction and premature death as a consequence of the coronary artery atherosclerosis and associated MI (Al-Jarallah et al., 2013b; Braun et al., 2002, 2003, 2008; Karackattu et al., 2006). SR-B1 is a high affinity cell surface receptor for high-density lipoprotein (HDL). It is abundantly expressed in liver and steroidogenic tissues (Rigotti et al., 2003). SR-B1 mediates HDL-dependent reverse cholesterol transport from peripheral tissues, including macrophages in the artery wall, to the liver for biliary excretion (Ji et al., 1997; Jian et al., 1998; Rigotti et al., 1997b; Y. Zhang et al., 2005). A study from human patients reported that rare inherited loss of function variants in the *SCARB1* gene resulted in early-onset and severe coronary artery disease (Koenig et al., 2021). This finding supports the use of *Sr-b1/ApoE dKO* mice as a useful preclinical mouse model to investigate underlying mechanisms of and novel therapeutics for CAD.

We have previously reported that treatment of *Sr-b1/ApoE dKO* mice with the HMG-CoA reductase inhibitor rosuvastatin, delayed the development of coronary artery atherosclerosis and myocardial infarction even though it resulted in increased PCSK9 levels in plasma (Yu et al., 2018). Therefore, we sought to test the contribution of PCSK9 to the development of occlusive coronary artery atherosclerosis and myocardial infarction in *Sr-b1/ApoE dKO* mice. To investigate this, we inhibited PCSK9 in *Sr-b1/ApoE dKO* mice by knocking out the *Pcsk9* gene. Here, we report that *Pcsk9* gene deletion protects *Sr-b1/ApoE dKO* mice against coronary artery atherosclerosis and myocardial infarction

in a cholesterol independent manner. Restoring circulating PCSK9 in *Pcsk9/Sr-b1/ApoE triple KO* (tKO) mice using a recombinant adeno associated virus 8 (AAV8) suggested that the cholesterol independent protective effects of genetic *Pcsk9* inactivation are driven by the absence of liver-derived, circulating PCSK9. On the other hand, treatment of *Sr-b1/ApoE dKO* mice with an anti-PCSK9 antibody, which disrupts the interaction between PCSK9 and LDLR (Ason, Van Der Hoorn, et al., 2014), increased circulating PCSK9 by ~10 fold and exhibited a sex-dependent reduction in coronary artery atherosclerosis in males but did not protect against myocardial fibrosis in *Sr-b1/ApoE dKO* mice. These results suggest that the effects of PCSK9 on coronary artery atherosclerosis, particularly in females, as well as effects on myocardial fibrosis in both males and females, are at least partially independent of the LDLR.

3.3 Materials and Methods:

***3.3.1 Mice*:** All procedures involving mice were approved by the Animal Research Ethics Board of McMaster University and followed the guidelines of the Canadian Council on Animal Care. Mice were bred and housed in the David Braley Research Institute (DBRI) animal facility and had free access to water and a normal diet containing 18% protein, 5% fat and 5% fiber (Teklad Global, Envigo, Madison, WI, USA).

3.3.2 Generation of Pcsk9 $^{ko/ko}$***Sr-b1*** $^{ko/ko}$***ApoE*** $^{ko/ko}$ **(PSEKO) *mice:*** *Pcsk9*$^{ko/ko}$ mice (Zaid et al., 2008a) were obtained from a colony at the DBRI animal facility maintained by Patricia Liaw (McMaster University). *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ mice (mixed C57BL/6J and 129Sv genetic background) were bred in-house from founders originally provided by

Monty Krieger, Massachusetts Institute of Technology, Cambridge, USA. *Pcsk9*$^{ko/ko}$ and *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ mice were mated to generate *Pcsk9*$^{wt/ko}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{wt/ko}$ offspring. These were mated together to generate *Pcsk9*$^{wt/ko}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ offspring, which were then crossed together to generate *Pcsk9*$^{ko/ko}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ founders and control *Pcsk9*$^{wt/wt}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ founders. PSEKO (*Pcsk9*$^{ko/ko}$ *Sr-b1*$^{ko/ko}$*ApoE*$^{ko/ko}$) mice used for experiments were generated by mating male and female *Pcsk9*$^{ko/ko}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ mice. Similarly, SEKO (*Pcsk9*$^{wt/wt}$ *Sr-b1*$^{ko/ko}$*ApoE*$^{ko/ko}$) and EKO (*Pcsk9*$^{wt/wt}$ *Sr-b1*$^{wt/wt}$*ApoE*$^{ko/ko}$) control mice were generated by mating control *Pcsk9*$^{wt/wt}$ *Sr-b1*$^{wt/ko}$*ApoE*$^{ko/ko}$ mice. Genotyping was carried out by PCR on DNA isolated from tail biopsies collected at weaning (3 weeks of age) as previously described (Braun et al., 2002; Denis et al., 2012; Yu et al., 2018).

3.3.3 Recombinant adeno associated virus serotype 8 (rAAV8) mediated Pcsk9 expression: rAAV8 containing wild type mouse *Pcsk9* cDNA under the control of a liver specific *hcrApoE/hAAT1* promoter was used to drive liver-specific *Pcsk9* expression. A control rAAV8 containing *eGFP* cDNA under the control of the same liver specific *hcrApoE*/*hAAT1* promoter was also used. rAAV8-*hcrApoE*/*hAAT1-Pcsk9* (AAV8-PCSK9) and rAAV8-*hcrApoE/hAAT1-eGFP* (AAV8-eGFP) viruses were purchased from Vector Biolabs (Malvern, PA, USA). A single retro-orbital injection of either AAV8-eGFP or AAV8-PCSK9 was conducted in PSEKO mice at 3 weeks of age or *Pcsk9*$^{ko/ko}$ *Sr-b1*$^{wt/wt}$*ApoE*$^{ko/ko}$ mice at 6 weeks of age to deliver 4 x 10^{10} genome copies (GC) of viral particles per mouse.

3.3.4 Antibody treatment: The antibody against PCSK9 (anti-PCSK9 antibody) (Ason, Van Der Hoorn, et al., 2014) was kindly provided by Amgen Inc. This anti-PCSK9 antibody is a chimeric monoclonal antibody containing the variable domains of human IgG against human PCSK9 [55] and the constant domains of mouse IgG (Ason, Van Der Hoorn, et al., 2014). Anti-AGP3 (4D2, kindly provided by Amgen Inc.) or human IgG (027102, Invitrogen Life Technologies Inc., Burlington, ON, Canada) were used as control antibodies. There were no differences observed between anti-AGP3 and human IgG on all parameters we measured in the study and anti-AGP3 and human IgG treated mice were grouped as control antibody treated mice. Female and male SEKO mice were treated with either control antibody or the anti-PCSK9 antibody in 0.9% saline (10 mg/kg body weight) by weekly subcutaneous injections.

3.3.5 ECG: A subset of mice at 6.5 weeks old of age were anesthetized with isoflurane (1.8% O_2, induction with 5% isoflurane, maintenance with 2.5% isoflurane). ECG tracings were collected from needle electrodes inserted into the skin with a green needle electrode in the left hind limb, a red needle electrode in the left lower chest and a black needle electrode in the right upper chest. ECG tracings were recorded for 3 mins using a PowerLab 8/35 ADInstruments and LabChart 8 software (ADInstruments, Inc., CO, USA).

3.3.6 PV loop analysis of left ventricular cardiac function: Hemodynamic analysis was conducted in a subset of mice as described previously (Durham et al., 2019; Pacher et al., 2008; Townsend, 2016). Briefly, mice at 5 weeks of age were anesthetized with 5% isoflurane, intubated, and connected to a rodent ventilator (Microvent 1, Hallowell EMC).

Isoflurane (3 %) was used for the maintenance of anesthesia through a 100% oxygen vaporizer. The apex of the heart was exposed via an open-chest surgery, allowing a 1.4-Fr pressure-conductance catheter (PVR-1030; Millar Instruments, Houston, TX, USA) to be inserted transmurally into the left ventricle. After the signal was stabilized, PV loop tracings were recorded. Then, inferior vena cava occlusions were performed by compression of the inferior vena cava. Catheter calibrations were performed by a bolus injection of 30% hypertonic saline into the external jugular vein. Cuvette calibration was performed with fresh heparinized warm blood. The data were analyzed with LabChart 8 software (ADInstruments, Inc., CO, USA).

3.3.7 Euthanasia and tissue and blood collection: Mice were monitored daily from 4 weeks of age until experimental endpoints (5 weeks of age for PV loop analysis or 6.5 weeks of age for tissue collection and analysis) or until a surrogate humane endpoint. Mice were considered to have reached a surrogate humane endpoint and were humanely euthanized when they exhibited the following signs: hunched posture/gait, slow movements in response to gentle stimuli, ruffled fur, and heavy labored breathing. Mice that reached experimental endpoints were fasted for 4 hrs and anesthetized with isoflurane/O_2. Blood for plasma analysis of creatine kinase-myocardial band (CK-MB) and cardiac troponin-I was collected from the submandibular vein into a heparinized tube. For all other analyses, blood was collected by cardiac puncture into a heparinized syringe. Mice were euthanized by thoracotomy. Heparinized blood was centrifuged at 5,500 xg for 10 min and the plasma was recovered and stored at -80 °C prior to analysis. Tissues were perfused in situ with phosphate buffered saline containing 10 U of heparin/ml. Hearts

were excised and cut transversely. The base was frozen in Shandon Cryomatrix (Fisher Scientific, Ottawa, ON, Canada) and the apex was frozen in RNAlater (Invitrogen Life Technologies Inc., Burlington, ON, Canada), and both were stored at -80 °C prior to further analysis. Livers were snap frozen in liquid nitrogen and stored at -80 °C for protein analysis or stored in RNAlater at -80 °C for mRNA analysis. Kidneys and spleens were stored in RNAlater at -80 °C for mRNA analysis. Aortas were harvested and excess tissues and fats were cleaned off under the microscope. Clean aortas were then stored in RNAlater at -80 °C for mRNA analysis.

3.3.8 Preparation of peritoneal macrophages: Peritoneal macrophages were prepared from a subset of mice as described previously (Al-Jarallah et al., 2014b). Mice at 9 weeks of age were injected intraperitoneally with 1 ml of 10% thioglycolate. Four days later, mice were anesthetized with isoflurane/O_2, euthanized by cervical dislocation while under anesthesia, and peritoneal macrophages were collected by flushing the peritoneal cavity with 10 ml of PBS containing 5 mM EDTA. Peritoneal macrophages were washed once in Dulbecco′s Modified Eagle′s Medium (DMEM). Cells were then cultured in DMEM containing 20% FBS with 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin for 2 hours. Peritoneal macrophages were harvested and lysed for mRNA analysis.

3.3.9 Liver total membrane preparation: Liver tissue (0.1 g) in 200 ul of ice-cold homogenization buffer (20mM Tris-HCl pH 7.5, 2mM $MgCl_2$, 0.25 M sucrose with the following protease inhibitors added just before use: 50uM PMSF, 2ug/ml pepstatin A,

5ug/ml leupeptin, and 10 ug/ml aprotinin) was homogenized using an Ultra-Turrax tissue homogenizer. The total liver homogenates were centrifuged at 3000 xg for 10 min at 4 °C to pellet nuclei and cell debris. Then, the supernatants were recovered and subjected to ultracentrifugation at 100,000 x g for 60 min at 4 °C using a Beckman Optima MAX-XP ultracentrifuge with an MLA-130 rotor to pellet total membranes. Membrane pellets were re-suspended in 200 µl of 10 mM sodium phosphate pH 7.0, containing the protease inhibitors listed above at the indicated concentrations and 0.1% SDS. Samples were then snap frozen in liquid nitrogen and stored at -80°C.

3.3.10 SDS – PAGE and immunoblotting: Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Hepatic total membrane protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on either 8% or 12% acrylamide separating gels. Proteins were then transferred onto PVDF membranes in transfer buffer (150 mM glycine, 18 mM Tris-Cl, and 20% methanol). Membranes were blocked with 5% skim milk (Carnation) in Tris-buffered saline (TBS: 137mM NaCl, 2.7 mM KCl, and 24.8 mM Tris-Cl, pH 7.4) for 1 hr at room temperature. They were then incubated with goat anti-LDLR (R&D Systems, AF2255, Minneapolis, MN, USA), rabbit anti-ε-COP (Novus Biologicals, NBP2-38512, Colorado, CO, USA) or mouse anti-Na^+/K^+ ATPase (Upstate Technology, 05-369, Lake Placid, NY, USA) primary antibodies diluted 1:1000 in 3% BSA in TBS at 4°C overnight. Membranes were then washed three times with TBST (TBS plus 0.1% Tween-20) and incubated with horseradish peroxidase (HRP) conjugated rabbit anti-goat (305-035-003), donkey anti-rabbit (711-005-152) or donkey anti-mouse (715-035-150) IgG (Jackson

Immunoresearch Laboratory, West Grove, PA, USA) secondary antibodies diluted 1:5000 in 5% skim milk in TBS for 1 hr at room temperature. Membranes were washed with TBST three times again. HRP was then detected using the Pierce ECL Western Blotting Substrate kit (Thermo Fisher Scientific, Rockford, IL, USA) using a Gel Doc instrument (Bio-Rad Laboratories, Hercules, CA, USA) and of the protein bands were quantified using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

3.3.11 Real time quantitative - PCR: Total RNA was extracted and purified using the RNeasy Mini kit (Qiagen Inc., Toronto, ON, Canada) from tissues stored in RNAlater or fresh cell lysates. RNA concentrations were measured using a Nanodrop ND -8000 spectrophotometer. cDNA synthesis was performed from 1 μg of total RNA using the QuantiTect reverse Transcription Kit (Qiagen Inc., Toronto, ON, Canada). Real-time quantitative PCR was performed using Platinum Sybr Green dye (Invitrogen Life Technologies Inc., Burlington, ON, Canada) in an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the housekeeping gene. Values were compared using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Primer sequences (supplementary table a) were selected using Pubmed's primer-BLAST. All primers were synthesized by Invitrogen Life Technologies (Burlington, ON, Canada).

3.3.12 Plasma protein analysis: Plasma PCSK9, CK-MB, IL-6 and MIP-1β were measured by the mouse proprotein convertase 9 Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA), the mouse creatine kinase MB ELISA Kit (Novus Biologicals, Colorado, CO, USA), the mouse IL-6 ELISA MAX™ Deluxe Set

(BioLegend, San Diego, CA, USA) or the mouse CCL4/MIP-1 beta Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA) respectively according to manufacturer's instructions. An array of 31 plasma cytokines and chemokines was analyzed by Eve Technologies (Calgary, AB, Canada) using the mouse cytokine/chemokine 31-plex discovery assay array (MD31).

3.3.13 Plasma lipid Analysis: Plasma was fractioned by gel filtration fast-protein liquid chromatography (FPLC). 100 µl of plasma was loaded into a Superose 6 10/300 GL column on an AKTA FPLC system (GE Healthcare Life Sciences, ON, Canada). Total cholesterol levels were measured by using the Infinity^{TM} Cholesterol kit (Thermo Fisher Scientific, Ottawa, ON, Canada) from FPLC fractions (100 ul) or whole plasma (1 ul). Plasma triglyceride levels were measured by using the L-Type Triglyceride M Enzyme Color A and B kit (Wako Diagnosis, Richmond, VA, USA). Plasma HDL levels were measured by using the HDL cholesterol E kit (Wako Diagnosis, Richmond, VA, USA). Plasma non-HDL cholesterol levels were calculated as the difference between plasma total cholesterol and plasma HDL-cholesterol for individual samples.

3.3.14 Atherosclerosis and myocardial fibrosis: Transverse cryosections (10 µm) of hearts were stained for lipids with oil red O. The aortic sinus atherosclerotic plaque volume was calculated as the area under the curve of the plaque area versus the distance in the aortic sinus. Coronary artery atherosclerotic burdens were measured in 7 sections (300 µm apart) in the upper portion of the heart, starting from the aortic annulus (0 µm) and moving towards the apex direction (covering 1800 µm). The coronary artery occlusion levels were averaged from those 7 sections. Transverse cryosections (10 µm) of

hearts were also stained for myocardial fibrosis with Masson's Trichrome (Thermo Fisher Scientific, Ottawa, ON, Canada), which stains healthy myocardium red and collagen blue. An Olympus BX 41 microscope with an automated XY stage was used to collect a gallery of images at 4X magnification for each Trichome-stained transverse section. Slidebook 6 software was used to create a composite image of each transverse section. Five sections (300 μm apart) starting from the aortic annulus (0 μm) towards the apical direction (covering 1200 μm) were analyzed to determine the average myocardial fibrosis levels from each mouse.

3.3.15 Lipid accumulation in the heart: Lipid accumulation in the heart was quantified in 10 transverse heart sections (10 μm) stained by oil red O, starting from the aortic annulus (0 μm) and moving towards the apex direction (covering 2700 μm). The total lipid accumulation was calculated as the area under the curve of the lipid accumulation profile (the ratio of the lipid accumulation area to the cross-sectional myocardial area versus distance 0 μm to 2700 μm).

3.3.16 Immunofluorescence staining: 10 μm of transverse heart sections were fixed in 4% formaldehyde for 10 mins and permeabilized in 2:1 ethanol/acetic acid at -20 °C for 5 mins. They were then blocked in either 5% or 10% goat serum in phosphate buffered saline (PBS) at room temperature for 1 hour or overnight at 4 °C. Sections were then incubated with rat anti-Mac-3 (1:50 in 5% goat serum in PBS, BD Biosciences, 553322, Mississauga, ON, Canada), rat anti-CD41 (1:200 in 10% goat serum in PBS, BD biosciences, 553847, Mississauga, ON, Canada), or rabbit anti-CD36 (1:200 in 5% goat serum in PBS, Novus Biologicals, NB400-144, Colorado, CO, USA) primary antibodies

overnight at 4 °C and followed with goat anti-rat Alexa Fluor 647 (1:500 in 5% goat serum in PBS, Thermo Fisher Scientific, A-21247, Mississauga, ON, Canada), goat anti-rat Alexa Fluor 488 (1:500 in 10% goat serum in PBS, Thermo Fisher Scientific, A-11006, Mississauga, ON, Canada), or goat anti-rabbit 594 (1:500 in 5% goat serum in PBS, Thermo Fisher Scientific, A-11012, Mississauga, ON, Canada) secondary antibodies at room temperature for 1 hour. Co-staining for Mac-3 and lipids was conducted by incubating sections with BODIPY 505/515 (1 mg/ml, Invitrogen, D3921, Fisher Scientific, Mississauga, ON, Canada) overnight at 4 °C and followed by rat anti-mouse Mac-3 staining as described above. Co-staining for CD36 and wheat germ agglutinin was conducted by first carrying out CD36 staining as described above followed by incubation of sections with Alexa Fluor 488 conjugated wheat germ agglutinin (1:100 in PBS, Thermo Fisher Scientific, W11261, Mississauga, ON, Canada) at room temperature for 1 hour. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) for 5 min. Fluorescent images were captured using either a Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss Canada Ltd. Toronto, ON, Canada) or a Stellaris 5 inverted confocal microscope (Leica Microsystems, Inc, Concord, Ontario, Canada).

3.3.17 Statistical Analysis: GraphPad Prism 9.4.1 software was used for statistical analysis. Data were subjected to a Grubb's outliner test first and cleaned data were then subjected to different statistical analysis. For the comparison of two groups, data were first subjected to the Shapiro-Wilk normality test. Those that did not pass the normality test were analyzed by the Mann-Whitney Rank Sum test. Those which passed the normality test were then subjected to the F-test for comparing variances. Data that had

significantly different variances were analyzed by the Student's t test with Welch's correction and data that had equal variances were analyzed by the Student's t test. For comparison of multiple groups, data were also first subjected to the Shapiro-Wilk normality test. Data that did not pass the normality test were analyzed by the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. Data that passed the normality test were then subjected to the Brown-Forsythe test for comparing standard deviations. Those that had significantly different standard deviations were analyzed by Welch's ANOVA test with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli post hoc test. Those that had equal standard deviations were analyzed by the ordinary one-way ANOVA with Tukey's post hoc test. Data are presented as mean ± standard deviation. NS: not statistically significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. For the Kaplan-Meier survival curves, data were subject to the log-rank test for significance. Results were considered statistically significant when $P < 0.05$.

3.4 Results:

3.4.1 *Pcsk9* gene deletion in SEKO mice does not alter plasma cholesterol levels, despite increased hepatic LDLR protein levels.

Previous studies have shown that at 5 weeks of age, and fed a normal chow diet from weaning, *Sr-b1*$^{ko/ko}$*ApoE*$^{ko/ko}$ (*Sr-b1/ApoE double knockout*; or SEKO) mice exhibit substantial coronary artery and aortic sinus atherosclerosis and myocardial fibrosis when compared with control *Sr-b1*$^{wt/wt}$*ApoE*$^{ko/ko}$ (*ApoE single KO*, or EKO) mice (Al-Jarallah et al., 2013b; Braun et al., 2002; Yu et al., 2018) . Male 5-week-old SEKO mice have

increased plasma levels of total cholesterol (TC) associated with VLDL-sized lipoproteins when compared to age matched male EKO mice (Supplementary Figure 1 A-B). SEKO mice also had significantly increased hepatic *Pcsk9* mRNA (Figure 1A) and plasma PCSK9 protein (Figure 1B) levels compared age matched male EKO mice. Hepatic *Ldlr* mRNA levels were not statistically significantly different (Figure 1C); however, hepatic LDLR protein levels were lower in SEKO compared to control EKO mice (Figure 1D), consistent with the expected effects of increased plasma PCSK9 protein levels.

These findings led us to hypothesize that elevated PCSK9 levels might contribute to the accelerated aortic sinus and coronary artery atherosclerosis that develops in SEKO mice. To test this, we generated *Pcsk9*$^{ko/ko}$*Sr-b1*$^{ko/ko}$*ApoE*$^{ko/ko}$ (*Pcsk9/Sr-b1/ApoE triple KO*; PSEKO) mice and compared them to matched *Pcsk9*$^{wt/wt}$ *Sr-b1*$^{ko/ko}$*ApoE*$^{ko/ko}$ (SEKO) mice. Since SEKO mice have previously been reported to exhibit reduced life span, we first examined survival of the PSEKO mice compared to matched SEKO and EKO mice. As reported previously (Braun et al., 2002; Karackattu et al., 2006), both female and male SEKO mice exhibited reduced lifespan compared to control EKO mice, with median survival to surrogate endpoint (see methods) of 47 and 46 days for female and male SEKO mice, respectively. In contrast, the control female and male EKO mice all survived past 80 days of age, as shown in Supplementary Figure 2A-B. Female but not male PSEKO mice exhibited somewhat increased survival (Supplementary Figure 2A; median survival of female PSEKO mice 54 days; Supplementary Figure 2B; medial survival of male PSEKO mice 48 days).

As expected, total LDLR protein levels were higher in membranes prepared from liver tissues of PSEKO mice as compared to SEKO mice for both females and males (Figure 1E). Despite the increased LDLR protein levels in liver membranes, there were no differences in plasma lipoprotein TC profiles or plasma TC, HDL-C, non-HDL-C or TG levels (Figure 1F-J). This is consistent with reports demonstrating that an intact apoE-LDLR axis is required for plasma cholesterol lowering mediated by PCSK9 inhibition (Ason, Van Der Hoorn, et al., 2014).

3.4.2 *Pcsk9* gene deletion reduces both aortic sinus and coronary artery atherosclerosis in SEKO mice.

Studies have indicated that PCSK9 might have an influence on systemic inflammatory responses which contribute to atherogenesis in a plasma cholesterol independent manner (Giunzioni et al., 2016c; Ricci et al., 2018b; Z.-H. Tang et al., 2017; Tavori et al., 2016). Therefore, we tested whether knocking out the *Pcsk9* gene in SEKO mice modulated systemic inflammation levels. To compare markers of systemic inflammation in these mice, we screened the levels of 31 cytokines in plasma from the EKO, SEKO and PSEKO mice. While the majority of the cytokines and chemokines analyzed were not different (Supplementary Figure 3), a subset (IL-6, MCP-1, and MIP-1β) were statistically significantly increased in plasma from the SEKO compared to the EKO mice for both females and males (Supplementary Figure 3). However, none of them was significantly reduced in both female and male PSEKO mice compared to corresponding SEKO mice (Supplementary Figure 3). Plasma analysis by ELISA further

demonstrated that knockout of *Pcsk9* did not affect circulating IL-6 or MIP-1β levels in either female or male SEKO mice (Supplementary Figure 4A-B). These results suggest that inactivation of the *Pcsk9* gene does not significantly reduce systemic inflammatory markers in SEKO mice.

Next, we investigated whether genetic *Pcsk9* inactivation in SEKO mice affected atherosclerosis development in the aortic sinus and coronary arteries, despite the absence of effects on plasma cholesterol and systemic inflammatory markers (Figure 1F-J; Supplementary Figure 3-4). Atherosclerotic plaque sizes in the aortic sinuses were analyzed in mice harvested at 6.5 weeks of age. By this age, the SEKO mice develop significant atherosclerosis in the aortic sinus (Figure 2A and E), consistent with previous reports (Al-Jarallah et al., 2013b; Braun et al., 2002, 2003; Karackattu et al., 2006; Yu et al., 2018). The corresponding male and female PSEKO mice developed significantly smaller atherosclerotic plaques in their aortic sinuses (Figure 2A-H). Similarly, both male and female PSEKO mice exhibited significantly lower proportions of coronary artery cross sections containing atherosclerotic plaques, and correspondingly higher proportions of coronary artery cross sections that were devoid of atherosclerosis compared to those in SEKO mouse hearts (Figure 2I-K). In female SEKO mice, 47% ± 9% of coronary artery cross sections were without and 53% ± 9% contained atherosclerotic plaques, whereas in female PSEKO mice, 66% ± 7% of coronary artery cross sections were without and only 34% ± 7% contained atherosclerotic plaques (Figure 2J). Similarly, in male SEKO mice, 41% ± 9% of coronary artery cross sections were without and 59% ± 9% contained

atherosclerotic plaques, whereas in male PSEKO mice, on average 57% ± 11% of coronary artery cross sections were without and 43% ± 11% contained atherosclerotic plaques (Figure 2K). However, knockout of *Pcsk9* in SEKO mice did not change the relative macrophage content of atherosclerotic plaques in either the aortic sinus (Supplementary Figure 5A-B) or coronary arteries (Supplementary Figure 5C-D).

We have previously reported platelet accumulation in coronary artery atherosclerotic plaques of SEKO mice (Yu et al., 2018). Platelet activation and accumulation is involved in both early- stages of atherogenesis and later- stages of advanced atherothrombosis. Studies have reported that PCSK9 regulates platelet activation and aggregation (Cammisotto et al., 2020; Qi et al., 2021b). Therefore, we tested whether knocking out the *Pcsk9* gene in SEKO mice affected platelet accumulation in coronary artery atherosclerotic plaques. We observed that the number of atherosclerotic coronary artery cross sections that were positive for CD41 (a marker for platelet accumulation) among the number of total atherosclerotic coronary artery cross sections, was not different between PSEKO and SEKO mice (Supplementary Figure 6A-C), suggesting platelet accumulation in coronary artery atherosclerotic plaques was not directly impacted by knocking out *Pcsk9* in SEKO mice.

3.4.3 *Pcsk9* gene deletion in SEKO mice reduces cardiac enlargement, myocardial fibrosis and left ventricular (LV) dysfunction.

SEKO mice develop cardiac enlargement, myocardial fibrosis and LV dysfunction, likely as a consequence of occlusive coronary artery atherosclerosis (Braun et al., 2002). Since we found that PSEKO mice exhibited reduced coronary artery atherosclerosis, we evaluated these parameters in the PSEKO and control SEKO mice. The heart weights (normalized to tibia lengths) were increased in both female and male SEKO mice at 6.5 weeks of age, compared to corresponding EKO mice (Figure 3A-B), suggesting cardiac enlargement in SEKO mice. Knockout of *Pcsk9* in SEKO mice did not change body weights (Supplementary Figure 7A-B); however, it significantly decreased heart weight: tibia length ratios in both females and males (Figure 3A-B), indicating *Pcsk9* inactivation reduced cardiac enlargement in SEKO mice. Quantification of collagen (blue) in trichrome-stained cross sections of hearts (Figure 3C-E; F-H), revealed a statistically significant reduction of cardiac fibrosis in both female and male PSEKO mice compared to corresponding SEKO mice. Similarly, we observed reduced plasma levels of cardiac damage marker CK-MB in both female and male PSEKO mice compared to corresponding SEKO mice (Figure 3I and J).

Lipid accumulation has previously been observed throughout the hearts of SEKO mice (Al-Jarallah et al., 2013b; Braun et al., 2002). Consistent with previous reports (Al-Jarallah et al., 2013b; Braun et al., 2002), oil red O staining revealed neutral lipid accumulation in the hearts from 5-week-old SEKO mice, particularly in regions closer to the base and this was reduced in PSEKO mice lacking *Pcsk9* (Figure 3K-M; Supplementary Figure 8). Consistent with previous reports [42,43], the majority of lipid

deposition, detected by BODIPY staining co-localized with macrophages as detected by Mac-3 immunofluorescence staining in the hearts of SEKO mice (Supplementary Figure 9). Analysis of Mac-3 staining revealed that hearts from both female and male PSEKO mice also had significantly decreased macrophage abundance than those from corresponding SEKO mice (Figure 3N-O).

The incidence of ECG abnormalities, including ST segment depressions and elevations, indicative of non-transmural and transmural myocardial ischemia, and missing QRS complexes, reminiscent of second-degree heart block, appeared to be diminished in both female and male PSEKO mice compared to SEKO mice at 6.5 weeks of age (Supplementary Figure 10A-B). No abnormal ECG patterns were detected in similarly aged control EKO mice (Supplementary Figure 10A-B). Analysis of cardiac LV pressure-volume relationships (Figure 3P-Q) revealed that LV-ejection fractions were substantially reduced in SEKO mice compared to control EKO mice, as reported previously (Braun et al., 2002). LV ejection fractions were improved in age matched PSEKO compared to SEKO mice (Figure 3Q). Similarly, we observed worsened cardiac output, end systolic volume, dP/dt_{max} and $-dP/dt_{min}$ in SEKO compared to EKO mice and trends (which did not reach statistical significance) towards improvements in cardiac output and end systolic volume in PSEKO mice (Supplementary Figure 11). This is consistent with *Pcsk9* knockout having attenuated the degree of myocardial fibrosis and left ventricular dysfunction although they were not fully corrected in the PSEKO mice.

The extent of reduction in myocardial fibrosis appeared unexpectedly high (Figure 3E and H; 38% for females and 49% for males), given the relatively modest reduction in coronary artery atherosclerosis by *Pcsk9* KO (Figure 2J and K; 36% for females and 27% for males). To test if *Pcsk9* KO might have direct effects on the heart, we examined cardiac CD36 protein levels in EKO, SEKO and PSEKO mice. Immunofluorescence staining revealed that CD36 appeared localized to the cell surface of cells in the heart and that levels were increased in hearts of SEKO compared to EKO mice, and that levels were further increased by knockout of *Pcsk9* in PSEKO mice (Supplementary Figure 12).

3.4.4 Restoring circulating PCSK9 by AAV8-PCSK9 treatment reverses CAD in PSEKO mice.

To determine if restoring circulating PCSK9 in PSEKO mice to levels similar to those in SEKO mice might reverse some or all of the protective effects of *Pcsk9* knockout, a recombinant AAV8 vector was used to direct mouse wild type *Pcsk9* expression in the liver under the control of a liver specific *hcrApoE/hAAT1* promoter (AAV8-PCSK9). As a control, we used an AAV8 to drive liver specific expression of *eGFP* under the same *hcrApoE/hAAT1* promoter (AAV8-eGFP).

Titration of the viral dose administered to PSEKO mice (data not shown) revealed that intravenous injection of 4×10^{10} GC of the AAV8-PCSK9 resulted in circulating PCSK9 levels (average 76 ± 66 ng/ml) in PSEKO mice that approached those measured in SEKO mice (164 ± 50 ng/ml) (Figure 4A). As expected, PCSK9 was undetectable in

plasma from either uninfected or AAV8-eGFP infected PSEKO mice. PSEKO mice infected with AAV8-PCSK9 exhibited significantly reduced hepatic LDLR protein levels than those infected with AAV8-eGFP (Figure 4B) but plasma total cholesterol levels were unaffected (Figure 4C), in agreement with the results from whole body *Pcsk9* genetic deficiency in SEKO mice (Figure 1G). Quantitative real time RT-PCR for *Pcsk9* expression using mRNA prepared from a variety of tissues as well as peritoneal macrophages confirmed that the recombinant AAV8-PCSK9 used drove liver specific *Pcsk9* expression (Figure 4D and E).

Restoration of circulating PCSK9 by AAV8-PCSK9 treatment in PSEKO mice significantly increased atherosclerotic plaque development in the aortic sinus (Figure 5A-D) and increased the proportions of coronary artery cross sections containing atherosclerotic plaques and correspondingly decreased the proportions of coronary artery cross sections that were devoid of atherosclerosis (Figure 5E). At 6.5 weeks of age, 61% ± 5% of coronary artery cross sections were without atherosclerotic plaques, and 39% ± 5% of coronary artery cross sections contained atherosclerotic plaques in PSEKO mice treated with the control AAV8-eGFP virus (Figure 5E). On the other hand, 50% ± 6% of coronary artery cross sections were without and 50% ± 6% contained atherosclerotic plaques in PSEKO mice treated with the AAV8-PCSK9 virus (Figure 5E). Therefore, restoring circulating PCSK9 by AAV8-PCSK9 treatment reversed the protective effects of whole body *Pcsk9* genetic deficiency on both aortic sinus and coronary artery atherosclerosis in a plasma cholesterol-independent manner.

Unexpectedly, PSEKO administered either AAV8-eGFP or AAV8-PCSK9 exhibited heart weights (normalized to tibia lengths) that were larger than uninfected PSEKO mice but were similar to those measured for SEKO mice (Figure 5F compared to Figure 3A and B), suggesting that treatment with the AAV8 virus alone triggered cardiomegaly in these mice. There we could not evaluate if restoring circulating PCSK9 per se reversed the protection against cardiomegaly observed when *Pcsk9* was knocked out. Nevertheless, AAV8-PCSK9 virus treatment significantly increased myocardial fibrosis (Figure 5H-J), plasma CK-MB levels (Figure 5K), and lipid deposition in the heart (Figure 5L-N; Supplementary Figure 13) compared to control AAV8-eGFP virus treatment in PSEKO mice. However, AAV8-PCSK9 treatment did not change myocardial macrophage accumulation (Figure 5O-P) or cell surface CD36 protein levels (Supplementary Figure 14) in the hearts compared to AAV8-eGFP treatment in either female or male PSEKO mice. Nevertheless, it increased the proportion of abnormal ECG patterns (Supplementary Figure 15), indicating restoring circulating PCSK9 enhanced abnormal myocardial electrical conduction in PSEKO mice. Thus, AAV8-PCSK9 virus infection restored circulating PCSK9 and reversed most of the protective effects of *Pcsk9* KO on atherosclerosis in the aortic sinus and coronary arteries, myocardial fibrosis and cardiac damage in PSEKO mice in a plasma cholesterol independent manner.

3.4.5 PCSK9 antibody treatment protects males but not females against coronary artery atherosclerosis development but does not protect against myocardial fibrosis in SEKO mice.

Starting from 3 weeks of age, female and male SEKO mice were treated weekly (10 mg/kg body weight in 0.9% saline) with either a control antibody or an anti-PCSK9 antibody that disrupts the interaction between PCSK9 and the LDLR. Analysis of mice at 6.5 weeks of age revealed that treatment of both female and male SEKO mice with the anti-PCSK9 antibody substantially increased hepatic LDLR protein levels (Figure 6B-C), despite dramatically increasing plasma PCSK9 levels (Figure 6A) as previously reported by others (Byun, Lebeau, Platko, Carlisle, Faiyaz, Chen, MacDonald, et al., 2022; Koren et al., 2015; Oleaga et al., 2021; Ridker, Tardif, et al., 2017; E. M. Roth et al., 2012; Shapiro et al., 2018; L. Zhang, McCabe, Condra, Ni, Peterson, Wang, Strack, Wang, Pandit, Hammond, Wood, Lewis, Rosa, Mendoza, Cumiskey, et al., 2012). Anti-PCSK9 antibody treatment did not affect body weights (Supplementary Figure 16), plasma or lipoprotein total cholesterol levels, or plasma TG levels in SEKO mice (Figure 6D-F), consistent with the results from genetic PCSK9 inactivation in SEKO mice (Figure 1F-J). The anti-PCSK9 antibody treatment of SEKO mice significantly reduced atherosclerosis development in the aortic sinus (Figure 7A, B, E, and F) and coronary arteries (Figure 7I) in males but not in females (Figure 7C, D, G, H and J). Despite the reduction in aortic sinus and coronary artery atherosclerosis in male SEKO mice, anti-PCSK9 antibody treatment did not affect myocardial lipid accumulation in male SEKO mice (Supplementary Figure 17). Furthermore, it did not protect either male or female SEKO

mice against cardiac enlargement (Figure 8A and B), myocardial fibrosis (Figure 8C-H), or increased plasma CK-MB levels (Figure 8I and J) or reduce cell surface CD36 protein levels in hearts (Supplementary Figure 18). Similarly, anti-PCSK9 antibody treatment did not prevent LV dysfunction measured in 5 week old male or female SEKO mice (Figure 8K-L; Supplementary Figure 19) or abnormal ECG patterns (data not shown) compared to control antibody treatment in SEKO mice. The results suggest that the effects of PCSK9 on coronary artery atherosclerosis in females and on cardiomyocyte damage, cardiac fibrosis and LV dysfunction, or in the regulation of cardiac cell surface CD36 protein levels in both males and female SEKO mice are not inhibited by the anti-PCSK9 antibody and therefore likely independent of PCSK9's interaction with the LDLR.

3.5 Discussion:

Because SEKO mice lack APOE, a ligand for the LDLR, and others have shown that PCSK9 inhibition did not regulate plasma cholesterol levels in mice lacking either the LDLR or APOE (Ason, Van Der Hoorn, et al., 2014), we reasoned that SEKO mice might represent a useful model system in which to examine the role of PCSK9 inhibition in coronary artery atherosclerosis independent of its effects on plasma cholesterol lowering. We demonstrate that SEKO mice exhibit increased hepatic expression and plasma levels of PCSK9 compared to control EKO mice (Figure 1A-B). By crossing these mice to *Pcsk9* KO mice, we generated PSEKO mice which lack *Pcsk9* expression. These mice show elevated levels of LDLR expression in livers compared to *Pcsk9* expressing SEKO mice but do not exhibit differences in plasma cholesterol levels or lipoprotein

profiles (Figure 1E-J), in agreement with the finding that PCSK9 mediated regulation of circulating cholesterol levels requires APOE (Ason, Van Der Hoorn, et al., 2014). *Pcsk9* knockout in these SEKO mice reduced atherosclerosis development (Figure 2A-K) in both the aortic sinus and coronary arteries as well as the development of myocardial fibrosis (Figure 3C-H), plasma levels of a cardiac damage marker (CK-MB) (Figure 3I-J) and cardiac enlargement (Figure 3A-B), and improved parameters of left ventricular function (Figure 3P-Q) in both male and female mice. Knockout of the *Pcsk9* gene also extended the survival of female but not male PSEKO mice (Supplementary Figure 2A-B).

The recent observation that *Pcsk9* KO mice are protected against myocardial infarction in a surgical model of left coronary artery ligation (Ding, Wang, et al., 2018) is in close agreement with our findings of reduced myocardial fibrosis and reduced left ventricular dysfunction in PSEKO compared to SEKO mice. In that study, it was reported that PCSK9 levels were increased in the ischemic border zone in the affected myocardium (Ding, Wang, et al., 2018). However, it is not clear if the protection afforded by *Pcsk9* KO was a consequence of the absence of liver derived, circulating PCSK9 or the absence of expression of PCSK9 in the heart itself. To test the involvement of liver derived, circulating PCSK9 in the development of atherosclerosis in the aortic sinus and coronary arteries, myocardial fibrosis and myocardial damage, we used an AAV8 vector to express recombinant *Pcsk9* specifically in livers and not other tissues/cell types, including macrophages and heart tissue, leading to near-restoration of plasma levels of PCSK9 in PSEKO mice. This increased atherosclerosis in both the aortic sinus and coronary arteries (Figure 5A-E) and exacerbated myocardial fibrosis, myocardial damage and myocardial

lipid accumulation in PSEKO mice (Figure 5H-N; Supplementary Figure 13), effectively reversing the effects of knocking out *Pcsk9*. Like the effects of *Pcsk9* KO, restoration of *Pcsk9* expression specifically in livers did not affect plasma cholesterol levels (Figure 4C). These results strongly suggest that the absence of liver derived, circulating PCSK9 is responsible for the lipoprotein-independent protective effects on coronary artery atherosclerosis, myocardial infarction and lipid deposition, and LV dysfunction in PSEKO mice.

Evaluation of plasma markers of inflammation revealed a subset (IL-6, MCP-1 and MIP-1β) that were elevated in both male and female SEKO compared to EKO control mice (Supplementary Figure 3 and 4). However, none of these were consistently reduced by *Pcsk9* KO in both male and female PSEKO mice (Supplementary Figure 3 and 4) or increased by restoration of circulating PCSK9 in PSEKO mice (data not shown). These results suggest that the plasma cholesterol independent effects of PCSK9 on atherosclerosis development, myocardial damage and myocardial fibrosis are not due to alterations in these systemic inflammatory factors. A recent study has shown that overexpression of circulating PCSK9 by AAV8 promoted the plasma cholesterol independent development of vein graft lesions (Katsuki et al., 2022), the late stage of which shares similar characteristics with atherosclerosis (Yahagi et al., 2016). These results are in close alignment with our finding that circulating PCSK9 promoted atherosclerosis in both the aortic sinus and coronary arteries independently of plasma cholesterol levels (Figure 4A and C; Figure 5A-E). Macrophage accumulation was suggested as the mechanism of circulating PCSK9 induced vein graft lesions (Katsuki et

al., 2022); whereas our study suggested the protection against atherosclerosis by *Pcsk9* KO was not driven by macrophage accumulation in atherosclerotic plaques (Supplementary Figure 5). We also found that platelet accumulation was not the driver of global *Pcsk9* KO mediated protection against coronary artery atherosclerosis (Supplementary Figure 6). PCSK9 inhibition in cultured SMCs and ECs has also been reported eliciting anti-atherogenic responses (Xiong et al., 2021). However, the extent to which circulating PCSK9 may impact these responses and their contribution to the mechanism by which eliminating circulating PCSK9 triggers plasma cholesterol-independent protection against aortic sinus and coronary artery atherosclerosis, myocardial fibrosis and myocardial damage remains to be explored. PCSK9 has been reported to mediate the degradation of MHC-I and reduce intratumoral $CD8^+$ T cell infiltration (X. Liu et al., 2020). $CD8^+$ T cells are reported to have both pro-atherogenic and atheroprotective effects (Saigusa et al., 2020b). It is possible that circulating PCSK9 might also regulate MHC-I levels in atherosclerotic plaques and affect $CD8^+$ T cell infiltration, which might contribute to its effects on atherosclerosis development. However, this hypothesis needs to be explored.

To understand whether the plasma cholesterol independent effects of circulating PCSK9 on coronary artery atherosclerosis and myocardial fibrosis involve its interactions with the LDLR, we treated SEKO mice with an anti-PCSK9 antibody, which disrupts the PCSK9-LDLR interaction (Ason, Van Der Hoorn, et al., 2014). This elevated hepatic LDLR protein levels in SEKO mice (Figure 6B and C). However, it also increased circulating PCSK9 levels by ~10 fold (Figure 6A), consistent with previous reports which

indicated that this was due to both delayed LDLR mediated PCSK9 clearance and a post-translational increase in PCSK9 secretion (Byun, Lebeau, Platko, Carlisle, Faiyaz, Chen, MacDonald, et al., 2022; Oleaga et al., 2021). Consistent with the results of *Pcsk9* KO, anti-PCSK9 antibody treatment of SEKO mice did not alter plasma cholesterol levels (Figure 6D). Nevertheless, the increased hepatic LDLR and circulating PCSK9 protein levels (Figure 6A-C) demonstrated that the anti-PCSK9 antibody treatment in SEKO mice successfully blocked the PCSK9-LDLR interactions.

Anti-PCSK9 antibody treatment protected male but not female SEKO mice against atherosclerosis in both the aortic sinus and coronary arteries (Figure 7A-J). The reason for this sex-dependent effect is not clear. However, the mice used in this study were harvested by 6.5 weeks of age and likely sexually immature. Therefore, the sex-dependent effects of PCSK9-antibody treatment on atherosclerosis are likely not mediated by sex hormones. The nature of the different effects in males versus females remains to be determined. In contrast, anti-PCSK9 antibody treatment did not attenuate myocardial fibrosis, plasma CK-MB levels or LV dysfunction in either male or female SEKO mice (Figure 8A-L). In female SEKO mice, this is consistent with the lack of protection against the extent of coronary artery atherosclerosis (Figure 7J, 8B, 8F-H, and 8J-L). However, the lack of protection against myocardial fibrosis or damage, or LV dysfunction observed in male mice (Figure 8A, 8C-E, 8I, and 8K-L) was unexpected given the observed, though modest, ~20% reduction in the extent of coronary artery atherosclerosis (Figure 7I). This suggests either that the 20 % reduction in coronary artery atherosclerosis observed in male SEKO mice was not sufficient to impact myocardial damage, fibrosis or

LV dysfunction, or that PCSK9 may exert an LDLR-independent effect on the myocardium. Consistent with this, we noted that *Pcsk9* KO in PSEKO mice resulted in what appeared to be greater protection against myocardial fibrosis than against coronary artery atherosclerosis and that AAV8-mediated hepatic expression of recombinant *Pcsk9* in PSEKO mice resulted in greater increases in the myocardial damage marker, CK-MB, and myocardial fibrosis than in coronary artery atherosclerosis. These observations are consistent with the previous demonstration that *Pcsk9* KO protected mice against myocardial fibrosis in a coronary artery ligation experimental MI model (Ding, Wang, et al., 2018; F. Wang et al., 2022).

CD36 is a known target of PCSK9 regulation (Seidah & Garçon, 2022) that plays an important role in cardiomyocytes. We observed that *Pcsk9* KO was accompanied by increased cell surface CD36 protein levels in cardiac cells (Supplementary Figure 12). However, neither AAV8-mediated expression of recombinant *Pcsk9* in PSEKO mice (Supplementary Figure 14), nor anti-PCSK9 antibody treatment of SEKO mice (Supplementary Figure 18) affected cell surface CD36 protein levels in hearts, suggesting that cell surface CD36 protein levels in hearts might not be regulated by circulating PCSK9. Further studies are required to determine if PCSK9 expressed in the cardiomyocytes or other cells in the heart, itself, might be responsible for the regulation of cardiac cell surface CD36 levels. Regardless, however, it does not appear that PCSK9's effects on myocardial damage or fibrosis are mediated through effects on cardiac cell surface CD36 protein levels, since AAV8-mediated hepatic expression of recombinant *Pcsk9* reversed the protection against myocardial damage and fibrosis (Figure 5H-K) but

did not alter myocardial cell surface CD36 levels (Supplementary Figure 14). The mechanisms by which circulating PCSK9 may exert direct effects on the heart to modulate myocardial damage, myocardial fibrosis, and myocardial macrophage and lipid accumulation remain unknown.

A study in human patients has shown that lipid deposition in the myocardium of the LV was associated with larger infarctions and worse LV function (Goldfarb et al., 2009). Another study reported that lipid deposition was associated both with cardiomyocytes and with macrophages in infarcted hearts of human patients (Sakurai et al., 1978). Consistent with these and other analyses (Al-Jarallah et al., 2013b; Braun et al., 2002), we observed extensive lipid deposition, associated primarily with macrophages in the myocardium of SEKO mice (Supplementary Figure 9) and reduced myocardial lipid deposition and cardiac macrophage content in PSEKO mice (Figure 3K-O; Supplementary Figure 8). These alterations corresponded to the attenuated cardiac fibrosis and damage (Figure 3C-J) and improved LV function (Figure 3P-Q; Supplementary Figure 11) in PSEKO compared to SEKO mice. Whether the reduced myocardial lipid deposition and macrophage accumulation are the cause or consequence of reduced cardiac fibrosis and damage and LV dysfunction in PSEKO mice, is unknown. However, one study has reported that *Pcsk9* KO mice were protected from coronary artery ligation induced myocardial infarction, potentially by regulating myocardial macrophage accumulation and polarization (F. Wang et al., 2022). Interestingly, restoration of circulating PCSK9 by AAV8-mediated liver specific *Pcsk9* expression increased myocardial lipid deposition but did not change cardiac macrophage content in

PSEKO mice compared to those which received the control AAV8-eGFP virus (Figure 5L-P; Supplementary Figure 13). It is possible that the recombinant AAV8 virus treatment alone induced local inflammation and macrophage accumulation in the heart in addition to triggering cardiomegaly (Figure 5F compared to Figure 3A and B), since it is known that this AAV strain exhibits tropism for cardiomyocytes; thus, the effects of restoring circulating PCSK9 on macrophage levels in the heart and heart size might be masked by the AAV8 virus treatment. Nevertheless, the increased myocardial lipid accumulation by restoring circulating PCSK9 was associated with enhanced myocardial fibrosis and damage in PSEKO mice (Figure 5H-N) and anti-PCSK9 antibody treatment did not affect either myocardial fibrosis and damage or myocardial lipid accumulation in male SEKO mice (Figure 8A, 8C-E, and 8I; Supplementary Figure 17). These results suggest that despite the absent effects on systemic, plasma lipid levels (Figure 1F-J and 4C), circulating PCSK9 affects local lipid accumulation in the heart that is independent of the interaction between the LDLR and PCSK9. It is possible that circulating PCSK9 may alter local myocardial lipid accumulation by regulating the levels of another receptor. Our data suggests that this is not CD36 on the surface of cardiac cells since restoration of circulating PCSK9 by AAV8-PCSK9 treatment reduced myocardial lipid accumulation without impacting myocardial cell surface CD36. The nature of the PCSK9 target, the cell type affected (cardiomyocyte versus macrophage) and the role of myocardial lipid accumulation in the progression of myocardial fibrosis and damage and LV dysfunction remain to be elucidated.

In conclusion, we have revealed that the absence of *Pcsk9* protected against coronary artery atherosclerosis, myocardial damage and fibrosis, and LV dysfunction in SEKO mice in a plasma cholesterol independent manner, and that the effects on atherosclerosis, myocardial damage and fibrosis can be reversed by hepatic expression of recombinant *Pcsk9*, leading to near-normal physiological levels of PCSK9 in circulation. Importantly, these effects appear to be largely independent of LDLR binding, particularly in females. The mechanisms by which circulating PCSK9 exerts these effects remain to be elucidated, however these findings suggest that strategies that target PCSK9 protein expression/secretion, particularly by the liver may have beneficial, plasma cholesterol-independent effects in reducing coronary artery atherosclerosis and associated myocardial infarction.

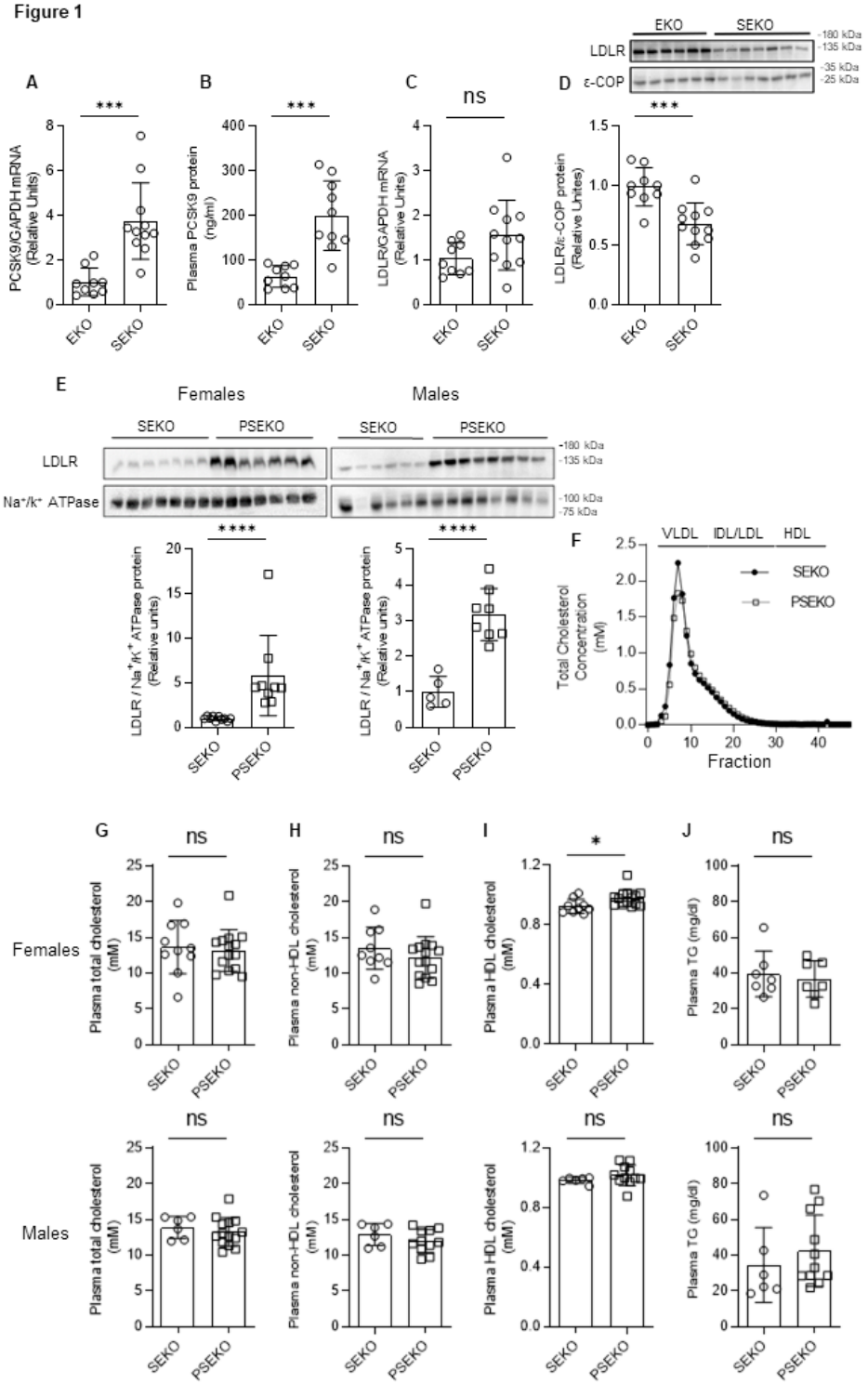


Figure 1
A
B
C
D
EKO
SEKO
LDLR
ε-COP
180 kDa
135 kDa
35 kDa
25 kDa
PCSK9/GAPDH mRNA (Relative Units)
Plasma PCSK9 protein (ng/ml)
LDLR/GAPDH mRNA (Relative Units)
LDLR/ε-COP protein (Relative Unites)
ns
***
E
Females
Males
SEKO
PSEKO
LDLR
Na+/k+ ATPase
100 kDa
75 kDa
****
LDLR / Na+/k+ ATPase protein (Relative units)
F
VLDL
IDL/LDL
HDL
Total Cholesterol Concentration (mM)
Fraction
G
H
I
J
Females
Males
Plasma total cholesterol (mM)
Plasma non-HDL cholesterol (mM)
Plasma HDL cholesterol (mM)
Plasma TG (mg/dl)
*

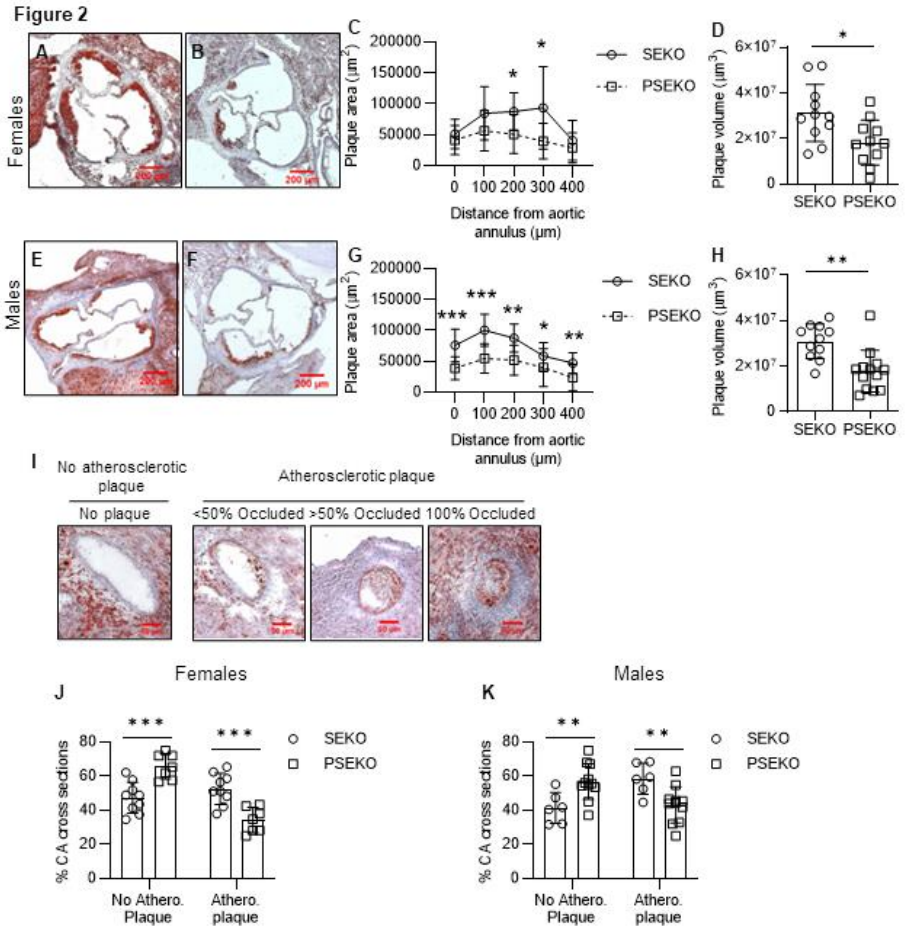


Figure 2
A
B
C
D
Females
Plaque area (μm²)
Plaque volume (μm³)
SEKO
PSEKO
Distance from aortic annulus (μm)
E
F
G
H
Males
I
No atherosclerotic plaque
Atherosclerotic plaque
No plaque
<50% Occluded
>50% Occluded
100% Occluded
Females
Males
J
K
% CA cross sections
No Athero. Plaque
Athero. plaque

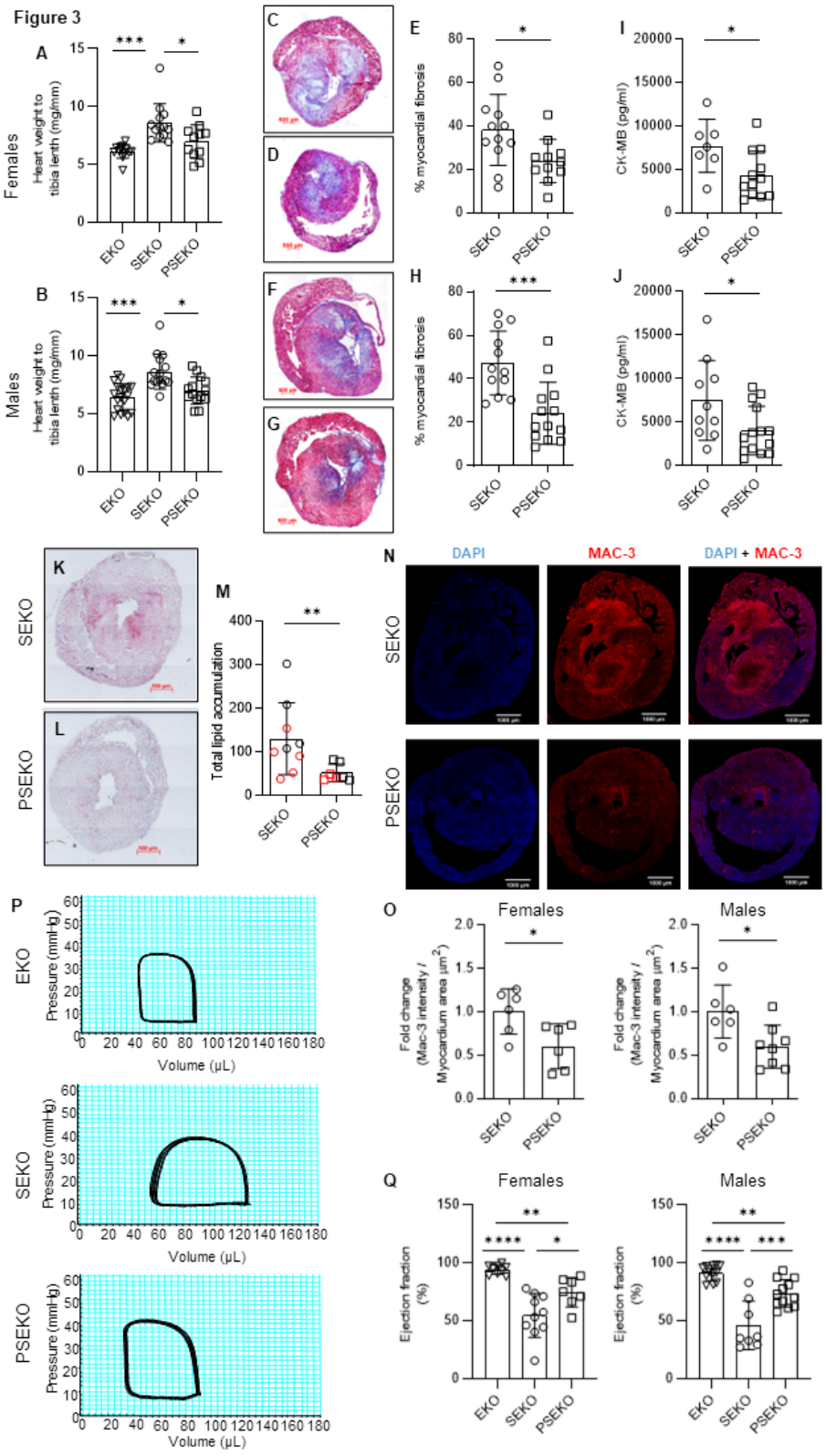


Figure 3
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
Females
Males
Heart weight to tibia lenth (mg/mm)
EKO
SEKO
PSEKO
% myocardial fibrosis
CK-MB (pg/ml)
Total lipid accumulation
DAPI
MAC-3
DAPI + MAC-3
Pressure (mmHg)
Volume (μL)
Fold change (Mac-3 intensity / Myocardium area μm²)
Ejection fraction (%)

Figure 4

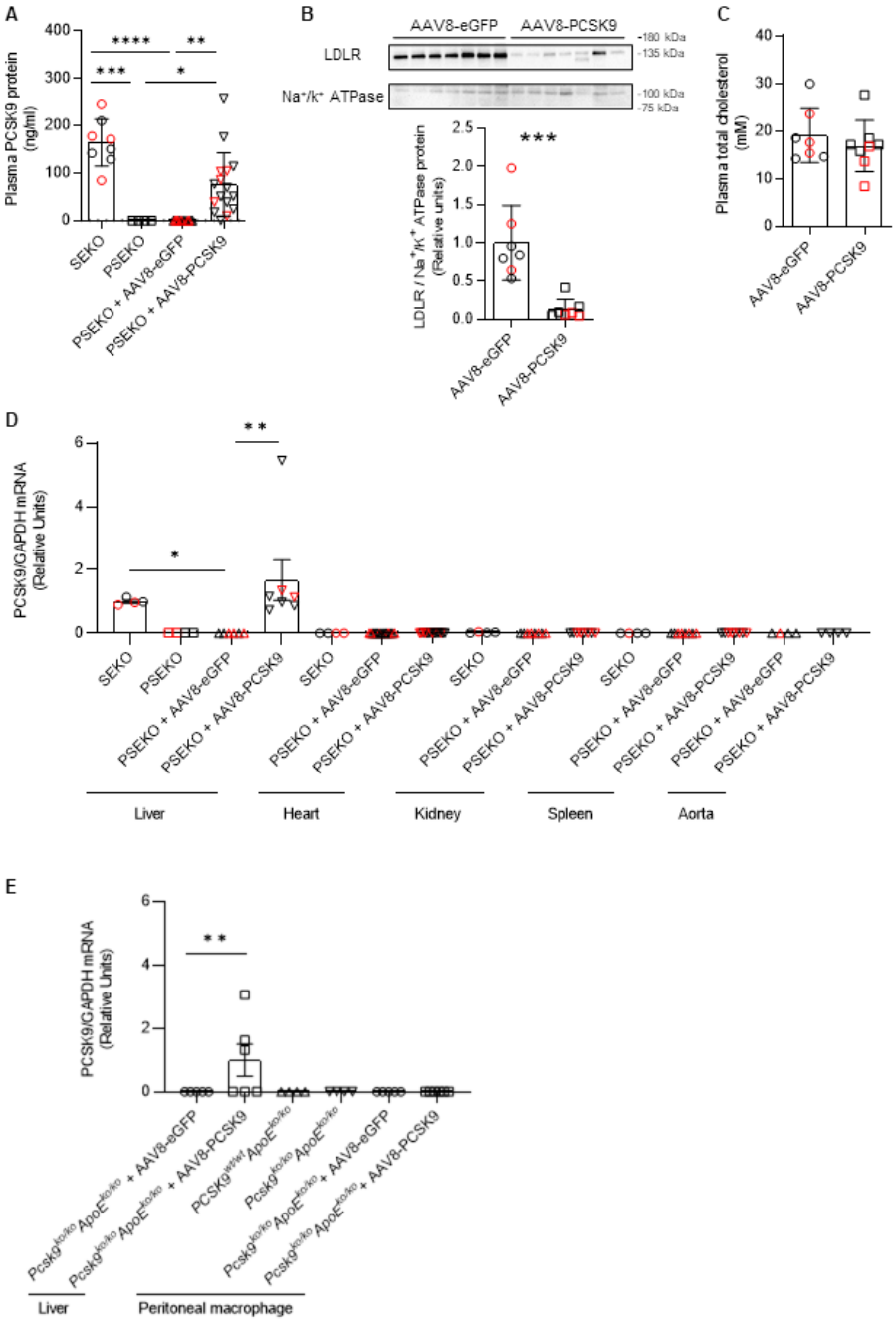

A
Plasma PCSK9 protein (ng/ml)
SEKO
PSEKO
PSEKO + AAV8-eGFP
PSEKO + AAV8-PCSK9
B
AAV8-eGFP
AAV8-PCSK9
LDLR
Na+/k+ ATPase
LDLR / Na+/K+ ATPase protein (Relative units)
C
Plasma total cholesterol (mM)
D
PCSK9/GAPDH mRNA (Relative Units)
Liver
Heart
Kidney
Spleen
Aorta
E
PCSK9/GAPDH mRNA (Relative Units)
Liver
Peritoneal macrophage

Figure 5

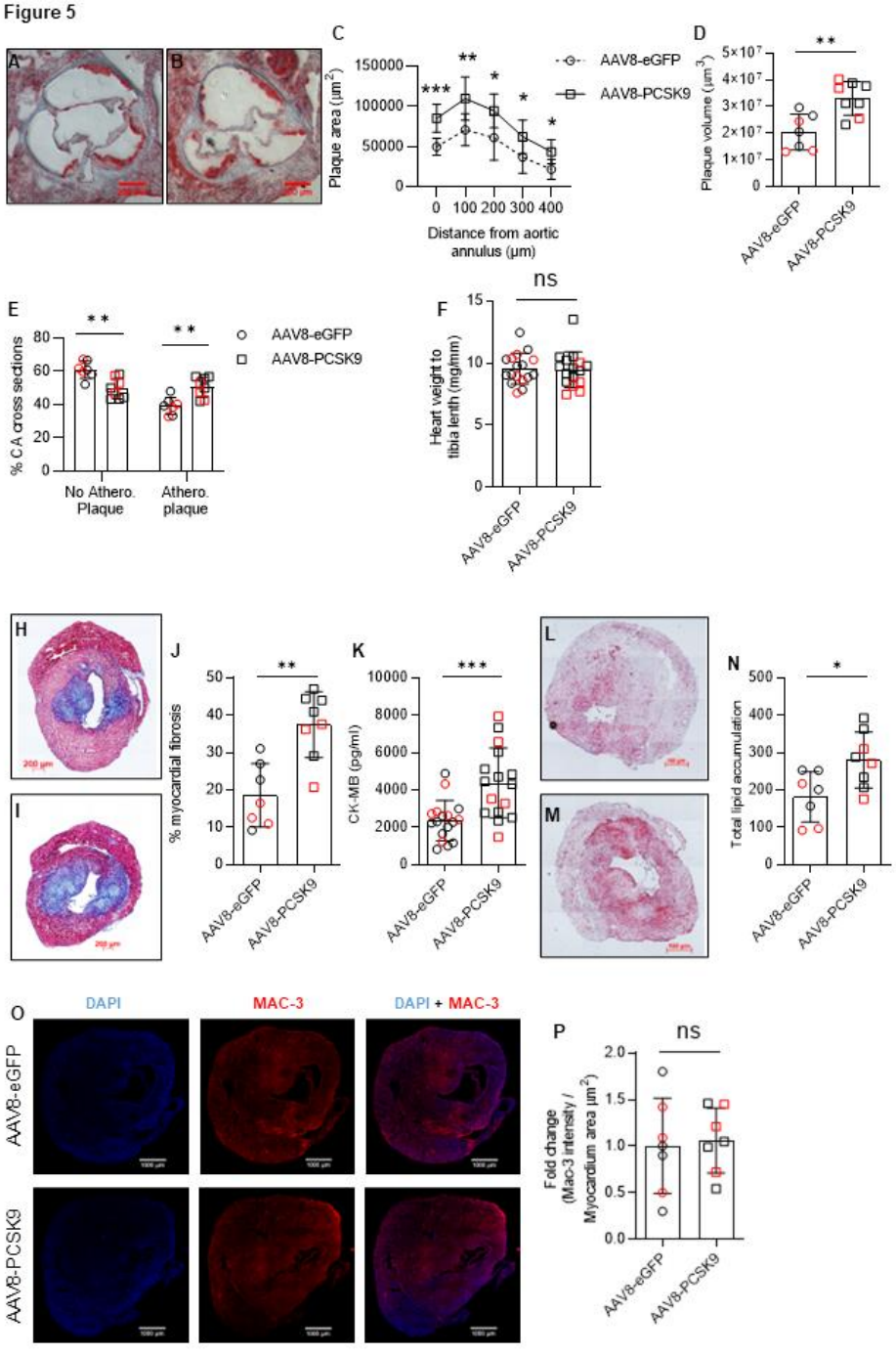
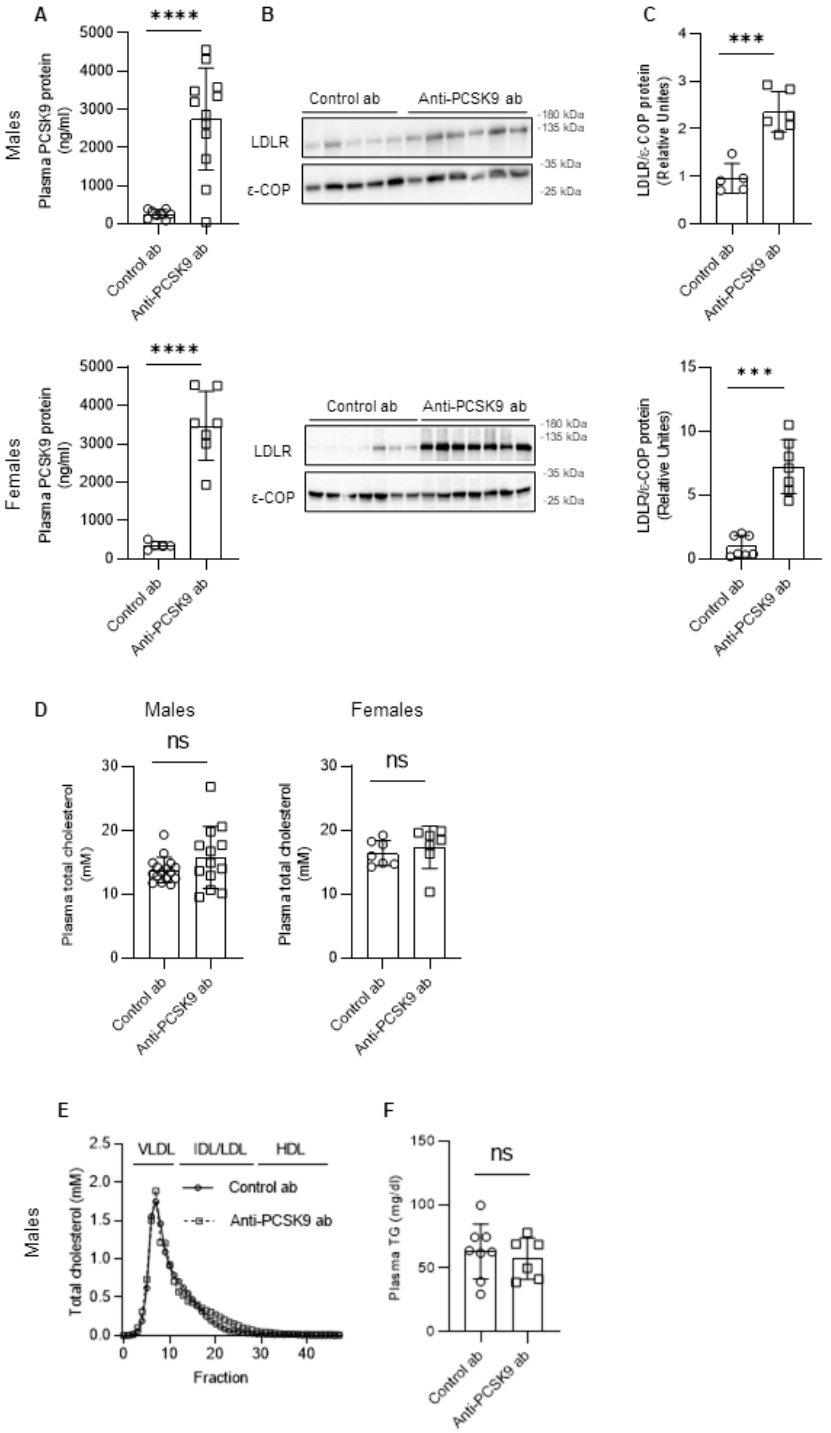

A
B
C
Plaque area (μm²)
150000
100000
50000
0
***
**
*
*
*
AAV8-eGFP
AAV8-PCSK9
0 100 200 300 400
Distance from aortic annulus (μm)
D
Plaque volume (μm³)
5×10⁷
4×10⁷
3×10⁷
2×10⁷
1×10⁷
0
**
AAV8-eGFP
AAV8-PCSK9
E
% CA cross sections
80
60
40
20
0
**
**
AAV8-eGFP
AAV8-PCSK9
No Athero. Plaque
Athero. plaque
F
ns
Heart weight to tibia lenth (mg/mm)
15
10
5
0
AAV8-eGFP
AAV8-PCSK9
H
I
J
% myocardial fibrosis
50
40
30
20
10
0
**
AAV8-eGFP
AAV8-PCSK9
K
CK-MB (pg/ml)
10000
8000
6000
4000
2000
0
***
AAV8-eGFP
AAV8-PCSK9
L
M
N
Total lipid accumulation
500
400
300
200
100
0
*
AAV8-eGFP
AAV8-PCSK9
O
DAPI
MAC-3
DAPI + MAC-3
AAV8-eGFP
AAV8-PCSK9
P
ns
Fold change (Mac3 intensity / Myocardium area (μm²))
2.0
1.5
1.0
0.5
0.0
AAV8-eGFP
AAV8-PCSK9

Figure 6


A
B
C
Males
Females
Plasma PCSK9 protein (ng/ml)
****
Control ab
Anti-PCSK9 ab
LDLR
ε-COP
180 kDa
135 kDa
35 kDa
25 kDa
LDLR/ε-COP protein (Relative Unites)
***
D
Plasma total cholesterol (mM)
ns
E
VLDL
IDL/LDL
HDL
Total cholesterol (mM)
Fraction
F
Plasma TG (mg/dl)

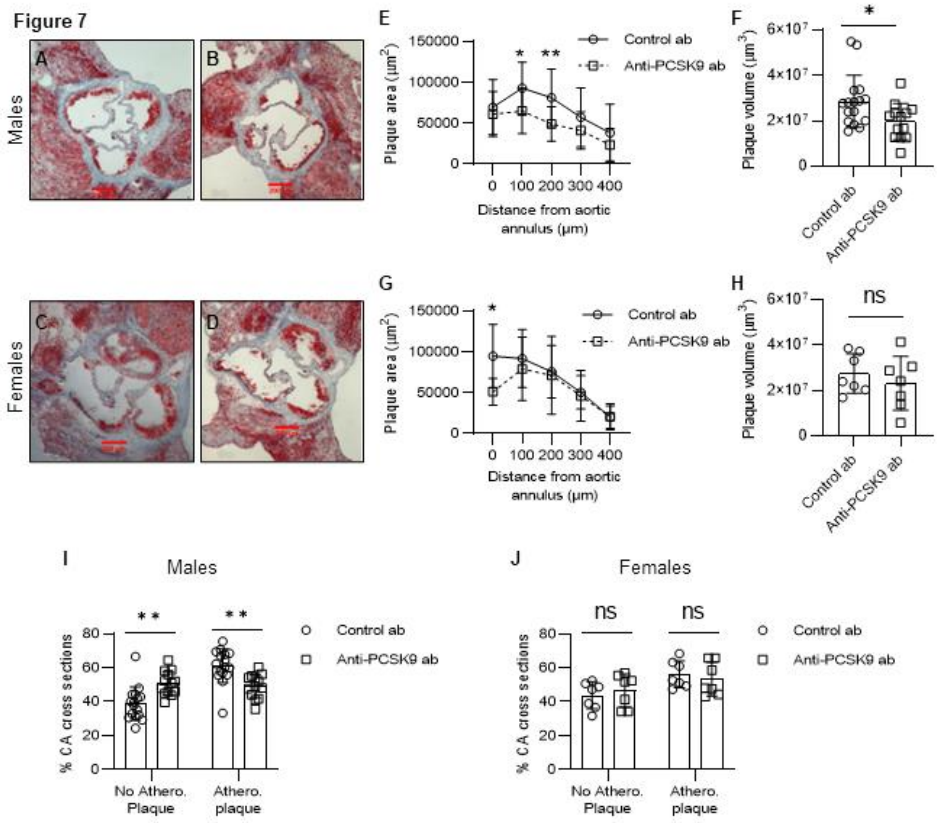


Figure 7
A
B
C
D
Males
Females
E
F
G
H
Control ab
Anti-PCSK9 ab
Plaque area (μm²)
Distance from aortic annulus (μm)
Plaque volume (μm³)
ns
I
J
Males
Females
% CA cross sections
No Athero. Plaque
Athero. plaque

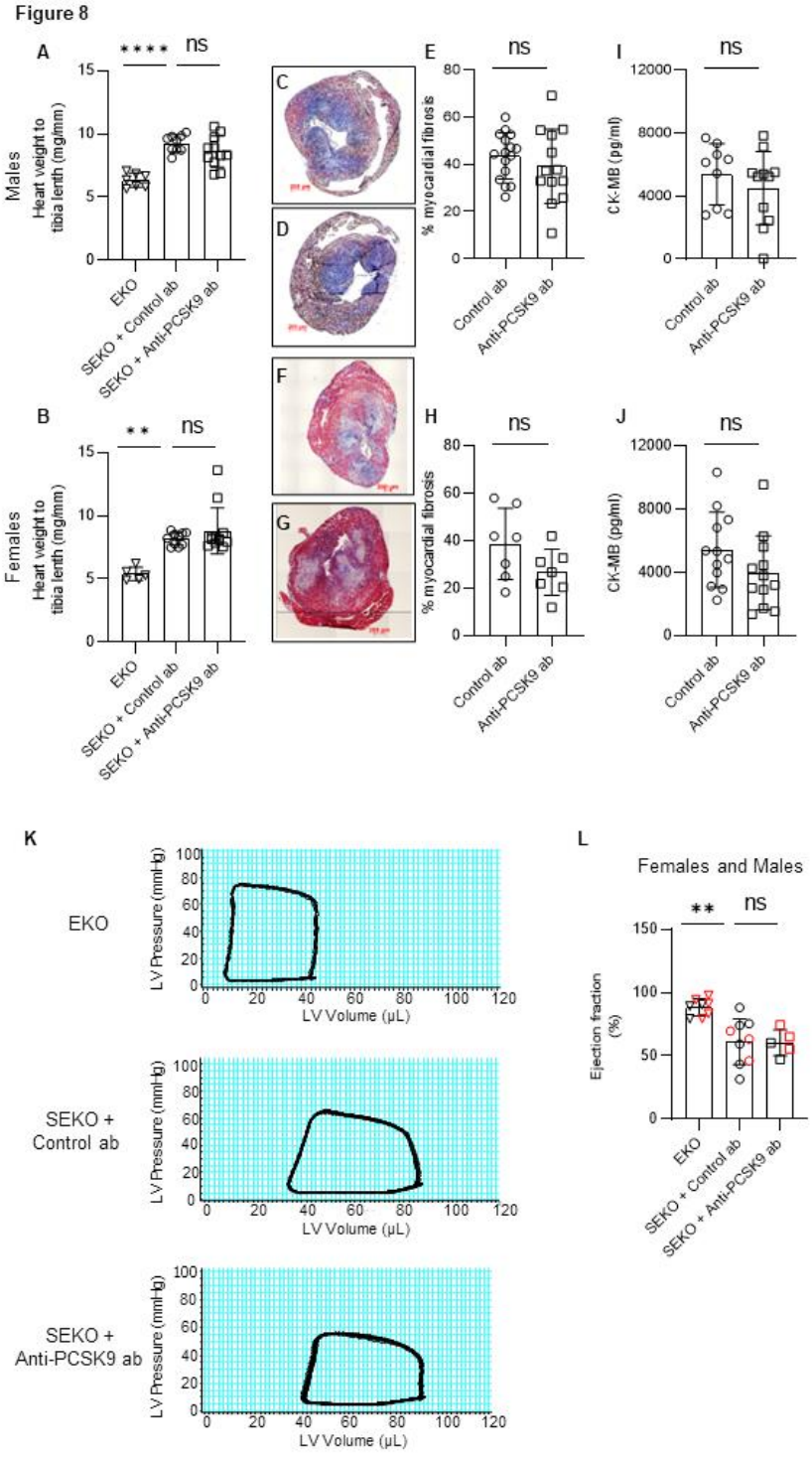


Figure 8
A
****
ns
Males
Heart weight to tibia lenth (mg/mm)
EKO
SEKO + Control ab
SEKO + Anti-PCSK9 ab
B
**
ns
Females
C
D
F
G
E
ns
% myocardial fibrosis
Control ab
Anti-PCSK9 ab
H
I
ns
CK-MB (pg/ml)
J
K
EKO
SEKO + Control ab
SEKO + Anti-PCSK9 ab
LV Pressure (mmHg)
LV Volume (µL)
L
Females and Males
Ejection fraction (%)

3.6 Figure legends:

Figure 1: *Pcsk9* gene deletion in SEKO mice does not alter plasma cholesterol levels, despite increased hepatic LDLR protein levels. Mice from panel A-D were analyzed at 5 weeks of age and mice from panel E- J were analyzed at 6.5 weeks of age. (A) Hepatic *Pcsk9* mRNA, (B) plasma PCSK9 protein and (C) hepatic *Ldlr* mRNA levels in SEKO and EKO mice. (D) Representative immunoblots and quantification for LDLR protein levels (normalized to ε-COP) in liver total membranes from SEKO and EKO mice. (E) Representative immunoblots and quantification of LDLR and Na^+/K^+ ATPase in liver total membranes from female (left) and male (right) SEKO and PSEKO mice. (F) Representative total cholesterol profiles of FPLC fractionated plasma from female SEKO and PSEKO mice (n=7, 7). Plasma (G) total cholesterol, (H) non-HDL cholesterol, (I) HDL cholesterol and (J) triglyceride levels from female (top) and male (bottom) SEKO and PSEKO mice. One outliner in female SEKO mice was excluded for the statistical analysis in panel J. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Figure 2. *Pcsk9* gene deletion in SEKO mice reduces atherosclerosis development both in the aortic sinus and coronary arteries. Mice were analyzed at 6.5 weeks of age. (A-B, E-F) Representative images of cross sections of aortic sinus stained with oil red O/hematoxylin, and (C and G) quantification of atherosclerotic plaque cross sectional

area profiles from (A and B) female and (E and F) male (A and E) SEKO and (B and F) PSEKO mice. (D and H) Quantification of atherosclerotic plaque volumes from (A-C) female and (E-G) male (A and E) SEKO and (B and F) PSEKO mice. (D and H) Plaque volumes are calculated as the areas under the curve of plaque cross sectional area versus distance over a 400 μm distance along the aortic sinus (C and G). (I) Representative images of transverse sections of coronary arteries stained with oil red O/hematoxylin with different extents of atherosclerosis (no plaque; <50% occluded; >50% occluded; and 100% occluded). Coronary artery (CA) cross sections with no plaque were considered non-atherosclerotic, while CA cross sections with atherosclerotic plaques occluding <50%, >50% or 100% of the artery lumen were considered as atherosclerotic. Quantification of the percentage of CA cross sections with no atherosclerotic plaque and with atherosclerotic plaques in (J) female and (K) male SEKO and PSEKO mice. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Figure 3: *Pcsk9* gene deletion protects against cardiac enlargement, myocardial fibrosis and LV dysfunction in SEKO mice. Mice were analyzed at 6.5 weeks of age except for panel K-M and P-Q. Mice were analyzed at 5 weeks of age for panel K-M and P-Q. (A, B) The ratios of heart weight to tibia length from (A) female and (B) male SEKO, PSEKO and control EKO mice. (C-D, F-G) Representative images of trichrome-

stained transverse sections of the upper portion of hearts from (C-D) female and (F-G) male (C and F) SEKO and (D and G) PSEKO mice. Healthy myocardium stains red and collagen containing myocardial fibrotic area stains blue. (E and H) Quantification of myocardial fibrosis levels in (E) female and (H) male SEKO and PSEKO mice. (I-J) Plasma levels of cardiac damage marker CK-MB for (I) female and (J) male SEKO and PSEKO mice. (K-L) Representative images of Oil red O/hematoxylin-stained transverse heart sections (900 μm away from the aortic annulus) in (K) SEKO and (L) PSEKO mice. (M) Total lipid accumulation in the heart of SEKO and PSEKO mice was calculated as the area under the curve of lipid accumulation profile (Supplementary Figure 8B), which shows the ratio of lipid accumulation area to cross-sectional myocardium area, starting at the cross-section of the aortic annulus until the cross-section 2700 um away from the aortic annulus (300 μm apart, 10 sections in total). (N) Representative images of immunostaining for Mac-3 in transverse heart sections of SEKO and PSEKO mice. (O) Quantification of Mac-3 intensity normalized by the total myocardium area in female and male SEKO and PSEKO mice. Left-ventricular pressure-volume analysis was determined using a solid-state pressure-volume catheter inserted transmurally into the left ventricle. (P) Representative left ventricle (LV) pressure-volume loop tracings from EKO, SEKO and PSEKO mice. (Q) Ejection fractions in female and male EKO, SEKO and PSEKO mice. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. In panel M, red symbols represent female mice and black symbols represent male mice. NS indicates not statistically significant ($P>0.05$); *

represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Figure 4. AAV8-PCSK9 treatment increases circulating PCSK9 levels without changing plasma total cholesterol levels in PSEKO mice. In panel A-D, PSEKO mice were injected with 4 x 10^{10} GC of either AAV8-eGFP or AAV8-PCSK9 at 3 weeks of age and analyzed at 6.5 weeks of age. (A) Circulating PCSK9 levels in female and male SEKO, PSEKO and AAV8-eGFP or AAV8-PCSK9 treated PSEKO mice. (B) Representative immunoblots and quantification of LDLR and Na^+/K^+ ATPase in liver total membranes from female and male PSEKO mice treated with either AAV8-eGFP or AAV8-PCSK9. The left three samples are females and right four samples are males for both AAV8-eGFP and AAV8-PCSK9 treatment in the immunoblots. (C) Plasma total cholesterol levels in female and male PSEKO mice treated with AAV8-eGFP or AAV8-PCSK9. (D) *Pcsk9* mRNA levels in the livers, hearts, kidneys, spleens and aortas of female and male SEKO, PSEKO and AAV8-eGFP or AAV8-PCSK9 treated PSEKO mice. (E) *Pcsk9* mRNA levels in the livers and peritoneal macrophages of male $Pcsk9^{wt/wt}ApoE^{ko/ko}$ mice, $Pcsk9^{ko/ko}ApoE^{ko/ko}$ mice or $Pcsk9^{ko/ko}ApoE^{ko/ko}$ mice treated with AAV8-eGFP or AAV8-PCSK9. $Pcsk9^{ko/ko}ApoE^{ko/ko}$ mice were treated with 4 x 10^{10} GC of either AAV8-eGFP or AAV8-PCSK9 at 6 weeks of age. Liver and thioglycolate-induced peritoneal macrophages were harvested at 9.5 weeks of age from $Pcsk9^{wt/wt}ApoE^{ko/ko}$ mice, $Pcsk9^{ko/ko}ApoE^{ko/ko}$ mice or $Pcsk9^{ko/ko}ApoE^{ko/ko}$ mice treated with AAV8-eGFP or AAV8-PCSK9. Each symbol represents an individual mouse. Bars represent means and

error bars represent standard deviations. Red symbols represent female mice and black symbols represent male mice. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Figure 5. AAV8-PCSK9 treatment reverses coronary artery disease compared to AAV8-eGFP treatment in PSEKO mice. PSEKO mice were injected with 4 x 10^{10} GC of either AAV8-eGFP or AAV8-PCSK9 at 3 weeks of age and analyzed at 6.5 weeks of age. (A and B) Representative images of cross sections of aortic sinus stained with oil red O/hematoxylin and (C) quantification of atherosclerotic plaque cross sectional area profiles from female and male PSEKO mice treated with (A) AAV-8 eGFP or (B) AAV-PCSK9. (D) Quantification of atherosclerotic plaque volumes in (A) AAV8-eGFP and (B) AAV8-PCSK9 treated PSEKO mice. Plaque volume was calculated as the area under the curve of plaque cross sectional area versus distance over a 400 μm distance along the aortic sinus (C). (E) Quantification of the percentage of CA cross sections with no atherosclerotic plaque and with atherosclerotic plaques in female and male PSEKO mice treated with AAV8-eGFP or AAV8-PCSK9. (F) The ratios of heart weight to tibia length. (H and I) Representative images of trichrome-stained transverse heart sections and (J) quantification of myocardial fibrosis levels in (H) AAV8-eGFP and (I) AAV8-PCSK9 treated PSEKO mice. (K) Plasma levels of cardiac damage marker CK-MB. (L and M) Representative images of Oil red O/hematoxylin-stained transverse heart sections (900 μm away from the aortic annulus) in (L) AAV8-eGFP and (M) AAV8-PCSK9 treated PSEKO mice. (N) Total lipid accumulation in the heart of AAV8-eGFP or AAV8-PCSK9

treated PSEKO mice was calculated as the area under the curve of lipid accumulation profile (Supplementary Figure 13B), which shows the ratio of lipid accumulation area to cross-sectional myocardium area, starting at the cross-section of the aortic annulus until the cross-section 2700 um away from the aortic annulus (300 μm apart, 10 sections in total). (O) Representative images of immunostaining for Mac-3 in transverse heart sections of AAV8-eGFP and AAV8-PCSK9 treated PSEKO mice. (P) Quantification of Mac-3 intensity normalized by the total myocardium area. Each symbol represents an individual mouse. Bars represent means and error bars represent standard deviations. Red symbols represent female mice and black symbols represent male mice. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

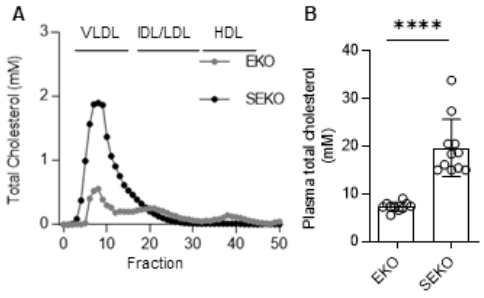
Figure 6: Anti-PCSK9 antibody treatment increases circulating PCSK9 and hepatic LDLR protein levels without affecting plasma cholesterol levels in both female and male SEKO mice. Mice were analyzed at 6.5 weeks of age. (A) Circulating PCSK9 levels in control or anti-PCSK9 ab treated male and female SEKO mice measured by ELISA. (B) Immunoblotting for liver total membrane LDLR (top) and ε-COP (bottom) and (C) quantification of immunoblotting of LDLR/ ε-COP protein levels in liver membranes of male and female SEKO mice with control or anti-PCSK9 antibody treatment. (D) Plasma total cholesterol levels in male and female SEKO mice with control or anti-PCSK9 antibody treatment. (E) Representative total cholesterol profiles of FPLC fractionated plasma from male SEKO mice with control or anti-PCSK9 antibody treatment (n=8,6). (F) TG levels in plasma from male SEKO mice with control or anti-

PCSK9 antibody treatment. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

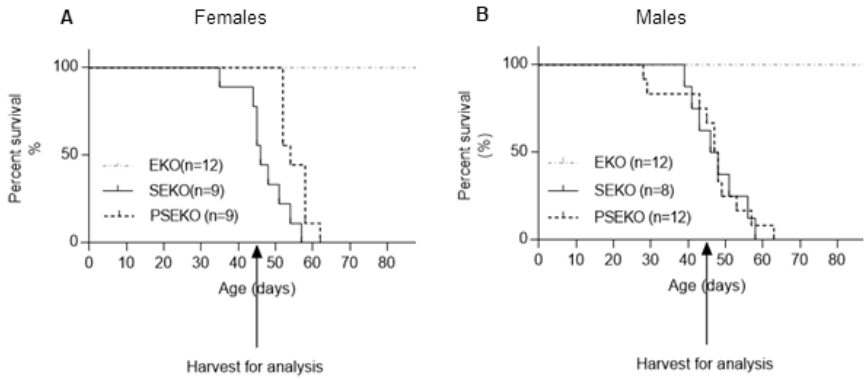
Figure 7: Anti-PCSK9 antibody treatment reduces aortic sinus and coronary artery atherosclerosis development in male but not female SEKO mice. Mice were analyzed at 6.5 weeks of age. (A-D) Representative images of cross sections of aortic sinus stained with oil red O/hematoxylin, and (E and G) quantification of atherosclerotic plaque cross sectional area profiles from (A, B and E) male and (C, D and G) female SEKO mice treated with (A and C) control or (B and D) anti-PCSK9 antibody. (F and H) Quantification of atherosclerotic plaque volumes from (A, B and E) male and (C, D and G) female SEKO mice treated with (A and C) control antibody or (B and D) anti-PCSK9 antibody. (F and H) Plaque volumes are calculated as the areas under the curve of plaque cross sectional area versus distance over a 400 μm distance along the aortic sinus (E and G). Quantification of the percentage of CA cross sections with no atherosclerotic plaque and with atherosclerotic plaques in (I) male and (J) female SEKO mice treated with control antibody or anti-PCSK9 antibody. Each symbol represents data from an individual mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Figure 8: Anti-PCSK9 antibody treatment does not protect against cardiac enlargement, myocardial fibrosis and cardiac dysfunction in SEKO mice. Mice were analyzed at 6.5 weeks of age except for panel K-L. Mice were analyzed at 5 weeks of age for panel K-L. (A, B) The ratios of heart weight to tibia length from (A) male and (B) female SEKO mice treated with control or anti-PCSK9 antibody and control EKO mice. (C-D, F-G) Representative images of trichrome-stained transverse sections of the upper portion of hearts from (C-D) male and (F-G) female SEKO mice treated with (C and F) control or (D and G) anti-PCSK9 antibody. Healthy myocardium stains red and collagen containing myocardial fibrotic area stains blue. (E and H) Quantification of myocardial fibrosis levels in (E) male and (H) female SEKO mice with control or anti-PCSK9 antibody treatment. (I-J) Plasma levels of cardiac damage marker CK-MB for (I) male and (J) female SEKO mice with control or anti-PCSK9 antibody treatment. Left-ventricular pressure-volume analysis was determined using a solid-state pressure-volume catheter inserted transmurally into the left ventricle. (K) Representative left ventricle (LV) pressure-volume loop tracings from SEKO mice with control or anti-PCKS9 antibody treatment and control EKO mice. (L) Ejection fractions in male and female SEKO mice with control or anti-PCKS9 antibody treatment and control EKO mice. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. In panel L, red symbols represent female mice and black symbols represent male mice. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Supplementary Figure 1



Supplementary Figure 2



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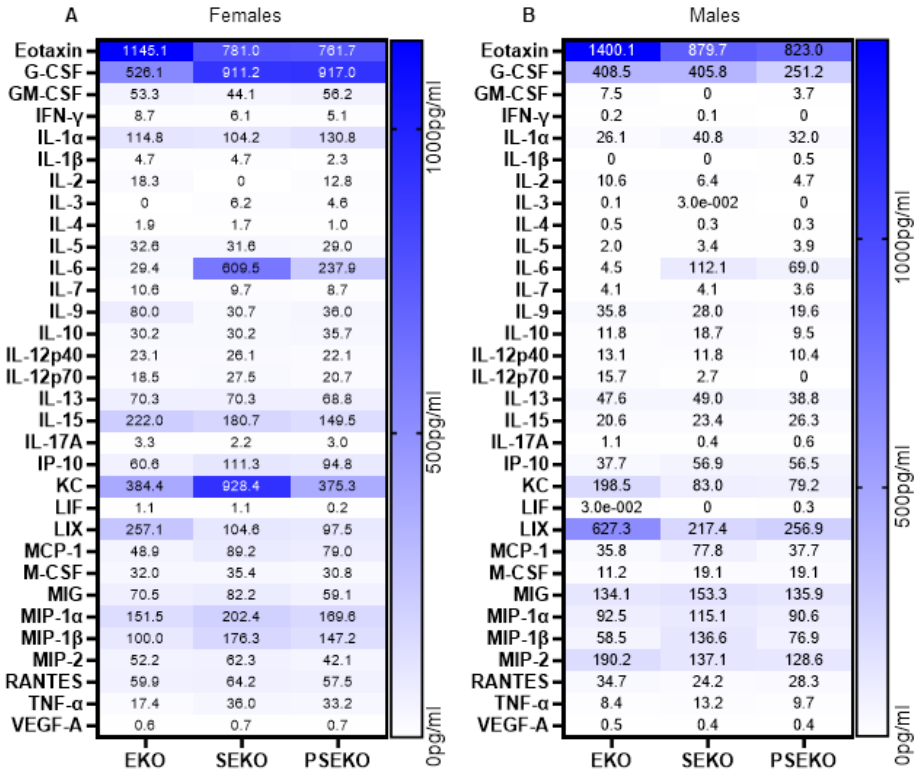

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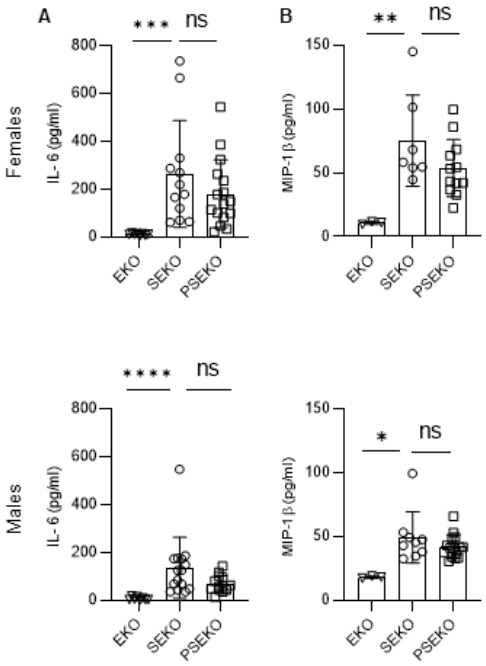

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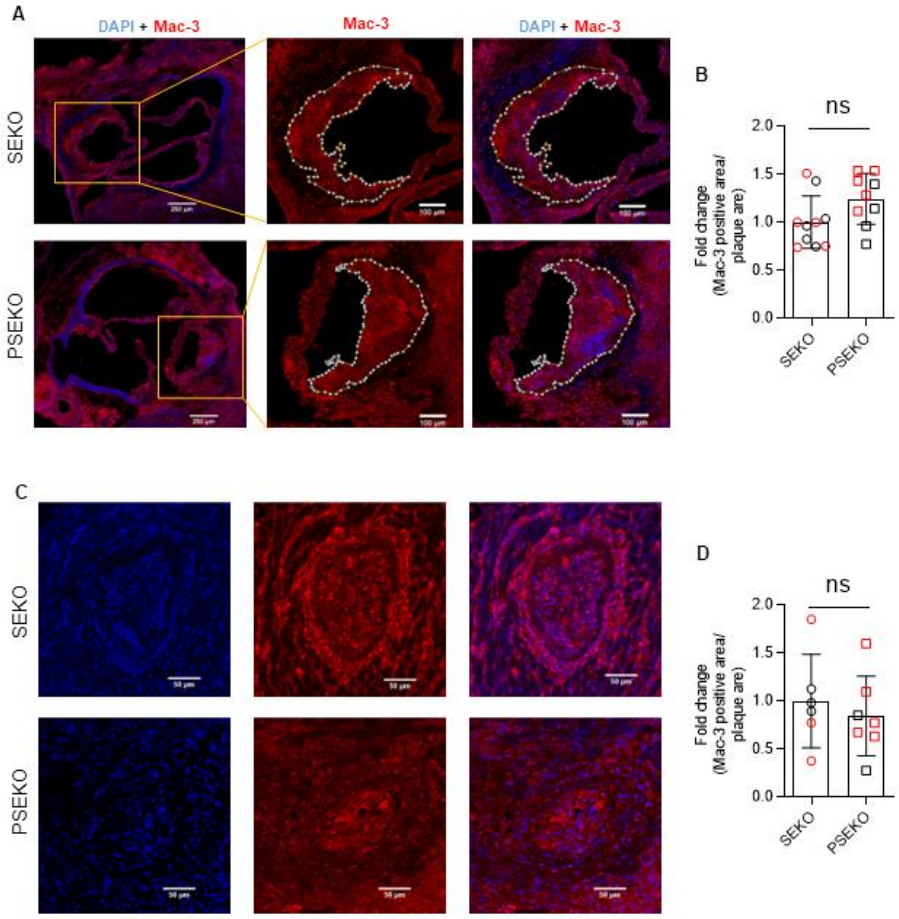

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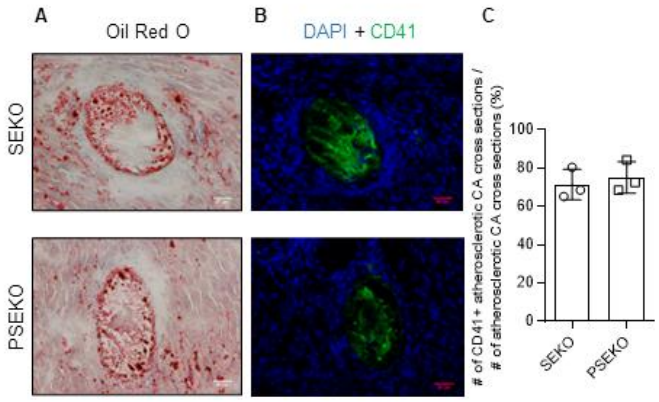
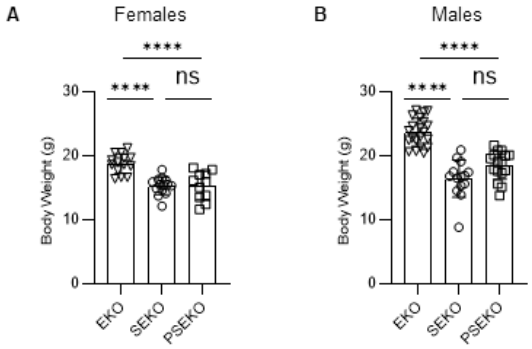
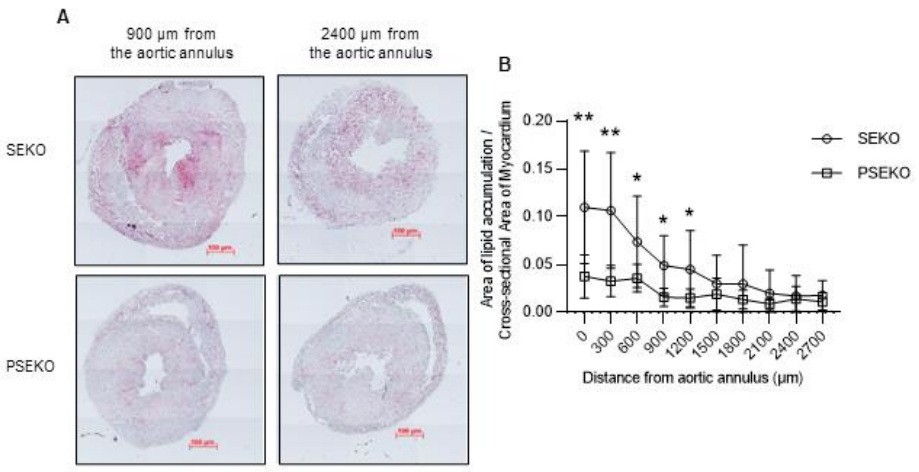


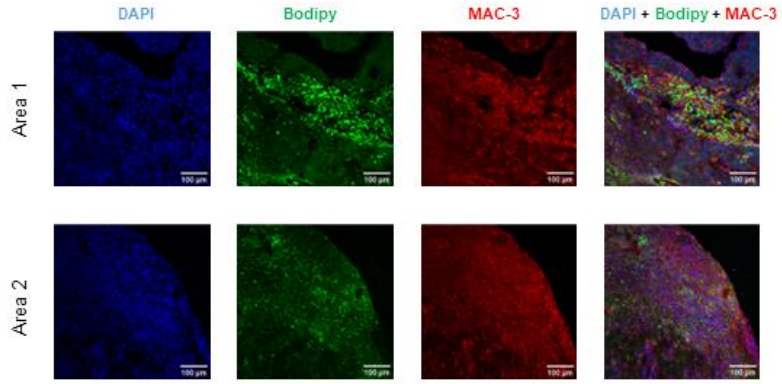
Supplementary Figure 7



Supplementary Figure 8

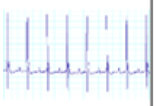
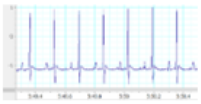
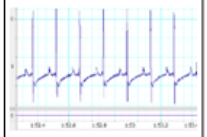
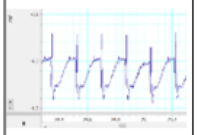


Supplementary Figure 9



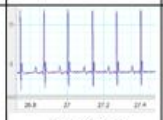
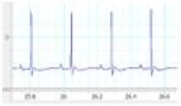
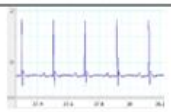
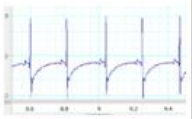
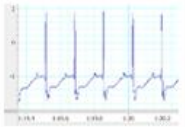
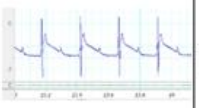
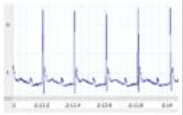
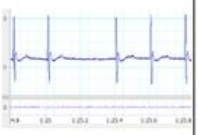
Supplementary Figure 10

A

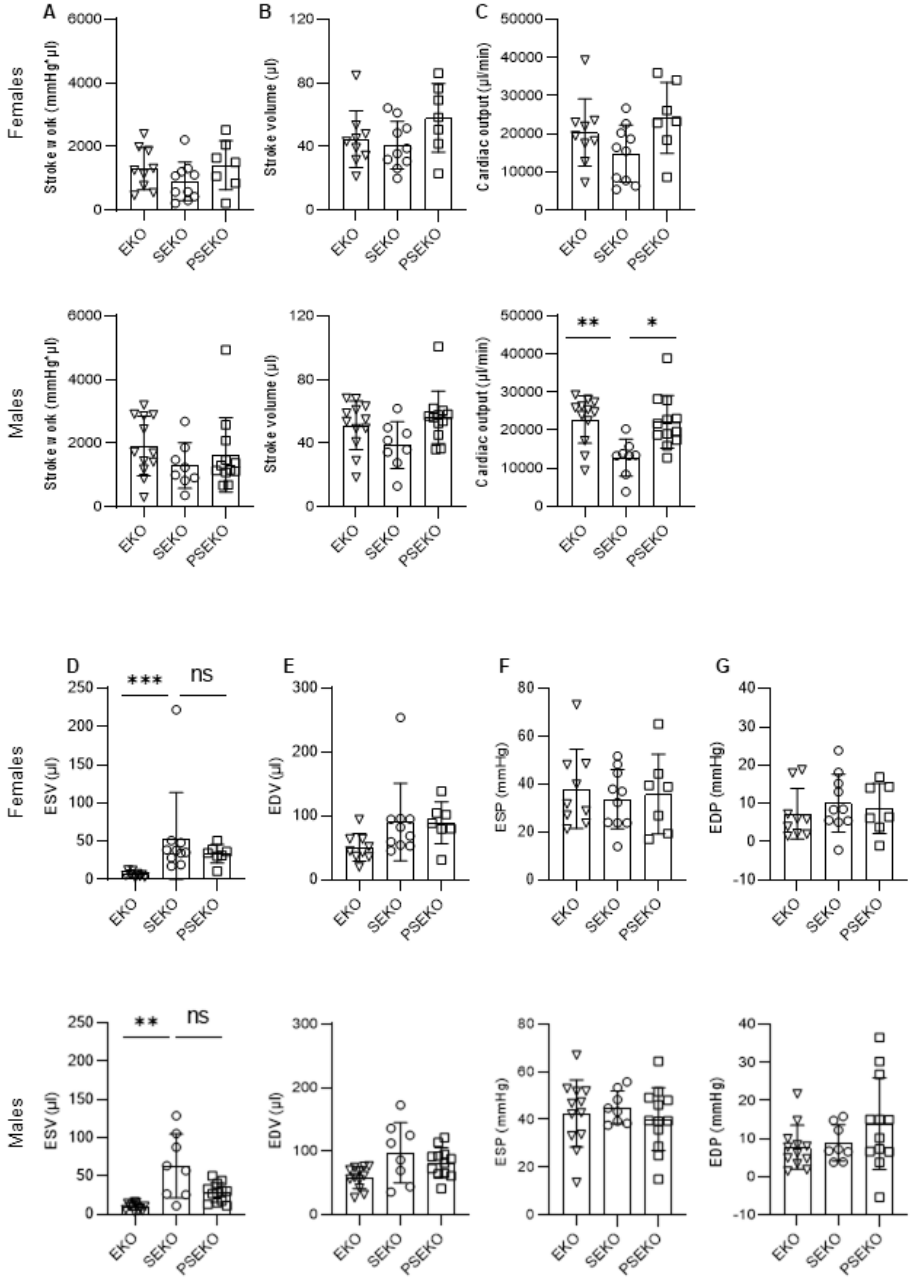
Female		EKO	SEKO		PSEKO	
Normal ECG patterns		 8/8=100%	 5/12 =42%		 8/13=62%	
Abnormal ECG patterns	ST depression		7/12 = 58%	 3/12	5/13 = 38%	 2/13
	ST elevation			 4/12		 2/13
	Missing QRS waves			0/12		 1/13

Supplementary Figure 10 - Continued

B

male		EKO	SEKO		PSEKO	
Normal ECG patterns		 10/10 =100%	 3/11 =27%		 11/18 =61%	
Abnormal ECG patterns	ST depression		8/11 = 73%	 2/11	7/18 = 39%	 4/18
	ST elevation			 3/11		 3/18
	Missing QRS waves			 3/11		0/18

Supplementary Figure 11



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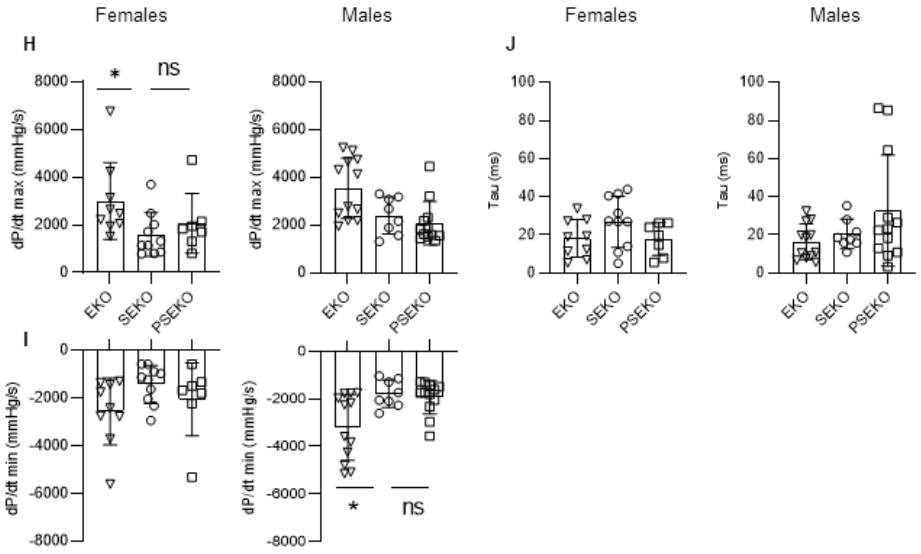

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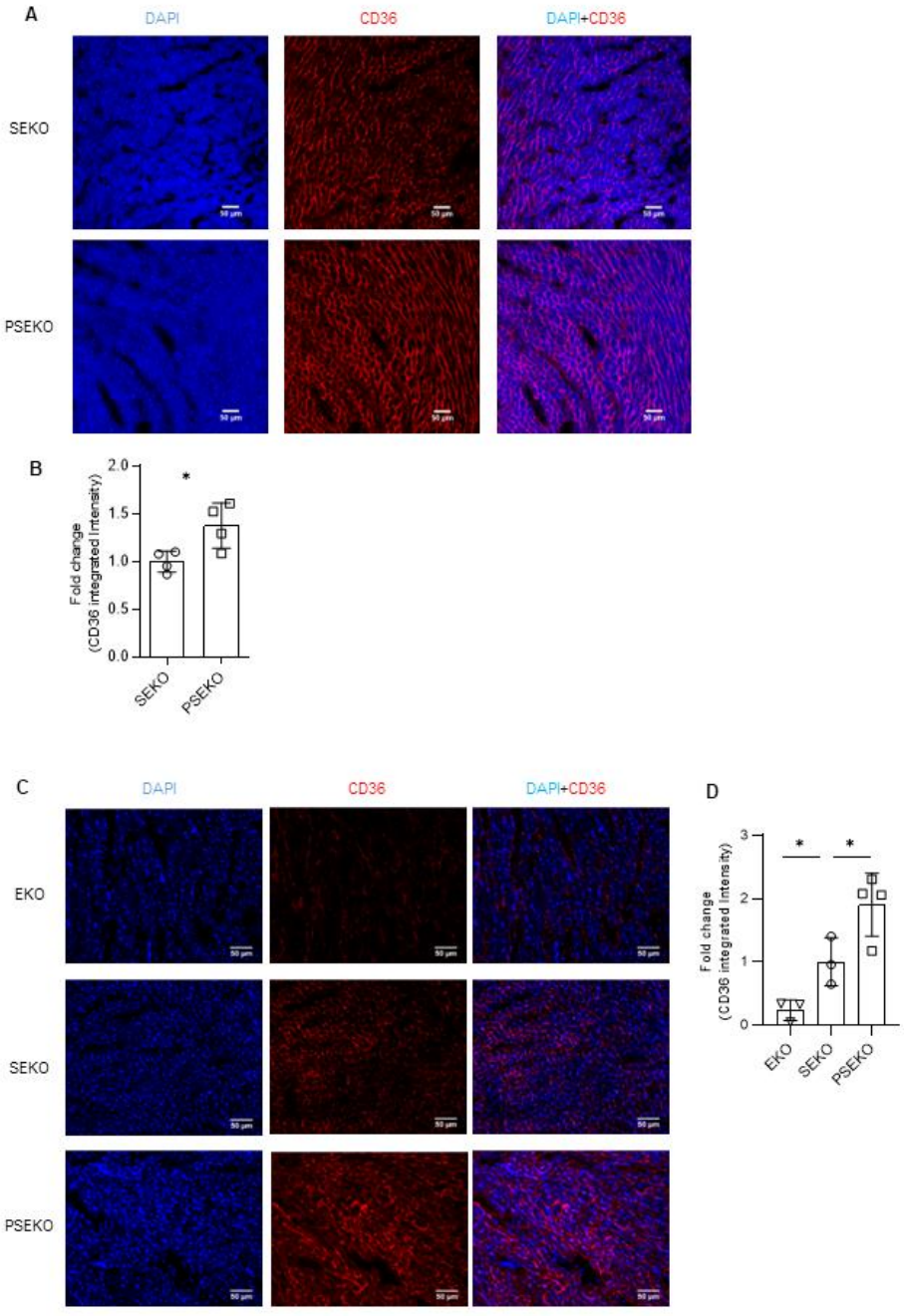
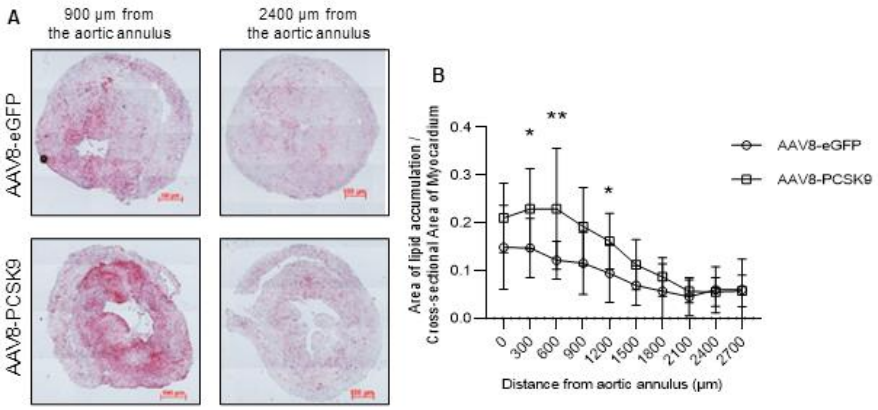
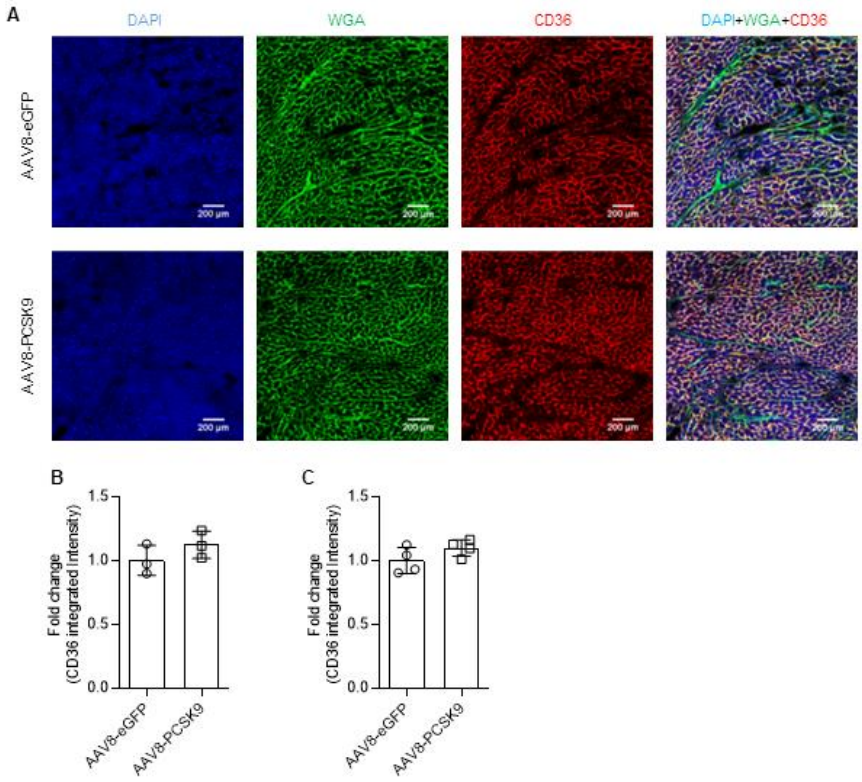


Supplementary Figure 13

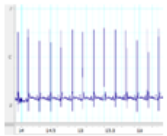
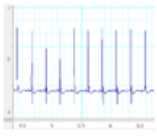
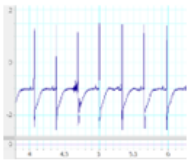
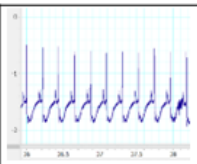
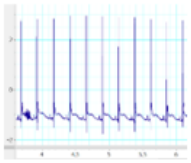


Supplementary Figure 14

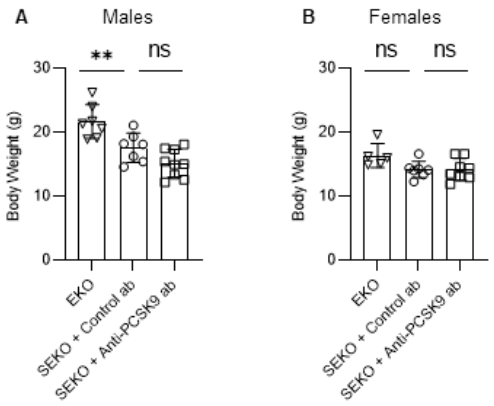


Supplementary Figure 15

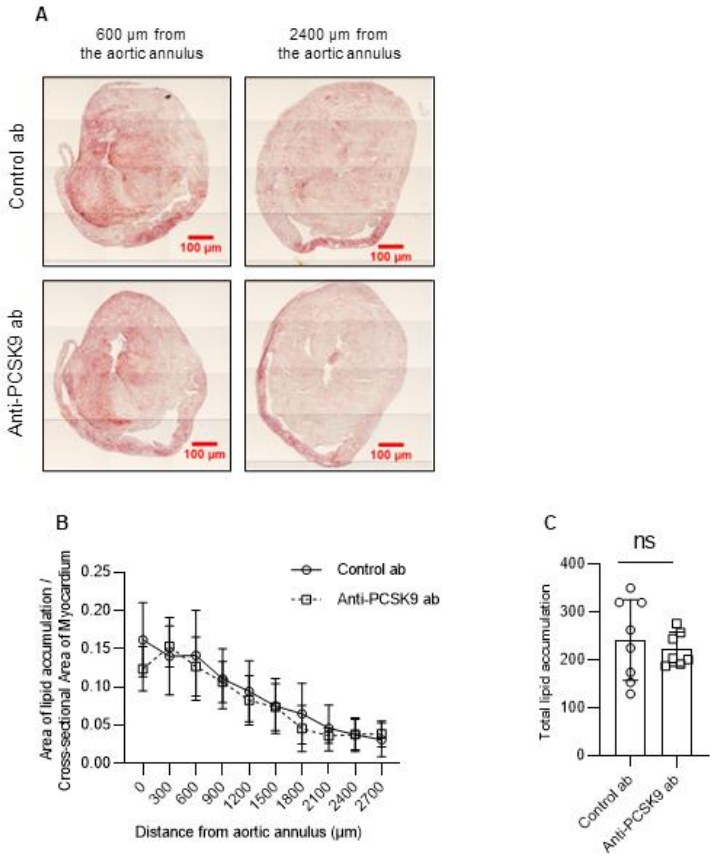
A

		AAV8-eGFP		AAV8-PCSK9	
Normal ECG patterns		 14/15 =93%		 7/14=50%	
Abnormal ECG patterns	ST depression	1/15 = 7%	 1/15	7/14 = 50%	 6/14
	ST elevation				 1/14
	Missing QRS waves				

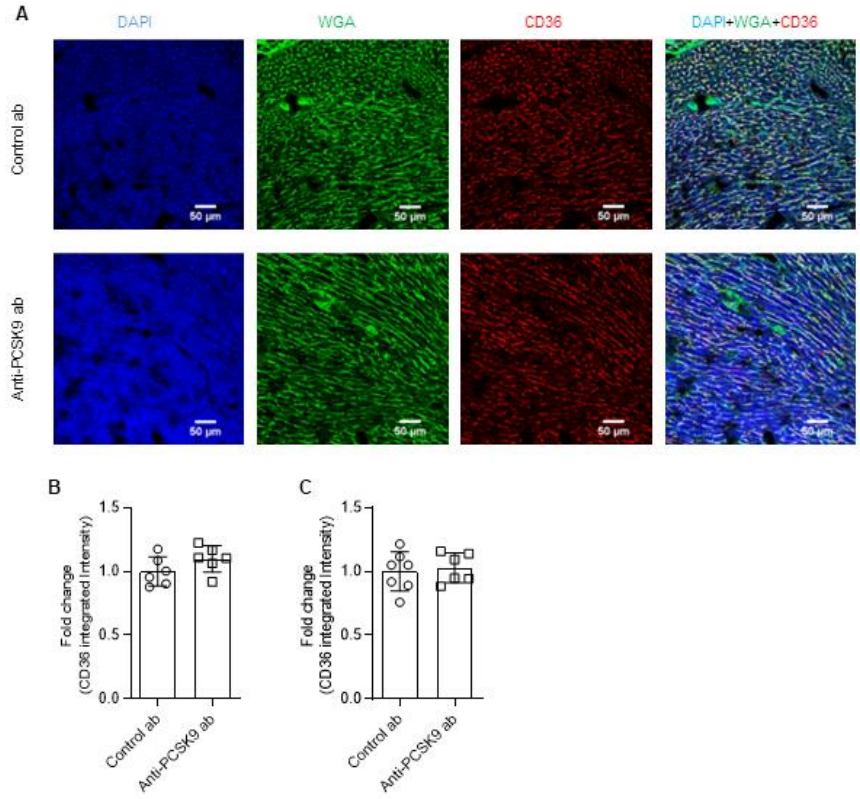
Supplementary Figure 16



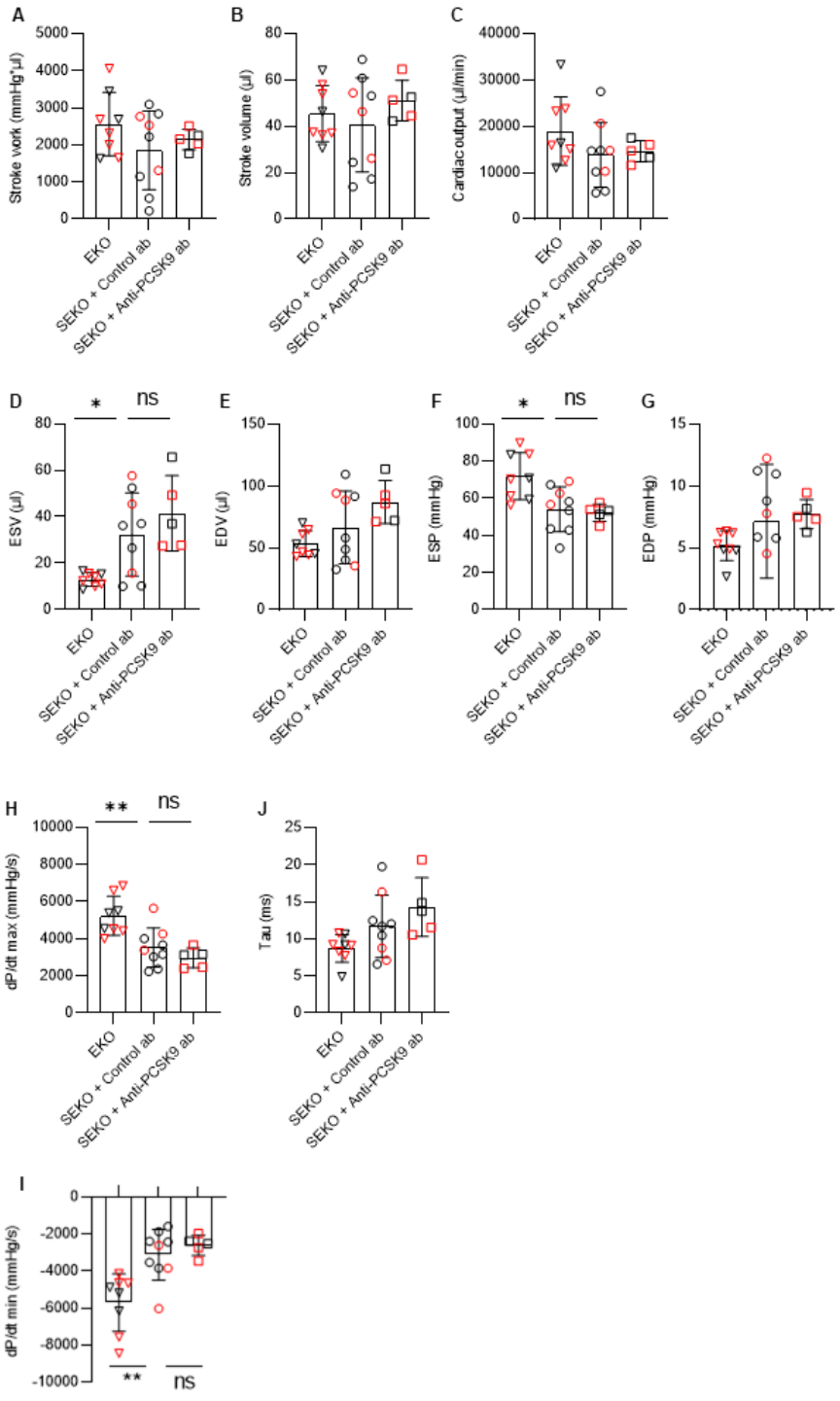
Supplementary Figure 17



Supplementary Figure 18



Supplementary Figure 19



Supplementary table a

Target gene	Primer Pair Sequence
Mouse LDLR (forward)	5'-TCCAATCAATTCAGCTGTG G-3'
Mouse LDLR (reverse)	5'-GAGCCATCTAGGCAATCTCG-3'
Mouse eGFP (forward)	5'-CTGCTGCCCGACAACCAC-3'
Mouse eGFP (reverse)	5'-TGTGATCGCGCTTCTCGTT-3'
Mouse PCSK9 (forward)	5'- TTGCAGCAGCTGGGAACTT-3'
Mouse PCSK9 (reverse)	5'-CCGACTGTGATGACCTCTGGA-3'
Mouse GAPDH (forward)	5'-ACCACAGTCCATGCCATCAC-3'
Mouse GAPDH (reverse)	5'-TCCACCACCCTGTTGCTGTA-3'

3.7 Supplementary figure legends:

Supplementary figure 1: Plasma lipoproteins levels in SEKO and EKO mice. Male mice were analyzed at 5 weeks of age. (A) Representative total cholesterol profiles of FPLC fractionated plasma from SEKO and control EKO mice. (B) Plasma total cholesterol levels from SEKO and EKO mice. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Supplementary figure 2: Effects of genetic *Pcsk9* inactivation on the survival of SEKO mice. Kaplan-Meier survival curves for (A) female and (B) male SEKO mice with intact *Pcsk9* expression (solid line) and PSEKO mice in which *Pcsk9* has been knocked out (black dashed line). Survival of control EKO mice (grey dashed line) is included for comparison. Log-rank (Mantel-Cox) tests indicated $P=0.0027$ for the comparison of SEKO and PSEKO data for females (panel A) and $P=0.884$ (not statistically significant) for the comparison of SEKO and PSEKO data for males (panel B). Arrows (Experimental endpoint) indicate the 6.5 weeks (45 days) of age point at which subsequent analyses were done.

Supplementary figure 3: Levels of plasma cytokines and chemokines from female EKO, SEKO and PSEKO mice. Mice were analyzed at 6.5 weeks of age. Plasma from (A) female and (B) male EKO, SEO and PSEKO mice (n = 10, 10, 10) was analyzed by

Eve Technologies using their mouse cytokine/chemokine 31-plex discovery array. Mean values for Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1-α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, 1L-15, 1L-17, KC, IP-10, LIF, LIX, M-CSF, MIG, MIP-1a, MIP-2, Rantes, TNF-α, and VEGF are shown in the heatmap.

Supplementary figure 4: Plasma levels of IL-6 and MIP-1β. Mice were analyzed at 6.5 weeks of age. Plasma (A) IL-6 and (B) MIP-1β levels in EKO, SEKO and PSEKO mice were measured by ELISA. One outliner in male SEKO mice and one outliner in male PSEKO mice were excluded for the statistical analysis in panel A. Each symbol represents data from an independent mouse. Red symbols represent female mice and black symbols represent male mice. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 5: *Pcsk9* gene deletion does not change macrophage accumulation in atherosclerotic plaques both in the aortic sinus and coronary arteries. Mice were analyzed at 6.5 weeks of age. (A) Representative images of immunostaining for Mac-3 in the aortic sinus of SEKO and PSEKO mice. (B) Quantification of Mac-3 positive area (normalized by the total plaque area) in female and male SEKO and PSEKO mice. (C) Representative images of immunostaining for Mac-3 in atherosclerotic coronary artery cross sections of SEKO and PSEKO mice. (D) Quantification of Mac-3 positive area (normalized by the plaque area in coronary arteries)

in female and male SEKO and PSEKO mice. Each symbol represents data from an individual mouse. Red symbols represent female mice and black symbols represent male mice. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 6: *Pcsk9* gene deletion in SEKO mice does not change platelet accumulation in coronary artery atherosclerotic plaques. Mice were analyzed at 6.5 weeks of age. Representative images of (B) immunofluorescence staining for DAPI and CD41 in atherosclerotic coronary artery cross sections and (A) oil red O/hematoxylin staining of corresponding atherosclerotic coronary artery in the adjacent sections from SEKO and PSEKO mice. (C) Quantification of the percentage of the number of CD41 positive atherosclerotic coronary artery cross sections among the total number of atherosclerotic coronary artery cross sections in male SEKO and PSEKO mice. Each symbol represents data from an independent mouse. Bars represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 7: *Pcsk9* gene deletion in SEKO mice does not change the body weight in SEKO mice. Mice were analyzed at 6.5 weeks of age. (A and B) Body weights from (A) female and (B) male SEKO, PSEKO and control EKO mice. Each

symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 8: *Pcsk9* gene deletion decreases lipid accumulation in the heart of SEKO mice. Mice were analyzed at 5 weeks of age. (A) Representative images of transverse sections 900 μm or 2400 μm away from the aortic annulus in the hearts of female and male SEKO and PSEKO mice. (B) Profiles of the ratio of lipid accumulation area to cross-sectional myocardium area, starting at the cross section of the aortic annulus (0 um) until the cross section 2700 um away from the aortic annulus from female and male SEKO and PSEKO mice. Symbols represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 9: Lipid accumulation is co-localized with macrophage in the heart of SEKO mice. Representative images of two selected areas of transverse heart sections co-stained with DAPI, BODIPY, and Mac-3 from SEKO mice.

Supplementary figure 10: *Pcsk9* gene deletion decreases the ratio of ECG abnormalities in SEKO mice. ECG of anesthetized (A) female and (B) male EKO, SEKO and PSEKO mice at 6.5 weeks of age. Representative ECG tracings are shown.

Numbers indicate the number of mice with ECG patterns shown vs. the number of mice analyzed and the ratio represented as percentage.

Supplementary figure 11: *Pcsk9* gene deletion attenuates left ventricular dysfunction in SEKO mice. (A) Stroke work, (B) stroke volume, (C) cardiac output, (D) end systolic volume, (E) end diastolic volume, (F) end systolic pressure, (G) end diastolic pressure, (H) maximum rate of pressure increase (dP/dt max), (I) minimum rate of pressure decrease (dP/dt min), and (J) Tau from female and male SEKO, PSEKO and control EKO mice. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Supplementary figure 12: *Pcsk9* gene deletion increases CD36 levels in the heart of SEKO mice. (A) Representative images of immunofluorescence staining for DAPI and CD36 in healthy myocardium (non-fibrotic area) of transverse sections of hearts from female SEKO and PSEKO mice. (B) Quantification of CD36 immunostaining in the transverse sections of hearts from female SEKO and PSEKO mice. (C) Representative images of immunofluorescence staining for DAPI and CD36 in healthy myocardium (non-fibrotic area) of transverse sections of hearts from male EKO, SEKO and PSEKO mice. (D) Quantification of CD36 immunostaining in the transverse sections of hearts from male EKO, SEKO and PSEKO mice. Each symbol represents an individual mouse.

Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 13: AAV8-PCSK9 treatment increases lipid accumulation in the heart of PSEKO mice compared to AAV8-eGFP treatment. PSEKO mice were injected with 4 x 10^{10} GC of either AAV8-eGFP or AAV8-PCSK9 at 3 weeks of age and analyzed at 6.5 weeks of age. (A) Representative images of transverse sections 900 μm or 2400 μm away from the aortic annulus in the hearts of PSEKO mice treated with AAV8-eGFP or AAV8-PCSK9. (B) Profiles of the ratio of lipid accumulation area to cross-sectional myocardium area, starting at the cross section of the aortic annulus (0 um) until the cross section 2700 um away from the aortic annulus from female and male PSEKO mice treated with AAV8-eGFP or AAV8-PCSK9. Symbols represent means and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 14: Restoring circulating PCSK9 by AAV8-PCSK9 treatment does not reduce CD36 levels in the heart of PSEKO mice. (A) Representative images of immunofluorescence staining for DAPI, wheat germ agglutinin (WGA), and CD36 in healthy myocardium (non-fibrotic area) of transverse sections of hearts from PSEKO mice treated with either AAV8-eGFP or AAV8-PCSK9. Quantification of CD36

immunostaining in the transverse sections of hearts from (B) female and (C) male PSEKO mice treated with either AAV8-eGFP or AAV8-PCSK9. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 15: AAV8-PCSK9 treatment increases the ratio of ECG abnormalities in PSEKO mice compared to AAV8-eGFP treatment. ECG of anesthetized female and male AAV8-eGFP or AAV8-PCSK9 treated PSEKO mice at 6.5 weeks of age. Representative ECG tracings are shown. Numbers indicate the number of mice with ECG patterns shown vs. the number of mice analyzed and the ratio represented as a percentage.

Supplementary figure 16: Anti-PCSK9 antibody treatment does not change the body weight in SEKO mice compared to control antibody treatment. Mice were analyzed at 6.5 weeks of age. (A and B) Body weights from (A) male and (B) female control or anti-PCSK9 antibody treated SEKO mice and control EKO mice. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 17: Anti-PCSK9 antibody treatment does not change lipid accumulation in the heart of male SEKO mice compared to control antibody treatment. (A) Representative images of transverse sections 600 μm or 2400 μm away from the aortic annulus in the hearts of male SEKO mice treated with control or anti-PCSK9 antibody. (B) Profiles of the ratio of lipid accumulation area to cross-sectional myocardium area, starting at the cross section of the aortic annulus (0 um) until the cross section 2700 um away from the aortic annulus from male SEKO mice treated with control or anti-PCSK9 antibody. (C) Total lipid accumulation in the heart of control or anti-PCSK9 antibody treated male SEKO was calculated as the area under the curve of lipid accumulation profile (B). Symbols represent means and error bars represent standard deviations. NS indicates not statistically significant ($P>0.05$); * represents $P<0.05$; ** represents $P<0.01$; *** represents $P<0.001$; **** represents $P<0.0001$.

Supplementary figure 18: Increased circulating PCSK9 levels by anti-PCSK9 ab treatment does not change CD36 levels in the heart of SEKO mice. (A) Representative images of immunofluorescence staining for DAPI, wheat germ agglutinin (WGA), and CD36 in healthy myocardium (non-fibrotic area) of transverse sections of hearts from SEKO mice treated with either control or anti-PCSK9 ab. Quantification of CD36 immunostaining in the transverse sections of hearts from (B) male and (C) female SEKO mice treated with either control or anti-PCSK9 ab. Each symbol represents an individual mouse. Bars represent the mean and error bars represent standard deviations.

NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

Supplementary figure 19: Anti-PCSK9 antibody treatment does not ameliorate left ventricular dysfunction in SEKO mice compared to control antibody. (A) Stroke work, (B) stroke volume, (C) cardiac output, (D) end systolic volume, (E) end diastolic volume, (F) end systolic pressure, (G) end diastolic pressure, (H) maximum rate of pressure increase (dP/dt max), (I) minimum rate of pressure decrease (dP/dt min), and (J) Tau from female and male control or anti-PCSK9 antibody treated SEKO and control EKO mice. Each symbol represents an individual mouse. Red symbols represent female mice and black symbols represent male mice. Bars represent the mean and error bars represent standard deviations. NS indicates not statistically significant (P>0.05); * represents P<0.05; ** represents P<0.01; *** represents P<0.001; **** represents P<0.0001.

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Chapter 4: General Discussion

4.1 Summary of Results in Chapters 2 and 3

This thesis focuses on studying the effects of PCSK9 inhibition on CAD in mouse models as well as the underlying mechanisms. In chapter 2, we examined the effects of PCSK9 antibody treatment on newly developing as well as continued development of pre-existing CAD in HFCC diet fed one year old male *Sr-b1 KO* mice. One year old *Sr-b1 KO* mice were switched from a normal chow diet to an HFCC diet to induce atherosclerosis development. By initiating PCSK9 antibody treatment one week before an HFCC diet feeding (7 weeks in total), we examined the effects of weekly PCSK9 antibody treatment on plasma cholesterol levels and the newly developing atherosclerosis in the aortic sinus and coronary arteries. More importantly, by initiating PCSK9 antibody treatment 7 weeks after the switch to HFCC diet (12 weeks in total), we examined the effects of PCSK9 antibody treatment (5 weeks in total) on plasma cholesterol levels and the continued growth of pre-existing atherosclerosis in both the aortic sinus and coronary arteries as well as cardiac fibrosis and damage. In both cases, anti-PCSK9 antibody treatment consistently increased plasma PCSK9 and hepatic LDLR protein levels and reduced plasma total cholesterol and IL-6 levels in one year old *Sr-b1 KO* mice. Anti-PCSK9 antibody treatment initiated one week before an HFCC diet feeding (7 weeks in total) in one year old *Sr-b1 KO* mice reduced the newly developing atherosclerosis in both the aortic sinus and coronary arteries. Furthermore, anti-PCSK9 antibody treatment during the last 5 weeks of a total of 12 weeks of HFCC diet feeding reduced the

continued growth of established atherosclerosis in both the aortic sinus and coronary arteries, and myocardial fibrosis and damage in one year old *Sr-b1 KO* mice. In conclusion, anti-PCSK9 antibody mediated plasma cholesterol lowering and reduced newly developing atherosclerosis in the aortic sinus and coronary arteries as well as the continued development of established CAD induced by an HFCC diet in one year old *Sr-b1 KO* mice.

In chapter 3, the effects of whole body genetic *Pcsk9* deletion on CAD were examined in *Sr-b1/ApoE dKO* mice. *Sr-b1/ApoE dKO* mice rapidly and spontaneously develop a variety of characteristics reminiscent of human CAD, including occlusive coronary artery atherosclerosis and extensive myocardial fibrosis associated with cardiomegaly, cardiac damage and dysfunction, and premature death. Whole body genetic *Pcsk9* deletion in female and male *Sr-b1/ApoE dKO* mice did not change plasma cholesterol levels despite increased hepatic LDLR protein levels. However, *Pcsk9* inactivation in both female and male *Sr-b1/ApoE dKO* mice reduced aortic sinus and coronary artery atherosclerosis, myocardial fibrosis and damage, cardiac enlargement and dysfunction, cardiac conduction abnormality and myocardial accumulation of macrophages and lipids. The plasma cholesterol independent protection against CAD by whole body *Pcsk9* inactivation in *Sr-b1/ApoE dKO* mice appears to be also independent of the systemic inflammation levels. Macrophage and platelet accumulation in atherosclerotic plaques do not seem to contribute to the *Pcsk9* inactivation mediated reduction in coronary artery atherosclerosis. Restoring circulating PCSK9 by a

recombinant AAV8 vector containing a liver-specific promoter reversed the plasma cholesterol independent protection against atherosclerosis in both the aortic sinus and coronary arteries, myocardial fibrosis and damage, and myocardial lipid accumulation in *Pcsk9/ Sr-b1/ApoE tKO* mice. Disrupting the interaction between the LDLR and PCSK9 by anti-PCSK9 antibody treatment in both female and male *Sr-b1/ApoE dKO* mice increased circulating PCSK9 levels by ~10 fold and enhanced hepatic LDLR protein levels. However, it did not change plasma cholesterol levels consistent with previous results. Anti-PCSK9 treatment in *Sr-b1/ApoE dKO* mice reduced aortic sinus and coronary artery atherosclerosis in males but not females. Despite the reduction of coronary artery atherosclerosis in males, anti-PCSK9 antibody treatment did not affect myocardial fibrosis and damage, cardiac enlargement and cardiac dysfunction in either male or female *Sr-b1/ApoE dKO* mice. The surprisingly significant promotion in myocardial fibrosis despite the only modest increases in coronary artery atherosclerosis mediated by restoring circulating PCSK9 in *Pcsk9/ Sr-b1/ApoE tKO* mice as well as the absence of effects on myocardial fibrosis despite reduced coronary artery atherosclerosis by anti-PCSK9 antibody treatment in male *Sr-b1/ApoE dKO* mice suggested a direct role of circulating PCSK9 on the myocardial fibrosis, potentially mediated by myocardial targets of circulating PCSK9. Nevertheless, the direct effects of circulating PCSK9 on the myocardium do not appear to involve cell surface CD36 in cardiac cells. In conclusion, circulating PCSK9 promotes coronary artery atherosclerosis and myocardial fibrosis in *Sr-b1/ApoE dKO* mice independently of plasma cholesterol levels and these plasma cholesterol independent effects of circulating PCSK9 are largely independent of the

LDLR-PCSK9 interaction, particularly in females. The results from chapters 2 and 3 revealed the critical effects of circulating PCSK9 on coronary artery atherosclerosis and myocardial fibrosis via affecting plasma cholesterol levels or a plasma cholesterol independent manner. Overall, this thesis provides a comprehensive investigation and understanding of PCSK9 inhibition on coronary artery atherosclerosis and myocardial fibrosis in mouse models of CAD.

4.2 Implications from the Studies Detailed in Chapters 2-3

4.2.1 Role of PCSK9 Inhibition on Pre-existing Atherosclerosis

Clinical trials using intravascular ultrasound have shown that intensive plasma LDL cholesterol lowering mediated by statin therapy in human patients with pre-existing CAD reduced the progression of coronary atherosclerosis (Nissen et al., 2004) and even resulted in coronary atherosclerosis regression (Nicholls et al., 2011; Nissen et al., 2006). PCSK9 antibody (evolocumab) mediated reduction in plasma LDL cholesterol levels also led to the regression of coronary atherosclerotic plaques in patients treated with statin, however, the extent of plaque regression was less than 1.5% (Nicholls et al., 2016). Using a mouse model that develops an HFCC diet induced coronary artery atherosclerosis and has intact expression of both the LDLR and APOE, we have shown that PCSK9 antibody mediated plasma cholesterol lowering and reduced the progression of newly developing atherosclerosis in the aortic sinus and coronary arteries in male *Sr-b1 KO* mice, consistent with the results from clinical studies. However, PCSK9 antibody intervention only slowed

down the progression of pre-existing atherosclerosis in the aortic sinus and coronary arteries when *Sr-b1 KO* mice were continued with an HFCC diet and no plaque regression was observed in our study. Anti-PCSK9 antibody treatments reduced plasma total cholesterol levels by ~37% in one year old *Sr-b1 KO* mice fed the HFCC diet. However, anti-PCSK9 antibody in clinical trials reduced plasma LDL cholesterol levels by ~ 60% (Robinson et al., 2015; Sabatine et al., 2015). It is possible that the extent of plasma cholesterol lowering mediated by PCSK9 antibody in our study is not sufficient to induce atherosclerosis regression. On the other hand, atherosclerosis regression has not been tested in this mouse model previously. It could be that the absence of plaque regression may have been due to the mouse model used (*Sr-b1 KO*) or the experimental conditions employed (continued HFCC diet feeding rather than switching to normal chow diet). HDL plays an essential role in atherosclerotic plaque regression (Di Bartolo et al., 2018). Deficiency of SR-B1 impairs HDL mediated RCT and atheroprotective pathways mediated by HDL signaling (Trigatti & Fuller, 2016). Therefore, it is possible that atherosclerotic plaque regression may be impaired in mice lacking SR-B1. Atherosclerosis regression in mouse models is often induced by switching diets from a high fat, high cholesterol atherogenic diet to a normal diet (Feig et al., 2011; Peled et al., 2017; Raffai et al., 2005; Reis et al., 2001). Our experimental approach that mice were kept with the challenge of HFCC diet feeding may be also a factor contributing to the absence of plaque regression. If the switch from an atherogenic to a normal diet could be thought of as a model of lifestyle changes accompanying an intervention, the approach we used may model the intervention alone, without lifestyle changes. Further research is

required to determine if the addition of lifestyle changes in the form of reducing dietary fat and cholesterol consumption, in alone or in combination with the PCSK9 inhibition are able to achieve atherosclerotic plaque regression in *Sr-b1 KO* mice.

4.2.2 Role of Circulating PCSK9 on Coronary Artery Atherosclerosis Independent of its Plasma Cholesterol Lowering Effects

While some studies reported that inhibiting PCSK9 in *ApoE KO* mice did not affect atherosclerosis development with the absence of plasma cholesterol lowering, other studies have reported that inhibiting PCSK9 in *ApoE KO* mice reduced aortic atherosclerosis in a plasma cholesterol independent manner (Ason et al., 2014; Denis et al., 2012; Tang et al., 2017). However, it was not known whether the plasma cholesterol independent effects of PCSK9 on aortic atherosclerosis came from circulating PCSK9 derived from the liver or PCSK9 expressed locally in the vascular wall. Our study of genetic *Pcsk9* deletion in *Sr-b1/ApoE dKO* mice in chapter 3 supports the idea that PCSK9 inhibition can reduce atherosclerosis development in a cholesterol independent manner. By restoring liver-derived, circulating PCSK9 in *Pcsk9/Sr-b1/ApoE tKO* mice, we demonstrated it was circulating PCSK9 that promoted coronary artery atherosclerosis development in a plasma cholesterol independent manner. This is consistent with another study reporting circulating PCSK9 promoted vein graft lesion development, the late stages of which exhibit similar characteristics with atherosclerosis, in a plasma cholesterol independent manner (Katsuki et al., 2022).

We further demonstrated that the plasma cholesterol independent effects of circulating PCSK9 on aortic sinus and coronary artery atherosclerosis were independent of the interaction between the LDLR and PCSK9 in female mice and in contrast primarily dependent of the PCSK9-LDLR interaction in male mice, indicated by the results from PCSK9 antibody treatment in *Sr-b1/ApoE dKO* mice. However, the effects of circulating PCSK9 on vein graft lesion was independent of the LDLR in male mice (Katsuki et al., 2022). Potentially, circulating PCSK9 might promote atherosclerosis and vein graft lesion development in a plasma cholesterol independent manner via different mechanisms in male mice. On the other hand, other studies suggested transgenic overexpression of PCSK9 promoted atherosclerosis development in a plasma cholesterol independent manner, mainly via a LDLR mediated mechanism in both sexes (Denis et al., 2012) or in females (Tavori et al., 2016). Our studies tested the inhibition and restoration of PCSK9 at physiological levels in mice. It is possible that overexpression of PCSK9 affected atherosclerosis progression via pathways that are not mediated by normal physiological levels of PCSK9 expression, and this may explain the differences in the LDLR dependence observed on our studies versus other studies (Denis et al., 2012; Tavori et al., 2016). On the other hand, while those studies used *Ldlr KO* mice to test the LDLR dependence (Denis et al., 2012; Tavori et al., 2016), our study used a PCSK9 antibody which disrupts the PCSK9-LDLR interaction (Ason et al., 2014; Chan et al., 2009) and increases circulating PCSK9 levels (Oleaga et al., 2021). The increased circulating PCSK9 bound by PCSK9 antibody in our study might target the degradation of other receptors, such as VLDLR in a sex-specific manner (Roubtsova et al., 2015). This might

have an influence on atherosclerosis development and mask the effects of the LDLR-PCSK9 interaction on atherosclerosis development.

PCSK9 interacts with the LDLR primarily through the catalytic domain of PCSK9 and the EGF-A domain of the LDLR (Hyock et al., 2008; Surdo et al., 2011; D. W. Zhang et al., 2007). In addition, there is a minor interaction between the prodomain of PCSK9 (Leu108) and the β-propeller domain of the LDLR (Leu626) (Abifadel et al., 2012; Surdo et al., 2011). Furthermore, it has also been suggested that the positively charged C-terminal region of PCSK9 interacts with the negatively charged ligand binding domain of the LDLR (Tveten et al., 2012; Yamamoto et al., 2011). Therefore, multiple domains of both PCSK9 and the LDLR contribute to the PCSK9-LDLR interaction. The PCSK9 antibody used in our study is thought to sterically hinder the binding between the EGF-A domain of the LDLR and the catalytic domain of PCSK9, therefore inhibiting LDLR degradation (Ason et al., 2014; Chan et al., 2009). Although PCSK9 antibody interacts with residues from both the catalytic domain and prodomain of PCSK9 (Ason et al., 2014; Chan et al., 2009), other residues of PCSK9 that are important for the PCSK9-LDLR interaction, such as Leu108 of PCSK9, might be still exposed for interacting with the LDLR. Therefore, our study of PCSK9 antibody treated *Sr-b1/ApoE dKO* mice might only reflect the effects of inhibiting interactions between PCSK9 and the LDLR at specific sites on atherosclerosis development, while the studies utilizing *Ldlr KO* mice reflected the effects of absence of all interactions between PCSK9 and the LDLR on atherosclerosis development.

4.2.3 Direct Role of Circulating PCSK9 on Myocardial Fibrosis and Cardiac Function

PCSK9 has been reported to reduce cardiomyocyte survival and function in vitro (Xiong et al., 2021). However, contradictory results have been reported about the effects of PCSK9 on myocardial fibrosis and cardiac function ex vivo or in vivo. Genetic *Pcsk9* deletion in mice reduced infarct sizes and improved the recovery of cardiac function of hearts subjected to ischemia/reperfusion injury ex vivo (Schreckenberg et al., 2022). However, Pep2-8, a small peptide that mimics structural of the EGF-A domain of the LDLR and inhibits the PCSK9-LDLR interaction (Y. Zhang et al., 2014), did not have any effects on infarct sizes or cardiac function ex vivo when it was administrated both before ischemia and at the beginning of reperfusion (Schreckenberg et al., 2022). Furthermore, no effects on infarct sizes and cardiac function were observed by genetic *Pcsk9* deletion in mice subjected to ischemia/reperfusion injury in vivo (Schreckenberg et al., 2022). On the other hand, Pep2-8 administrated before ischemia, reduced infarct sizes and improved cardiac function in rats subjected to ischemia/reperfusion injury in vivo (Palee et al., 2019). It has also been reported that the PCSK9 antibody, evolocumab from Amgen Inc., which disrupts the interaction between the LDLR and PCSK9, reduced cardiac hypertrophy and improved cardiac function in C57BL/6J mice subjected to intraperitoneal injection of ox-LDL (Li et al., 2022). Another study reported that both genetic *Pcsk9* deletion and pretreatment with Pep2-8 reduced the development of myocardial fibrosis and cardiac dysfunction induced by left coronary ligation (Ding et al., 2018). In contrast, it was reported that whole body *Pcsk9* deficiency, but not the absence

of circulating PCSK9, promoted heart failure with preserved ejection fraction independently of the LDLR, presumably via regulating myocardial CD36 levels and lipid metabolism in the heart (Da Dalt et al., 2021). The studies above, therefore, provide contradictory evidence for PCSK9's impact and the role of the LDLR-PCSK9 interaction on cardiac fibrosis/function. Furthermore, the role of circulating PCSK9 versus PCSK9 expressed in the heart in these processes remain unclear. Importantly, our study in chapter 3 indicated that liver-derived, circulating PCSK9 might promote myocardial fibrosis and cardiac dysfunction that is independent of the LDLR-PCSK9 interaction. We have also provided evidence that the direct impacts of PCSK9 on myocardial fibrosis are not due to its regulation of CD36 in the myocardium. Other reported targets of PCSK9, such as MHC-I, VLDLR, and ApoER2 in the heart remains to be tested for their involvement.

The absence of effects on myocardial fibrosis and damage and cardiac enlargement despite reduced coronary artery atherosclerosis in male *Sr-b1/ApoE dKO* mice highlight the importance of utilizing mouse models that develop myocardial fibrosis and dysfunction instead of only focusing on traditional atherosclerosis mouse models (e.g. *ApoE KO* and *Ldlr KO* mice) for CAD research. Moreover, the absence of effects on myocardial fibrosis and damage, cardiac dysfunction and cardiac enlargement in both female and male *Sr-b1/ApoE dKO* mice when treated with PCSK9 antibody suggest the beneficial outcomes of PCSK9 antibodies on reducing CVEs in clinical trials may be largely contributed by the significant plasma cholesterol lowering. It would be important to look forward to the phase 3 clinical outcomes of *PCSK9* siRNA (inclisiran) and to see whether it would provide greater beneficial effects on reducing CVEs compared to

PCSK9 antibodies, since the *PCSK9* siRNA diminishes PCSK9 production in the liver and circulating PCSK9.

4.2.4 Importance of Analyzing Atherosclerosis in Both Sexes

Clinical studies have shown that PCSK9 mAbs lowered plasma LDL cholesterol levels with a greater extent in men compared to women, although whether it leads to a significant difference in long term cardiovascular risk reduction is unclear (Cordero et al., 2022; Paquette et al., 2023; Robinson et al., 2015; Sever et al., 2021). Furthermore, studies in mice indicated that *Pcsk9* deficiency resulted in a lower increase of hepatic cell surface LDLR levels in females compared to males, an effect attributed to estrogen (Roubtsova et al., 2015, 2022). Sex differences in humans are associated with the incidence of the modifiable risk factors for CVD, including smoking, dyslipidemia, hypertension, and diabetes mellitus, which might have an impact on atherogenesis (Man et al., 2020). In addition, sex hormones also contribute to modulate atherosclerosis development (Villablanca et al., 2010). Although sex is a critical biological variable for atherosclerosis development, preclinical studies on atherosclerosis that utilize both sexes of animal models are limited (Man et al., 2020). Ramirez and Hibbert examined 771 preclinical studies on atherosclerosis and other vascular disease that were published between 2006 and 2016 (Ramirez & Hibbert, 2018). Of those 771 studies, 18.8% did not specify the sex of animal models (Ramirez & Hibbert, 2018). Of those studies that specified the sex, only 24.1% used both sexes in their studies (Ramirez & Hibbert, 2018).

In chapter 3, anti-PCSK9 antibody treatment in *Sr-b1/ApoE dKO* mice did not affect plasma cholesterol levels in either females or males. However, the extent of atherosclerosis was differently modulated by anti-PCSK9 antibody treatment between female and male *Sr-b1/ApoE dKO* mice. Since *Sr-b1/ApoE dKO* mice used in our studies were only 6.5 weeks of age, the sex-dependent effects of anti-PCSK9 antibody on atherosclerosis may not be mediated by sex hormones, because mice only likely reached sexual maturity at 6.5 weeks of age. Nevertheless, our results and previous studies highlight the importance of analyzing both sexes in atherosclerosis studies and studying the interactions between sex and therapeutic interventions for CVD.

4.3 Limitations and Future Directions

Due to the limited availability of one year old female *Sr-b1 KO* mice in our lab, we used one year old male *Sr-b1 KO* mice for chapter 2 while simultaneously carrying out experiments for chapter 3. However, chapter 3 has indicated that treatment of PCSK9 antibody in *Sr-b1/ApoE dKO* mice protected against atherosclerosis in both the aortic sinus and coronary arteries independently of plasma cholesterol levels in males but not females. Therefore, the protection against pre-existing atherosclerosis in the aortic sinus and coronary arteries in male *Sr-b1 KO* mice by PCSK9 antibody could be driven by both plasma cholesterol lowering and factors independently of plasma cholesterol levels. If we had used one year old female *Sr-b1 KO* mice, we could evaluate the effects of PCSK9 antibody on pre-existing atherosclerosis mediated by plasma cholesterol lowering

exclusively. Furthermore, the majority of coronary artery occlusions was less than 50% and there was not substantial myocardial fibrosis development (only 0.4% to 2.9%) in one year old male *Sr-b1 KO* mice fed an HFCC diet for 12 weeks. We could have examined the survival of male *Sr-b1 KO* mice switched to an HFCC diet at one year old of age. If the survival of male *Sr-b1 KO* mice allows, we could have maintained one year old male *Sr-b1 KO* mice on an HFCC diet for a longer period than 12 weeks. This would probably allow one year old male *Sr-b1 KO* mice to develop increased extents of more than 50% and/or fully occluded coronary artery atherosclerotic plaques and substantial myocardial fibrosis. Although atherosclerotic plaque sizes in the aortic sinus and coronary arteries were measured in chapter 2, indexes of plaque stability were not examined, such as the ratio of macrophage to SMCs in the plaque. Measurements of plaque stability would give a more comprehensive characterization of the effects of PCSK9 antibody on pre-existing atherosclerosis.

The treatment of rAAV8 vector expressing eGFP alone in *Pcsk9/Sr-b1/ApoE tKO* mice resulted in cardiomegaly and we were not able to evaluate the effects of restoring circulating PCSK9 on cardiomegaly in chapter3. rAAV8 exhibit tropism for the liver, skeletal and cardiac muscles, central nervous system, and pancreas (Nakai et al., 2005). However, recombinant AAV-DJ developed recently by using a gene shuffling method, has liver specific tropism (Grimm et al., 2008). Therefore, unlike AAV8, AAV-DJ vector may do not result in cardiomegaly in *Pcsk9/Sr-b1/ApoE tKO* mice and could be used with a liver-specific promoter to evaluate the effects of restoring circulating PCSK9 on cardiomegaly.

The naturally occurring mutations in *PCSK9* affect plasma LDL cholesterol levels via different mechanisms, including PCSK9 synthesis, PCSK9 processing, PCSK9 secretion, PCSK9 stability and the affinity of PCSK9 to the LDLR (Horton et al., 2007). Y142X and C679X, loss of function mutations in *PCSK9*, were associated with a 28% reduction in LDL cholesterol levels (absolute reduction in LDL cholesterol levels: 39 mg/dL (1 mmol/L)), however, they were associated with an 88% reduction in coronary heart disease (CHD) (Cohen et al., 2006). Another loss of function mutation in *PCSK9*, R46L, was associated with a 15% reduction in LDL cholesterol levels (absolute reduction in LDL cholesterol levels: 21 mg/dL (0.54 mmol/L)), and a 47% reduction in CHD (Cohen et al., 2006). These associations were striking as the Cholesterol Treatment Trialist has estimated that 1 mmol/L of LDL-C reduction by statin therapy was associated with a 21% reduction in cardiovascular risk (Mihaylova et al., 2012). However, life-long LDL cholesterol lowering (1 mmol/L) has been associated with a greater extent of reduction in CHD (54.5%) (Ference et al., 2012), which might partially explain the greater reduction in CHD in association with *PCSK9* variants (Y142X, C679X and R46L) that only results in a modest LDL cholesterol lowering. However, it is also possible that those *PCSK9* variants have an effect on CHD and MI independent of the LDL cholesterol levels.

The AAV-DJ mediated expression of *PCSK9* variants in the liver of *Pcsk9/Sr-b1/ApoE tKO* mice could be used to test the effects of those *PCSK9* variants on coronary artery atherosclerosis and myocardial fibrosis in a plasma cholesterol independent manner. The Y142X variant occurs early in the transcript and results in no PCSK9 protein

produced and the C679X variant interferes with the secretion of PCSK9 (Zhao et al., 2006). On the other hand, R46L variant does not interfere with PCSK9 synthesis, processing or secretion (Zhao et al., 2006). Therefore, AAV-DJ could be used to express R46L variant in the liver of *Pcsk9/Sr-b1/ApoE tKO* mice to study the structural basis of circulating PCSK9 on coronary artery atherosclerosis and myocardial fibrosis.

Our results in chapter 3 have indicated that PCSK9 might directly promote myocardial fibrosis. However, the downstream target(s) of circulating PCSK9 in the heart that might be involved in the myocardial fibrosis development is unclear. In the future, if VLDLR, ApoER2 or MHC-I are identified as potential downstream targets of PCSK9 in the heart, treatment of *Pcsk9/Sr-b1/ApoE tKO* mice with AAV-DJ expressing *Pcsk9* variants that disrupt the interaction between PCSK9 and the potential target could be used to test the effects of PCSK9 mediated degradation of the potential target on myocardial fibrosis development.

4.4 Conclusions

Chapter 2 of this thesis demonstrates that PCSK9 antibody mediated plasma cholesterol lowering protects against both newly developing and pre-existing atherosclerosis in the aortic sinus and coronary arteries induced by an atherogenic HFCC diet in one year old male *Sr-b1 KO* mice. Furthermore, PCSK9 antibody also protects against myocardial damage and fibrosis in one year old male *Sr-b1 KO* mice fed the HFCC diet. Chapter 3 of this thesis demonstrates that liver-derived, circulating PCSK9

promotes atherosclerosis in the aortic sinus and coronary arteries, myocardial damage and fibrosis, cardiac dysfunction and cardiomegaly in a plasma cholesterol independent manner in *Sr-b1/ApoE dKO* mice. Those plasma cholesterol independent effects of circulating PCSK9 are largely independent of the LDLR-PCSK9 interaction, especially in females. The results from this thesis highlight circulating PCSK9 promotes CAD development via both cholesterol-dependent and cholesterol-independent pathways. Future studies should focus on uncovering the downstream target of PCSK9 in the heart that might be involved in myocardial fibrosis development and also the structural basis of PCSK9 on affecting coronary artery atherosclerosis and myocardial fibrosis in a plasma cholesterol independent manner utilizing *Pcsk9/Sr-b1/ApoE tKO* mice with the treatment of AAV-DJ expressing different *PCSK9* variants.

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