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Characterizing the expression and the function of *CARMN* lncRNA and miR-143/145 in human and mouse atherosclerosis.

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Author's Declaration

I declare that this PhD thesis has been written entirely by myself as a summary of the work I have performed during my PhD, under the direct supervision of Professor Andrew H. Baker, Dr. Laura Denby and Professor Judith Sluimer. This research was carried out at the Queen Margaret Research Institute, Centre for Cardiovascular Science in Edinburgh, with the exception of histological analysis of *CARMN* knock-out and wild type mouse plaque which were carried out by me in Maastricht University (Maastricht) under the supervision of Professor Sluimer with the help from Dr. Kim van Kuijk, Jenny de Bruijn and Dr. Marion Gijbels. The Fluorescent In Situ Hybridization was carried out with the professional help of Dr. Amira Mahmoud. In addition, laser capture microdissection was carried out by me at Columbia University (New York), under the supervision of Professor Ira Tabas and the professional guidance from Dr. Amanda C. Doran and Kuriakose George. The *in vivo* study design and experiment was performed under the supervision of Dr. Laura Denby and Professor Judith Sluimer in the animal house facility in Edinburgh. Optical projection tomography, as well as following histological analysis of mouse tissue were performed under the supervision of Professor Patrick Hadoke and the professional help from Dr. Eileen Miller. The RNA sequencing design and analysis were performed under the supervision of Dr. Julie Rodor. This PhD thesis has not previously been submitted for a higher degree.

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Determination, passion, creativity and versatility are the best tools to draw the path to your goals.

List of publications and presentations

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Summary

Atherosclerosis is a progressive and chronic condition that belongs to the group of Cardiovascular Diseases (CVDs) which represents the first cause of death worldwide. Although recent advances in strategies for studying the mechanisms underlying the disease progression have led to better understanding of the pathological phases, there is still a lot that needs to be done in order to have a complete knowledge of the molecular factors implicated in the pathophysiology of atherosclerosis. In particular, considering the complexity of atherosclerosis, understanding the processes involved in the advancement from early to advanced plaque phenotype, constitutes an essential step towards monitoring the disease progression.

Noncoding RNAs, including small noncoding RNAs (miRNAs) and long noncoding RNAs (lncRNAs), have been characterised as important molecules involved in the regulation of physiological cellular processes and the pathology of diseases. MiR-143/145 is a conserved microRNA cluster involved in the maintenance of vascular smooth muscle cells (vSMCs), whose dysregulation can affect the vSMC phenotype. This is one of the key cellular components involved in the pathophysiology of vascular disease, and specifically in atherosclerosis. Studies have shown that the dysregulation of miR-143/145 affects vSMC function during vascular injury and repair, leading to vSMC phenotypical transition towards a pro-pathological state. *CARMN* is a conserved lncRNA host gene for miR-143/145, whose role was not explored in vSMC phenotypic transition.

In this project, *CARMN* axis role, including *CARMN* and miR-143/145, was explored in dysregulated vSMC *in vitro* and *in vivo* during atherosclerosis. Key findings of this study have shown the downregulation of *CARMN* and miR-143/145 during the advancement from early to advanced plaque, in both human and mouse atherosclerosis. Functional studies performed in vSMC, identified dysregulated pathways, - following *CARMN* axis depletion, which were mostly involved in the regulation of vSMC proliferation, migration, inflammation, and lipid homeostasis. In

addition, the expression of miR-143/145 was affected following *CARMN* depletion. In this study, *CARMN* downregulation was identified as primary regulator of vSMC proliferation independently from the regulation of miR-143/145, whereas the dysregulation of miR-143/145 expression was found involved in the modulation of vSMC migration and de-differentiation towards a macrophage-like phenotype. Herein, *CARMN* axis role was also explored in an atherosclerotic mouse model of the disease. In particular, the genetic depletion of *CARMN*, and associated miR-143/145, induced a more advanced plaque phenotype characterised by increased plaque volume and area versus wild type controls. In addition, the knock-out of *CARMN* enhanced the content of pro-inflammatory cells, increased cell proliferation of cells within the plaque and reduced the collagen content compared to wild type mice.

Collectively, this study identified *CARMN* loss as a detrimental key event occurring in early plaques, which primes vSMC to a dysregulated phenotype *in vitro*, and leading to more advanced lesions *in vivo*.

Lay summary

The pathology of atherosclerosis is characterised by progressive accumulation of lipid particles in the blood vessels of the cardiovascular system, local inflammation of the arteries, and dysregulation of cells of the arterial wall. In particular, cells composing the arterial wall start to increase in number, process known as cell proliferation, move within the layers of the vessel wall, referred to as cell migration, and acquire new capabilities from their differentiated state, process therefore known as cell de-differentiation. The combination of these processes leads to disorganised vessel structure and formation of a plaque within the vessel which in turn impedes the correct flow of the blood through the vascular system. Clinical events associated with the formation of the plaque within the vessel can lead to acute cardiovascular pathologies such as myocardial infarction or stroke. Nowadays, atherosclerosis is the leading cause of death worldwide and understanding the processes that are critical during the development of the plaque is fundamental. Importantly, recent discoveries have shown that a group of cellular components belonging to the RNA family and do not encode proteins, referred to as non-coding molecules, are important in the development of the disease and control cellular processes. Moreover, the expression of these noncoding molecules within the cells has been identified enhanced or decreased during the progression of the plaque formation.

This PhD project studied the role of three noncoding molecules, *CARMN*, miR-143 and miR-145 (miR-143/145), located in close proximity to each other in the human and mouse genome. Herein, the cellular levels of *CARMN* and miR-143/145 were found decreased in human and mouse plaque compared to healthy controls. By using cell models of disease in which vascular smooth muscle cells, abundant cells composing the vascular wall, are treated with inflammatory molecules, *CARMN* and microRNAs were found downregulated in their expression. However, when cells were induced to migrate, the expression of the axis was not significantly affected.

To study the function of this genetic locus, *CARMN* and miR-143/145 were artificially decreased in their expression levels. This strategy led to enhanced cellular proliferation, migration and de-differentiation. In particular, in this study the downregulation of *CARMN* noncoding RNA regulated cell proliferation, whereas the downregulation of miR-143/145 controlled cell migration and de-differentiation. When *CARMN* and miR-143/145 were artificially cut from the mouse genome in a mouse model of atherosclerosis, mice developed bigger and more complex plaques versus mice expressing the three noncoding RNAs, therefore suggesting that the loss of *CARMN* and miR-143/145 expression can have adverse effect during the progression of atherosclerosis.

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List of abbreviations

AAV: adeno-associated virus

ABCA1: ATP-binding transporter cassette 1

ACTA2: Smooth muscle actin 2

ApoE: apolipoprotein E

ASO: antisense oligonucleotides

CAGE: cap analysis gene expression

CARMN: cardiac mesoderm enhancer-associated noncoding RNA

CARMN^{-/-}: *CARMN* knock-out

CARMN^{+/+}: *CARMN* wild type

CD68: Cluster of Differentiation 68

CMBCD: Cholesterol-Methyl- β -Cyclodextrin

CNN1: Calponin 1

COL: Collagen

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

DsiRNAs: Dicer-substrate Short Interfering RNAs

ECM: Extracellular Matrix

EdU: 5-ethynyl-2'-deoxyuridine

ELN: Elastin

FACS: Fluorescence-activated Cell Sorter

Gap: GapmeR

HCASMCs: Human Coronary Artery Smooth Muscle Cell

HCD: High Cholesterol Diet

HCPCs: Human Cardiomyocytes Progenitor Cells

ISH: In-Situ Hybridization

IP: Intraperitoneally

KLF4: Kruppel-like factor 4

LCM: Laser Capture Microdissection

LDLR^{-/-}: Low Density Lipoprotein Receptor Knock-out

LGALS33: Galectin 3

LXR: Liver X Receptor
LncRNA: long noncoding RNA
MYH11: Myosin Heavy Chain 11
MiRNAs: miRNAs
NHEJ: Non-homologues End Joining
OPT: optical projection tomography
ORO: Oil Red O
Ox-LDL: Oxidised Low-Density Lipoprotein
PCSK9: proprotein convertase subtilisin/kexin type 9
PDGF: platelet-derived growth factor
PDGFRa: platelet-derived growth factor receptor alpha
qRT-PCR: quantitative real-time polymerase chain reaction
RACE: Rapid amplification of cDNA ends
RNA-seq: RNA sequencing
RXR: Retinoid X Receptor
TAGLN: Transgelin
TALEN: Transcription activator-like effector nucleases
TSS: Transcriptional Start Sites
UBC: Ubiquitin C
VSMCs: Vascular Smooth Muscle Cells
ZFN: Zinc Finger Nucleases

Chapter 1 Introduction

1.1 Cardiovascular diseases

The group of Cardiovascular Diseases (CVDs) (according to the International classification of Diseases, 11th Revision, ICD-11) includes a wide range of pathologies affecting the circulatory system and the heart, including hypertensive diseases, ischemic heart diseases, congenital heart diseases, and cerebrovascular diseases. This large group of pathologies is the leading cause of deaths and financial burden globally¹. Indeed, as reported by the most current global statistics on the causes of deaths worldwide, the estimated number of deaths for CVDs is 17.8 million, representing 30% of all recorded deaths, of which 9 million are due to ischaemic heart diseases² (Figure 1). According to the European Social Survey, CVD causes more than 4 million deaths (45% of all deaths) in Europe every year, of which 1.8 million stem from ischemic heart disease (IHD)³. Moreover, despite the decrease in the mortality rate observed from 2010 to 2017 due to improvements in prevention strategies, the absolute number of deaths increased by 22.7% during the same period of time. This has a large impact on the total healthcare costs across Europe and globally. The global burden of disease project updated the list of behavioural risk factors that can be monitored to achieve a decline in cases, which includes unhealthy diet, smoking, physical inactivity, and alcohol consumption. In addition to this, existing medical conditions, such as hypertension, hypercholesterolemia, obesity, and hyperglycaemia have a cumulative effect and critical role in determining cardiovascular diseases⁴. The current strategies to manage CVD include pharmaceutical treatments and surgical interventions. Secondary prevention includes therapeutic approaches aimed at managing risk factors by lowering low-density-lipoproteins (Statins⁵, Protein convertase subtilisin kexin type 9⁶), blood pressure (Beta-blockers, Calcium channel blockers, Angiotensin converting enzyme inhibitors⁷), glucose levels (Metformin, Sodium-glucose co-transporter-2 inhibitors⁸), and platelet aggregation (Aspirin, Clopidogrel⁹). In addition to pharmacotherapies used for secondary prevention, patients with more severe symptoms undergo

surgical revascularization of blocked coronary or carotid arteries (through coronary artery bypass grafting, angioplasty, atherectomy, stent placement) or replacement of damaged valve or heart tissue (through cardiomyopathy, artificial heart valve surgery, or heart transplant) or procedures for the treatment of arrhythmias (such as radiofrequency ablation, or pacemaker implantation). Despite recent progress in secondary prevention and therapeutic intervention strategies, CVDs each year account for €210 billion of the total healthcare costs in Europe, of which €56.7 billion (27%) is attributable to IHD. Therefore, CVD is not only a health problem, but it represents a global economic challenge that is expected to become more pronounced in the next few years. Indeed, it is expected that the global costs for CVDs will exponentially increase by 2030¹⁰. Importantly, these statistics emphasize the need for improvements in CVD prevention strategies, in the policies aiming at improving cardiovascular health and treatments, and importantly highlight the exigence to explore more molecular pathways underlying the development of disease.

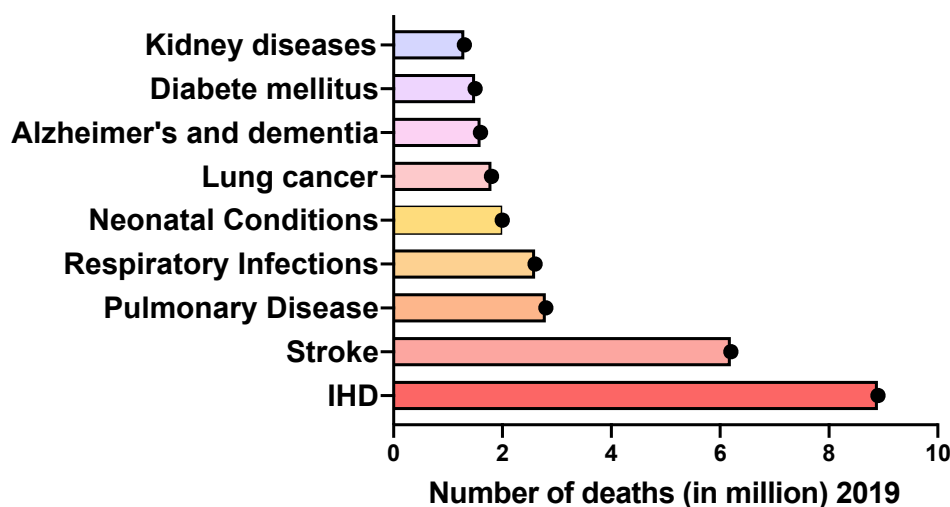


Figure1.1 Leading causes of death globally.

The top causes of death globally can be divided into categories and are represented in this histogram, adapted from WHO statistics (webpage below), which counts the millions of deaths registered in 2019. In 2019, these categories of deaths accounted for the 55% of the 55.4 million of deaths. IHD represents the world's top category, being responsible for the 16% of total deaths, followed by stroke and pulmonary

obstructive disease. Source: WHO Global Health Estimates,
<https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death>

1.2 Physiology of systemic arteries

The vascular system is composed by extensive network of different vessels (arteries, arterioles, capillaries, venules, and veins) which display homologies in their structural organisation and regulate blood flow and circulatory system homeostasis. Globally, the vessel structure and function has evolved to provide distal organs with oxygen supply and nutrients, as well as to favour the removal of unneeded metabolic products¹¹. Generally, arteries and veins of the systemic circuit are in charge of blood transportation in opposite directions: systemic arteries transport oxygenated blood to the peripheral tissues and organs, whereas following oxygen delivery to cells composing peripheral tissues, systemic veins return blood to the heart¹². Therefore, these vessels are subjected to different pressures and have developed specific anatomical features which confer to arteries more structure and thicker layers, while veins are generally characterised by thinner walls and larger lumen. In regard of the anatomical homologies of the different blood vessels, these are represented by layers of cells forming membrane or “*tunica*” surrounding the internal lumen (Figure 1.1).

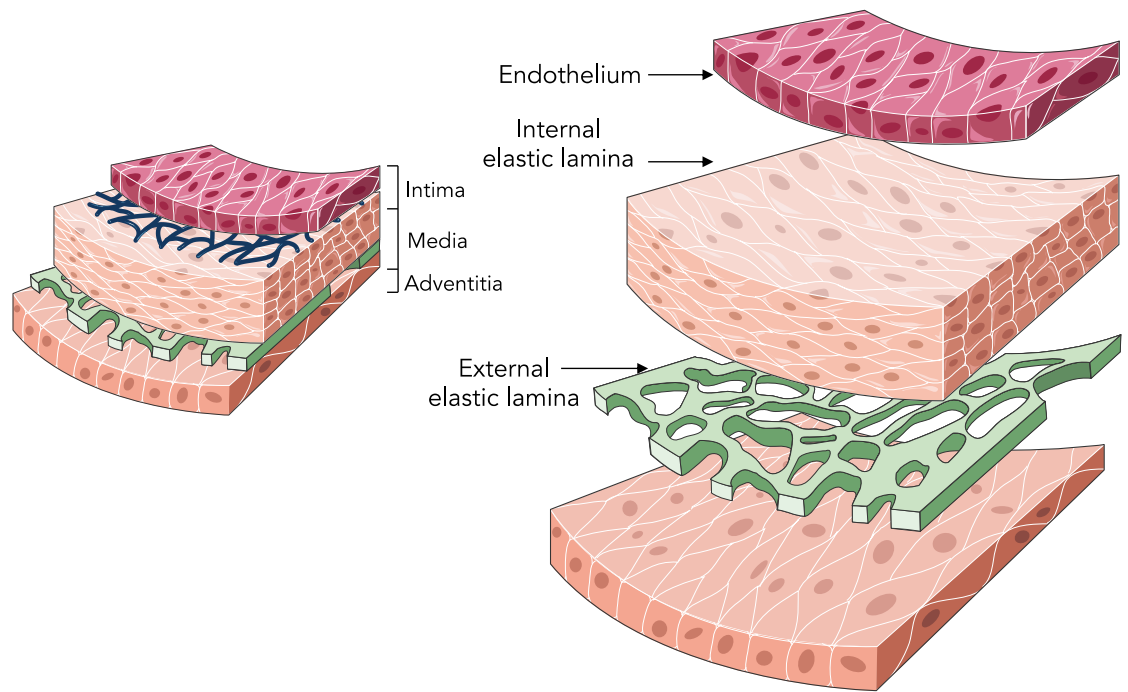


Figure 1.2 Anatomic organisation of systemic arteries.

Systemic arteries are organised in multi-layer structure composed by specialised *tunica* membranes surrounding the arterial lumen. The *tunica intima* is the innermost layer in direct communication with the systemic circulation composed by a monolayer of endothelial cells embedded in connective tissue referred to as internal elastic lamina. This layer of extracellular material connects the *tunica intima* with the *tunica media* mainly composed by multilayers of smooth muscle cells which provides the vessel with contractility and relaxation abilities to respond to changes in blood flow pressure. Smooth muscle cells are in contact with the external *tunica adventitia* by a layer of connective elastic tissue, external elastic lamina. This represents the outermost layer of the vessel wall, and it is composed mostly by fibroblasts which confer structural strength to the vessel. Image adapted by Servier Medical Art <https://smart.servier.com/>.

The most inner layer of cells in contact with the vessel lumen is referred to *tunica intima* composed by a single layer of endothelial cells embedded in a basal membrane constituted by connective extracellular material. The basal lamina binds to the

endothelium conferring structure and provides a conjunction with the sub-endothelial connective tissue which provides stability to the endothelium and flexibility to the vessel wall¹³. Endothelial cells are the most abundant cell type forming this layer, which specialised cells in the physiological exchange of nutrients and cellular/chemical interactions of the vessel wall with the systemic circulation. Pericytes, contractile multipotent cells with the ability to differentiate to different cell types (such as fibroblasts, smooth muscle cells, osteoblasts) are also present in conjunction with endothelial cells. Damage of this layer due to mechanical and chemical stress, can have important consequences to the correct functioning of the vessel and pathological remodelling observed in cardiovascular diseases, such as oxidative stress and promotion of local inflammation. In large arteries, a layer of elastic fibres referred to as *internal elastic lamina* confers structure and provides a connection platform for the exchange of chemical mediators with the above layer of smooth muscle cells known as *tunica media*¹⁴. This is generally the thickest layer of the arteries and it is predominantly constituted by organised layers of contractile smooth muscle cells and extracellular matrix components including collagen and elastin. The contraction and relaxation of smooth muscle cell in response to external stimuli regulates the vasodilatation and vasocontraction functions of the vessel blood modulating blood pressure. Dysfunction of these tightly regulated cellular processes in response to mechanical and chemical cues, can cause smooth muscle cell de-differentiation, disruption of the structural organisation of the media and maladaptive remodelling characterizing cardiovascular disease. The medial layer is connected with the outer layer by a distinct external elastic lamina which appears as layers of connective tissue. This layer confers structural strength as well as flexibility to the vascular wall¹¹. The *tunica adventitia* is instead the outmost layer of the arteries mostly composed by fibroblasts immersed into fibro-elastic connective tissue which protects and holds the entire vessel in relative position. This layer is considered one of the key components for the storage and release of chemical mediators regulating vascular cell function. In addition, the presence of multipotent progenitors results critical during the phases of repair mechanisms in vascular injury¹⁵.

1.2.1 vSMCs function in the maintenance of vascular tone.

During embryogenesis, vSMC can derive from two main sources, the mesoderm and ectoderm of the neural crest¹⁶. VSMCs composing the cardiac tract, including aorta and aortic arch vessels, differentiate from the neuroectoderm, from which fibroblasts also differentiate¹⁷. In the adult differentiated state, vSMC are characterised by a quiescent, non-proliferative, contractile phenotype. In this context, vSMCs retain a characteristic fusiform morphology, with a central nucleus, oriented in circular layers composing the tunica media. This phenotype is defined by a specific transcriptional profile and associated cellular functions. To absolve contractility functions, vSMC are provided with a contractility apparatus which includes calcium (Ca^{2+}) uptake/release pumps located in the sarcoplasmic reticulum and a cytoskeleton composed by the polymerization of a variety of filaments interconnected comprehending tubulin, actin, myosin and intermediate filaments of desmin and vimentin. When stimulated by humoral factors, vSMC activate their contractility functions and transmit these signals to adjacent cells using gap junctions¹⁸. The reception of external stimuli and the contraction/relaxation response is mediated by a vSMC marker protein and modulates vascular tone. The degree of differentiation is a gradual process in vSMC during embryonic development, which can be divided into phases, where vSMC start to express the markers required for contractility function: i) early differentiation markers (smooth muscle alpha actin, myocardin, smooth muscle 22 alpha¹⁹), ii) mid-term (h-caldesmon and smooth muscle calponin²⁰) and iii) late (desmin, myosin heavy chain, smoothelin²¹). These markers are involved in the regulation of fundamental vSMC physiological functions such as cell cycle, cell growth, contraction, cytoskeleton assembly, and their expression is dynamically modulated during vascular development in response to external stimuli. The capability to dynamically express these differentiation markers constitutes a source of tissue heterogeneity for vSMC and characterise this cell type with their high plasticity. Together with their contractility function, this cell type maintains vascular structure and stability through the secretion of extracellular matrix components (EMC), such as collagen and elastin, which in turn mediate the reception of external signals for cell proliferation, adhesion

or migration²². Extracellular matrix organisation and shear stress regulate vSMC behaviour. Under high shear stress conditions, vSMCs are induced to align with a parallel orientation to the direction of the flow, show a low proliferation rate, low synthetic activity and specific ion channel expression²³ required to fulfil contractile functions (Figure 1.3).

1.3 Atherosclerosis: underlying cause of ischaemic heart diseases.

The word ischemia derives from the Greek *iskhaimos* “stopping blood” and refers to the condition of a tissue or organ lacking adequate blood and oxygen supply. IHDs are the prevailing causes of CVDs and are a group of debilitating pathologies caused by inadequate perfusion of the heart and circulatory system, and include myocardial infarction, angina pectoris, stroke, vascular dementia, and peripheral vascular diseases. Atherosclerosis constitutes the primary underlying condition of all the above-mentioned clinical manifestations, and can be described as a chronic, progressive, and multifactorial disease of the vascular system, affecting middle and large-size arteries (including the aorta, coronary and carotid arteries or peripheral arteries). The pathologist Felix Jacob Marchand (1846-1928) was the first to use the term “atherosclerosis”, derived from the Greek words *athero* = gruel and *sclerosis* hardening in his book *Transplantation (Handbuch der Allgemeinen Pathologie)*, to indicate the combination of different factors including prolonged inflammation^{24,25}, dysregulated vascular cells^{26–29,30}, accumulation of circulating lipids^{31,32}, oxidation and genetic predisposition^{33,34}. Studies from two pathologists with opposite view, Carl von Rokitansky and Rudolf Virchow, elucidated the role of inflammation and cholesterol accumulation in the disease. While Rokitansky attributed a secondary importance to the development of inflammation, Virchow supported the idea that inflammation was a driver factor to the development of pathological arterial remodelling characterising atherosclerosis³⁵. The perspective that atherosclerosis is not a disease itself but a response of the vasculature to external stress conditions, initially as a protective adaptative mechanism, was comprehensively described in the late 1980s³⁶. In particular, the evidence that a gradual cascade of phenomena was

involved in the plaque's formation in childhood, derived from observational studies in necropsies of aortas from individuals of different ages (1 to 40 years old) where formation of initial accumulation of fat and immune cells, defined as "fatty streak", was found in children at the age of 1 year³⁷. Since then, progress in the study of the cellular processes, chemical and mechanical factors involved has allowed a more detailed description of the stages of plaque formation and progression from early to advanced lesions (Figure 1.2). During the first stages of plaque formation, the activation of the endothelium has been described as the critical factor initiating the cascade of events leading to the initial fatty streak's formation. The prolonged exposure of endothelial cells to excess lipids in the circulation and hemodynamic conditions in arterial branches induces the expression of adhesion molecules (such as vascular cell adhesion molecule 1) and secretion of vasodilatory agents (nitric oxide synthase), which in turn recruits blood monocytes to the site of injury³⁸. Monocytes adhering to the endothelium surface differentiate into macrophages, which extravasate to reach the subendothelial space. Infiltrated macrophages favour the recruitment of other inflammatory cells (e.g. monocytes, T cells) by secreting chemotactic and pro-inflammatory molecules (such as monocyte chemotactic protein 1), and internalising lipoproteins through the expression of scavenger receptors (such as CD36, scavenger receptor A1 and lectin-like oxidised LDL receptor-1)³⁹. Therefore, the activation of endothelium, formation of cytotoxic lipid-laden macrophages (foam cells) and recruitment of T cells are key factors contributing to the development of the first visible early lesion, fatty streak⁴⁰, which can stabilise and regress or develop to plaque⁴¹. If the pro-inflammatory and atherogenic stimuli persists over years (<20 year of age), this initial streak of foam cell can gradually develop into early fibroatheroma. Following exposure to pro-inflammatory cytokines and growth factors (such as platelet-derived growth factor), smooth muscle cells activate a reparative response. These cells migrate from the medial to the intimal layer of the arterial wall, start to proliferate and secrete a collagen-rich matrix, forming a protective layer named the fibrotic cap, which confers stability to the plaque. Increasing accumulation of blood lipids causes a disbalance in the

macrophages' cholesterol clearance, wherein foam cells enhances the inflammatory cascade, promoting lipid retention and secreting extracellular matrix degradation. The transition of the plaque to a more complex phenotype (generally occurring in individuals of > 55 years old) is characterised by the apoptosis and necrosis of lipid-laden foam cells, release of apoptotic toxic debris, enhanced oxidative stress⁴² and formation of a large necrotic core within the plaque, which progressively contributes to luminal narrowing⁴³. Studies investigating the factors influencing the progression to vulnerable lesions observed that the erosion of the fibrous cap by matrix metalloproteases (thin-cap fibroatheroma (TCFA)⁴⁴), a large necrotic core, a compensatory outward remodelling of the arterial wall⁴⁵, calcification⁴⁶, neovascularization and intraplaque haemorrhage^{47,48} have a determining role in the formation rupture-prone plaques, precursors of coronary syndrome. These complex and advanced lesions appear later in the clinical history of patients (individuals between 55 to 65 years), and silently localise mostly in the proximal region of the coronary artery⁴⁹. Their rupture was reported in the 60% of autopsies from sudden cardiac deaths⁵⁰. A simplified representation of the cascade of events above described can be found in Figure 1.3.

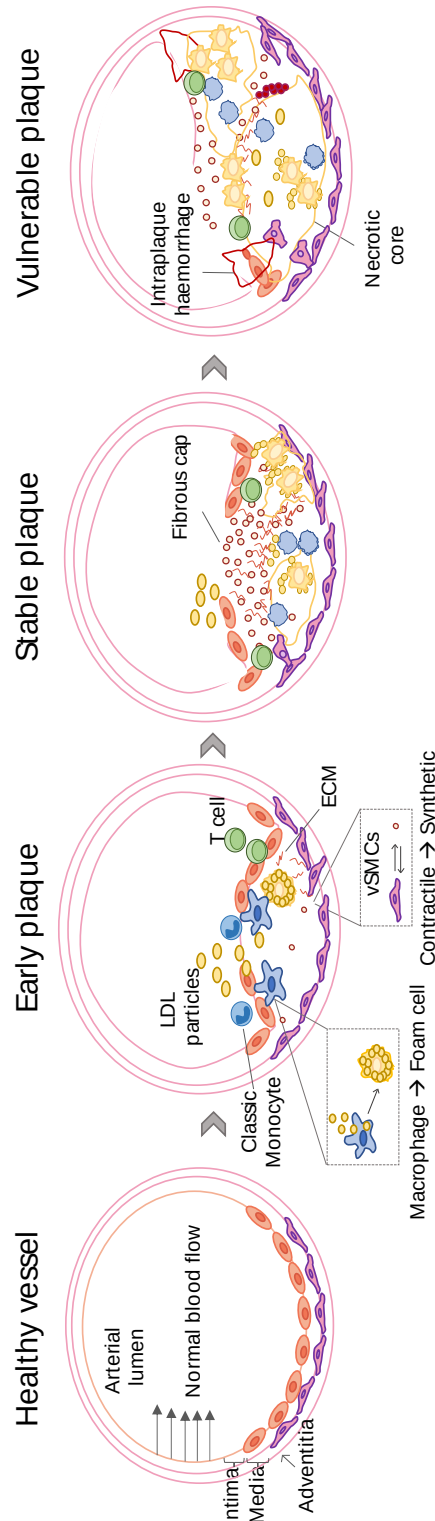


Figure 1.3 Simplified cartoon representing key phases in human atherosclerotic plaque development.

Atherosclerosis is a multi-step process which starts early in life in susceptible regions of the vasculature. Healthy vessels are characterised by a very well organised structure in layers where cells composing each layer contribute to the maintenance of vessel contractility and homeostatic blood flow. The increase in systemic cholesterol was identified as one of the first events initiating the early formation of fatty streak characterised by accumulation and interactions of lipid particles with the endothelium. This stimulation increases endothelial cell's ability to express adhesion molecules, which in turn recruit monocytes and T cells to the endothelial surface. Monocyte adhesion is moreover favoured by the secretion of pro-inflammatory and chemotactic molecules by dysregulated endothelium. Dysfunctional vascular endothelium is moreover characterised by elevated levels of permeability which allow monocytes to penetrate to the sub-endothelium space, together with LDL molecules, and activate into phagocytic macrophages. The uptake of LDL and oxidised LDL molecules by macrophages in turn leads to the formation of foam cells and enhancement of pro inflammatory cascade. The pronounced stimulation of VSMC with pro-pathological stimuli (nitric oxide, PDGF-BB, ILS, TGF- β) induce VSMC to phenotypically switch into pro-migratory and pro-proliferative phenotype. Pro-pathological stimuli favour the progression from early stages to stable plaque mainly characterised by the formation of initial necrotic core by foam cell and EC death and a thick fibrous cap surrounding the core, rich in proteoglycan. Prolonged inflammation and secretion of metalloproteases lead to degradation of extracellular matrix components (ECM) and thinning of the fibrous cap, key hallmark of advanced and vulnerable plaque. The continuous increase in ox-LDL particles moreover favour VSMC-derived foam cell formation with consequent increase in VSMC apoptosis contributing to the necrotic core pool. Erosion of the fibrous cap, intraplaque haemorrhage and calcification in turn contribute to the exposure of necrotic core whose exposure to the systemic blood flow might ultimately lead to a release of toxic molecules, thrombosis and cardiac death.

Figure 1.3 Simplified cartoon representing key phases in human atherosclerotic plaque development.

1.3.1 Morphological classification of human plaques.

The pathophysiological features characterising the phases of atherosclerotic plaque formation have been classified by observational studies using histological examination of human lesions, and divided into gradual stages (designed by Roman numerals) reflecting the temporal succession of processes from the initial appearance and accumulation of immune cells to the advanced lesions causing clinical syndromes^{51,52}. In particular, the committee on Vascular Lesions of the Council of Atherosclerosis (American Heart Association^{40,51,52}) described a total of six lesions (from type I to type VI) which they are now commonly used for the identification and diagnosis of disease.

1.3.1.1 Intimal thickening and Fatty streak (Type I-II lesion, AHA).

The intima is the innermost layer of the vascular wall and in and it is mainly composed of a thin proteoglycan layer populated by endothelial cells (ECs) in monolayer. This structure does not have uniform thickness, and thickening of this layer has been observed at the level of bifurcation branches as a physiological adaptative response to blood flow pressure and wall tension⁵¹. In the pathological context, it was observed that advanced atherosclerotic plaques develop mostly in correspondence with these more susceptible regions of the vasculature, therefore described also as atherosclerosis-prone regions (coronary arteries, aorta, femoral arteries). Type I lesions have been described in infants and children and are mainly characterised by increase of apolipoprotein A and B content at the level of the intimal thickening before the formation of abnormal lipids deposits and formation of foam cells. Type II lesions are instead characterised by multiple adjacent layers of macrophage-derived foam cells, a few T lymphocytes and disperse cholesterol droplets which have been largely internalised by macrophages⁴⁰. These lesions are also described as progression-prone type II lesions which differ from progression-resistant lesions for their localisation in vascular regions subjected to mechanical forces and shear stress which increase the time of interaction between circulating cholesterol particles and

the endothelium⁵³. At this stage, endothelial cells enhance their expression of adhesion molecules favouring the interaction and attachment of monocytes to the luminal side of the arterial wall. In addition, the secretion of chemotactic and inflammatory cytokines propagates the inflammatory cascade to activate medial smooth muscle cells⁵⁴.

1.3.1.2 Type III lesions and atheroma (or lesion type IV).

Lesions of type III are described as a morphological bridge between the formation of a visible fatty streak and the progression to pronounced atheroma (type IV). Type III lesions are also referred to as Pathological Intimal Thickening (PIT) and are primarily characterised by layers of dysregulated (rough-surfaced endoplasmic reticulum rich) smooth muscle cells (SMCs) attached to a matrix composed by proteoglycan and collagen⁵⁵. Moreover, SMCs containing lipid droplets have been observed at this stage of lesion formation⁴⁰. Macrophages accumulate in the luminal side of the plaque, outside of the lipid pool, together with cholesterol clefts interspersed at the edges of the neo-formed plaque. At this stage, neither the intimal, medial nor the adventitial layer is diseased or affected by morphological changes in their architecture. This is one of the main differences from type IV lesions (also known as atheroma), which instead show severe intimal disorganisation due to the formation of large necrotic cores derived from the enlargement and merging of the single cholesterol pools characterising type III lesions. Importantly, the pathological arterial remodelling response causes the enlargement of the arterial wall to compensate the progressive growth of the plaque⁵⁶. Cells populating type IV plaque are macrophages and foam cells, located mainly next to the inner core of the plaque, while dysregulated smooth muscle cells with unusual attenuated elongated morphology have been identified either next to the lipid core or in the tissue between the endothelium and the necrotic core immersed in a thick extracellular matrix layer (fibrous cap of the plaque). Traces of microcalcification and neovascularization have also been described at this stage⁵².

1.3.1.3 Type V and VI lesions

Type V lesions are characterised by thick fibrous connective tissue; therefore they are also referred to as fibroatheroma. These lesions can be clinically significant as they are associated with high levels of calcification and vascularization in regions surrounding the necrotic core, and microhaemorrhages. Large necrotic cores, formed mostly by apoptotic macrophage, are separated by thick fibrotic tissue composed by collagen I and III and occupy the inner core of the plaque expanding till the medial layer. At this stage, the high degree of structural remodelling can cause a progressive narrowing of the arterial lumen. Type V lesions have also been divided into subcategories (Va, Vb, Vc) based on the degree of disease progression and presence of specific features, such as thick layers of connective tissue (Va), mineralisation (Vb), reduction of lipids core (Vc)⁵². In particular, one of the features of “late” type V lesions is the reduction of necrotic cores and presence of free cholesterol aggregates resulting from defective lipid clearance by macrophages⁵⁵. However, although advanced, this plaque may be more stable than the rupture-prone type VI lesion. Type VI lesions are morphologically characterised by gradual erosion of fibrous cap into thin fibroatheroma (TCFA) which drives the progression into ruptured plaque. This plaque is characterised by relatively few SMCs still forming the weak fibrous cap, mostly composed only of collagen type I, which instead populate the necrotic core as vSMC-derived foam cells. In addition, this type of lesion displays haemorrhage and thrombotic deposits developed in the surface of the plaque⁵⁷. The combination of these features, disruption of the surface (mostly identified near to the shoulder region of the plaque but also in the mid fibrous cap⁵⁸), haemorrhage and thrombus contributes to the possibility of clinic syndromes.

Since the development of the first AHA-recommended classification, further up-to-date histological classification of plaque stages, obtained with a variety of new imaging and molecular analysis available in clinical practice, was formulated. This includes a more detailed explanation of advanced plaques, which are more difficult

to be studied and clearly defined in their associated cellular and molecular processes. Plaques defined by traditional classification as Va, Vb, Vc were replaced by VI, VII, VIII plaque types in the updated annotation. The last updated document revealed that although the development of the plaque is generally characterised by sequences of events that can be referred to by numerical stages, some plaques might not develop all of these stages in the described order which is a factor to be considered during the investigation of associated causative factors⁵⁹. A more recent classification proposed from Virmani and colleagues, classifies advanced and vulnerable plaques with specific features such as presence of rupture, erosion, and calcified nodules. In particular, this description expands the knowledge of critical biological markers of plaque rupture⁵⁵. Virmani and colleagues have identified that plaques precursors of rupture referred to as TCFA, are characterised by thin fibrous cap at the site of the rupture which exposes the lipid-rich core to the luminal thrombus. The process of plaque erosion follows the thrombus formation and occurs in plaques rich in smooth muscle cells and extracellular matrix components. While calcified nodules represent plaques in which calcified nodules are exposed to in communication with the luminal thrombus caused by lack of endothelial cells at the site of thrombus⁴⁶. Hereafter, I will discuss the role of endothelial cells, immune cell biology, and matrix and mesenchymal cells in atherogenesis.

1.3.2 Cellular components and processes characterising plaque formation.

1.3.2.1 Endothelial cells (ECs).

The endothelium in healthy vessels represents a “sensing” surface with the important role of capturing and transmitting signals received from humoral factors in circulation to other cell types and molecular components of the vessel wall⁶⁰. Therefore, it represents a critical component for the maintenance of vascular homeostasis. Endothelium dysfunction refers to morphological, functional and molecular changes associated with the response to stress during vascular injury and atherosclerosis⁴⁰. During the initial stages of atherosclerosis, endothelial cells residing in

atherosclerosis-prone regions of the arteries are subjected to mechanical forces, such as alteration in shear stress associated with irregular blood flow, and chemical signals represented by increase in blood lipids which in concert activate the initial response⁴⁰. The expression of adhesion molecules in the surface of endothelial cells (e.g., VCAM1, ICAM1, Selectins) and secretion of chemotactic cytokines (such as MCP1 or GM-CSF) favours the initiation of inflammatory cascade with the recruitment of circulatory monocytes and T lymphocytes, which in turn migrate and adhere to the endothelium layer. Moreover, signals transmitted by inflammatory cells and the prolonged exposure to increased circulating lipids favour the endothelial production of Nitric Oxide, prostacyclin, and endothelin, which transmits vasodilatory signals to the smooth muscle layer. The relaxation of the vSMC layer is responsible for the initial compensatory outward remodelling (Glagov's phenomenon) characterising plaques type I-III⁶¹. In this context, ECs gradually lose their cell junctions-organised structure, modify their morphology (shape and orientation) and increase permeability favouring the rapid influx of monocytes, circulating cholesterol and platelets in the sub-endothelial layer, therefore causing inflammation, lipid oxidation and activation of thrombogenic factors^{38,62}. Dysfunctional endothelial cells not only interact and transmit signals to immune cells, but also promote the recruitment and activation of smooth muscle cells. In particular, the biochemical crosstalk between these two cell types can be mediated by the release of secreted molecules and growth factors, such as transforming growth factor-beta (TGF-beta) and platelet-derived growth factor signalling (PDGF) and extracellular matrix components, which in turn induces the migration, proliferation and phenotypical switch of SMCs within the plaque³⁸.

1.3.2.2 Macrophages and immune cells.

The inflammatory response constitutes an important process during plaque formation. In the initial stages of lesion development (Type I/II plaques), monocytes and a few T lymphocytes have a central role in initiating the inflammatory cascade. The recruitment of circulating blood monocytes to the endothelial surface is followed

by the transmigration of these into the sub-endothelial space, a process that is primarily regulated by CC-chemokine ligands receptors (CCR2-CCL2, CCR5-CCL)⁶³. In addition to these, CD31 and VCAM1 have been described as important during the adherence and monocyte rolling phases. The interaction of monocytes and leucocytes with activated and permeable endothelium is primarily mediated by chemoattractant proteins such as MCP1 and Interleukin 8 and their receptors expressed on the surface of monocytes and leucocytes recognised with the name of scavenger receptors (SRA, CD36, MARCO, LOX1)⁶⁴. These receptors, in combination with secreted factors such as macrophage-colony stimulating factor (MCSF)⁶⁵, are in turn responsible for the activation of monocytes (LY6C expressing pro-inflammatory subset) into macrophages which are then able to internalise excessive lipid particles accumulated in the luminal side of the endothelium forming lipid laden foam-cells⁶⁶. The products of the modification of low-density lipoproteins (LDL) by acetylation or oxidation are then stored as cholesterol fatty acid esters, toxic for macrophages, therefore causing metabolic dysregulation. This process is initially balanced by pathways promoting lipids efflux, mediated by macrophages transporters expressed in the membrane of these cells, including ATP-binding cassette transporter A (ABCA1) or ABCG1⁶⁷. However, defects in cholesterol metabolism and efflux pathways lead to cytotoxicity and macrophage apoptosis with consequent release of foam cell content. This cascade of events, including defective cholesterol clearance and macrophage apoptosis, may promote the formation of a necrotic core and the progression to advanced lesions of type IV and V⁵². Macrophage turnover characterises each stage of plaque development and newly recruited monocytes-derived macrophages approach the site of injury. The local proliferation of macrophages within the plaque also contributes to the continuous replenishment of the lesional macrophage pool⁶⁸. In addition to this, vSMC can contribute to the enforcement of the macrophage-like pool of cells within the plaque by assuming a macrophage-like phenotype⁶⁹. Macrophages constitute a plastic cell type with a high degree of heterogeneity. In the pathological context of atherosclerosis several sub-populations with different functional profiles contribute to the pathology of disease⁶⁵. In this regard, the

dynamic balance of these sub-populations plays an important role in determining the severity and progression of atheroma. Advances in RNA single cell high-throughput analysis gave researchers the opportunity to characterise the transcriptomic profile of these different groups of monocytes-activated macrophages and revealed their ability to work in synergy or having different roles⁷⁰. Functionally, this cell type can be widely divided into macro-categories⁷¹ that can be differentiated *in vitro* through stimulation with specific cytokines, including M1 characterised by a pro-inflammatory phenotype (classically activated macrophages) or M2 which constitutes a non-inflammatory phenotype (known as alternatively activated macrophages). Studies of plaques *in vivo* have shown as these two populations are dynamic and differences in their prevalence might lead to plaque regression with increased M2 macrophages, or plaque advancement and erosion driven by the pro-inflammatory M1 group⁷². In particular, the advancement to rupture-prone plaque is driven by pro-atherosclerotic M1 subpopulation which display enhanced secretion of pro-inflammatory cytokines (IL6, IL-12, tumour necrosis factor alpha) and increased production of metalloproteases favouring the degradation of collagen-rich fibrous cap and the advancement to unstable plaque (type VI lesions)⁵⁵. Considering the plasticity of these cell types and their ability to display a “rainbow” of multiple phenotypes, questions around the roles of these different signatures and their clinical relevance in specific stage of disease progression represent an appealing area of study. Moreover, the recruitment of lymphocytes, particularly T cells, at the site of injury and inflammation has been described as a factor contributing to plaque initiation and progression. Similar to monocytes, T lymphocytes are recruited and are able to adhere to the dysfunctional endothelium where they activate following stimulation with inflammatory interleukins (IL-12 or IL-18). The activity of these immune cells in the plaque has been associated with secretion of cytokines, macrophage stimulation through the production of interferon gamma, and reduction collagen formation, thus favouring pro-atherogenic stimuli⁷³. In addition, naïve T lymphocytes can be activated through the antigen-presenting process by dendritic cells. These cells have been associated with the plaque’s advancement to a more

complex phenotype. In particular, following monocytes' recruitment by endothelial cells, they are induced to initial differentiation into macrophages with following activation into activated macrophages or into tissue dendritic cells⁶⁶. Functionally, they not only activate lesional T lymphocytes, but are also able to sense and internalise accumulated cholesterol, therefore contributing to foam cell formation and pro-pathological stimuli²⁹. The group of innate response inflammatory cells involved in plaque formation moreover includes blood neutrophils, which accumulate at the atherosclerosis-prone site of the arterial wall and help monocyte adhesion to the endothelium by releasing granular proteins which in turn intensify the inflammatory response, enhances endothelial adhesiveness and increases vascular permeability⁷³.

1.3.2.3 Extracellular matrix (ECM) and mesenchymal cells.

Although an acellular component, the extracellular matrix represents an important constituent of the plaque whose role can be associated with scaffolding surface of interaction and a combination of chemical and mechanical forces and stimuli⁵¹. Studies focusing on the composition of ECM by comprehensive matrix analysis of extracellular components in healthy vessels^{74, 75} identified the key components of the matrix including collagen fibres, proteoglycans and elastin which have been shown to influence viscoelastic functions of vascular cells, such as migration, proliferation, adhesion and permeabilization. Moreover, significant changes in the structure and abundances of ECM components have a critical role in atherosclerosis. Collagen fibres represent the ECM's most abundant component, which confer integrity and stability to the healthy vascular wall. In the pathological context of atherosclerosis, collagen content (collagen type I and III) increases in the initial stages of plaque formation (plaque type II to IV)⁷⁶. The main cell types secreting collagen fibres are SMCs and fibroblasts, which in turn increase or decrease the production of this component in response to external stimuli such as growth factor and cytokines. These stimuli moreover control the production of elastin fibres, which confer elasticity to the developing plaque after being secreted mainly by SMC and macrophages within the

plaque. Proteoglycans instead are mostly associated with the intimal layer where they interact through their negatively charged chains with the positive charge of apolipoproteins and can facilitate the uptake of LDL particles by macrophages and their chemical oxidation into ox-LDL⁷⁷. The complex network of ECM components is actively involved in both early and advanced stages of plaque formation. Following the initial scaffolding role during leucocyte-lipid interaction at the level of the intimal layer, ECM plays a critical role in the formation of the fibrous cap which stabilises and protects the plaque and necrotic core from rupture. However, during the latest stages of plaque development (lesions type V and VI), the enhanced secretion of proteolytic metalloproteases by macrophages within the necrotic core, impairs the synthesis/degradation balance of the ECM component, therefore inducing the gradual thinning of fibrous plaque and its consequent destabilization⁷⁷. Therefore, evaluating the proteomic composition of ECM in plaques can reveal important information on plaque stages and associated cellular phenotypes. A study interrogating proteomic changes in ECM isolated from plaques of symptomatic versus asymptomatic patients, using a proteomic approach⁷⁴, demonstrated that ECM acquires a specific proteomic signature which can be associated with vulnerable plaque at risk of rupture. In particular, the pathological, symptomatic-associated signature was characterised by increased metalloproteases content, calprotectin which in turn promotes phagocytes migration and endothelium activation, and pro-inflammatory LGALS3BP, therefore demonstrating the critical role played by ECM during plaque formation and development to more advanced phenotypes⁷⁸.

1.3.2.4 Fibroblasts.

Fibroblasts are the main cell type composing the complex layer of vascular adventitia, the outermost layer of the vascular wall, which also includes immune cells, nerves, and resident progenitor cells. This structure exhibits an important function for the maintenance of vascular homeostasis and response to vascular injury⁷⁹. Similarly to the intimal layer, the adventitia “senses” chemical stimuli and biomechanical forces and contributes to the complex network of cellular responses. During embryogenesis

and differentiation, fibroblasts show similar gene expression patterns to the medial vascular smooth muscle cell (VSMC) to which, in the adult arterial wall, is interfaced at the elastic lamina layer. Indeed, studies have reported that vascular fibroblast progenitors can arise from re-programmed vSMCs⁸⁰, in addition to three further processes, including bone-marrow derived progenitors, endothelial-to-mesenchymal transition of local ECs and from mesenchyme during embryogenesis. The heterogeneity of their origin translates to morphological, structural, and functional diversity of fibroblasts after differentiation. During vascular injury and atherosclerosis, fibroblasts retain the capability to phenotypically “activate” into vSMC-like myofibroblasts with fibro-proliferative response to prolonged stress stimuli⁸¹. The activation of tissue fibroblasts includes the acquisition of phenotypical features in common with vSMCs, such as high expression of smooth muscle α actin and contractile microfilaments, and an increase of cell-to-matrix junctions together with cell-to-cell junctions⁸². Lineage-tracing experiments using Gli expressing cells, a marker for adventitial and pericyte progenitor cells, have shown the existence of a plastic adventitial progenitor cell able to migrate into the neo-intimal layer and acquire SMC-like and osteoblast-like signature contributing to vascular remodelling. In this regard, adventitial progenitor cells expressing mesenchymal stem cell antigen Sca1 have been identified as potential vSMC progenitors able to origin a vSMC-like “niche” within the adventitial layer contributing to vessel repair as well as pathophysiological remodelling⁸³. Functionally, activated myofibroblasts can contribute with the compensatory mechanisms of adaptation activated in response to change in blood pressure and vascular stenosis, processes known as positive remodelling. However, in the case of sustained and prolonged mechanical stress and inflammatory mediators, these mechanisms can result in maladaptive arterial remodelling⁸⁴. In this context, one of the main characteristics of activated fibroblasts is the enhanced production of ECM components, such as collagen, elastin, and fibronectin. Importantly, the fibroblasts-matrix interactions are tightly regulated, and their dysregulation is determinant during the development of vascular pathology. The adventitial layer moreover includes dendritic cells and tissue residential

macrophages, which during vascular injury and inflammation generate a variety of inflammatory cytokines and metalloproteases, therefore unbalancing the synthesis/breakdown of ECM components. The loss of the structural cell-ECM crosslink, therefore, promotes myofibroblasts' migration towards the media or the intima⁸¹. Moreover, the expression of adhesion molecules, which facilitates the recruitment of leucocytes to the adventitial compartment, the production of ROS and the increase in the expression of growth factor receptors, such as Transforming Growth Factor beta receptor (TGFR- β), induce myofibroblasts' conversion to a pro-fibrotic and pro-proliferative and migratory phenotype⁸⁴. Prolonged pro-survival/anti-apoptosis and pro-inflammatory stimuli ultimately cause chronic fibrosis therefore affecting the stiffness of the vessel wall.

1.4 vSMCs phenotypical switching in atherosclerosis: proliferation, migration, de-differentiation.

vSMC are not terminally differentiated and undergo phenotypical transition towards a synthetic phenotype in response to prolonged biomechanical and chemical stress. Numerous studies have demonstrated that the high degree of plasticity in vSMC reflects the high level of heterogeneity of this cell type *in vivo*^{55,85-89}. Following vascular injury, endothelial denudation exposes vSMC to altered blood flow, imbalanced release of growth factors and chemotactic molecules at the site of injury. In particular, low shear stress induces vSMC to acquire a perpendicular orientation to the blood flow which predisposes cells to a pro-pathological phenotype in specific regions of the arterial branches⁹⁰. vSMCs can sense the structural rearrangements of the surrounding matrix and are in turn stimulated to increase collagen and fibronectin production, lose their contractile phenotype and exert mechanical tension on the ECM²². In this context, vSMC change their transcriptional profile losing the expression of their typical contractility markers and de-differentiate to a proliferative and migratory phenotype⁹¹. Studies demonstrated that this process is tightly regulated by a network of interactions which involves the transcriptional activator Serum Responsive Factor and its co-activator myocardin, which are central

regulators of vSMC identity⁹². Moreover, inflammatory mediators such as interleukins (such as TNF- α , IL-1, IL-6) induce the expression of adhesion molecules (such as ICAM-1) favouring the interaction with pro-inflammatory leucocytes. The concomitant release of MMPs at the site of injury enhances the ECM rearrangement which in turn amplifies vSMC phenotypic switch. Importantly, because of the high degree of plasticity, vSMC can transdifferentiate into a wide spectrum of phenotypes during vascular remodelling, which increases the heterogeneity of this cell type in different diseases. Local factors, such as elevated levels of calcium, phosphate or oxidative stress, induce vSMC to acquire an osteogenic-like phenotype in the medial and intimal vascular layers⁹³. Moreover, vascular calcification has been also observed during ageing and premature vSMC senescence, which is characterised by irreversible loss of mitotic division as a result of telomere shortening, secretion of pro-inflammatory cytokines (such as IL-6), and metalloproteases (MMPs)⁹⁴. Among the vSMC-derived sub-population in plaques, recent studies have also characterised a population with a fibroblast-like signature, also referred to as fibromyocyte, which display a unique transcriptional repertoire associated with beneficial effects in plaque stability and fibrous cap formation. These specialised, de-differentiated vSMC might de-differentiate from a small pre-existing population of dysregulated vSMC of the healthy vessel wall which can infiltrate at the site of injury promoting plaque stability⁸⁸. During phenotypical transition in atherosclerosis, vSMCs can also transdifferentiate into a foam-cell like during the pathological vascular remodelling characterising the pathophysiology of atherosclerosis, hypertension and hyperlipidaemia^{26,95}. The accumulation of low-density lipoproteins (LDL) in the subendothelial space and their oxidation by exposure to reactive intermediates, favours the production of toxic oxidised-LDL⁹⁶. vSMCs in contact with these reactive particles are induced to change their transcriptional profile and express receptors for oxidised-LDL particles uptake⁹⁷. *In vitro* and *in vivo* studies have demonstrated foamy-cells like vSMCs upregulate macrophage-related markers (CD68, Lgals3, ABCA, MMPs), inflammatory cytokines production (IL-1b, IL-6, TNF) and downregulate vSMC

contractility genes (ACTA2, M11), collectively exerting pro-pathological functions^{98,99} (Figure 1.4).

Figure 1.4 Schematic representation of vascular smooth muscle cell phenotypic switch in atherosclerosis.

Vascular smooth muscle cell (VSMC) is the main cell type composing the medial layer of the vascular wall. In physiologic conditions, differentiated VSMCs are embedded in a layer of extracellular matrix components, and display a contractile phenotype characterised by expression of structural protein such as alpha actin, myosin heavy chain for contractile functions therefore conferring stability to the vessel wall. The organization in layers is favoured by cell-cell junctions. Adult differentiated VSMC are moreover characterised by an elongated shape with a large central nucleus and provided with contractility apparatus which includes a sarcoplasmic reticulum for calcium influx/efflux. Following prolonged exposition to extracellular stress signals, such as increase of pro-inflammatory cytokines (ILs), nitric oxide, growth factors (such as PDGF-BB or TGF- β), metalloproteases (MMPs), inorganic phosphate (Pi) and cholesterol, VSMC are induced to phenotypically switch to a synthetic state with rhomboid shape, developed synthetic apparatus and decreased contractility proteins. Synthetic VSMC display increased proliferation, migration and release of inflammatory molecules. Dysfunctional VSMC are moreover able to de-differentiate into foam cells by ox-LDL uptake and show a calcified and senescent phenotype following stimulation with calcium phosphate and oxidative reactive species (ROS).

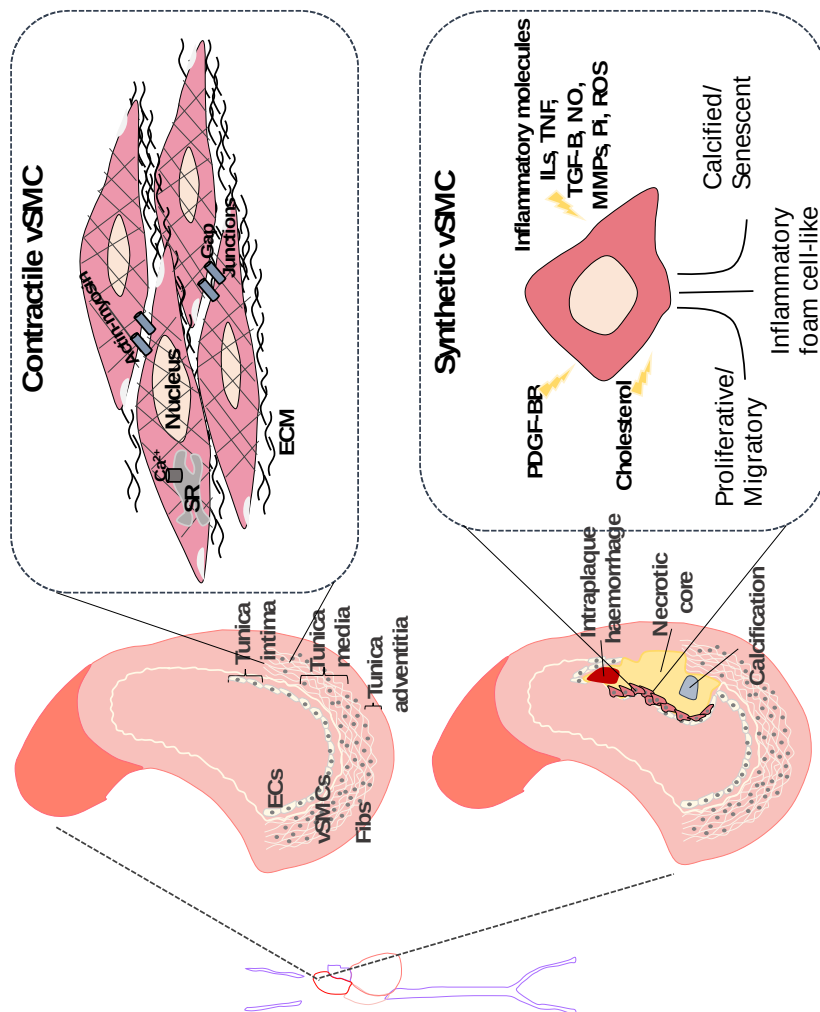


Figure 1.4 Schematic representation of vascular smooth muscle cell phenotypic switch in atherosclerosis.

1.4.1 vSMC proliferation.

The proliferation of vSMC of the vasculature is a dynamic process if we consider the multiple environmental conditions in which vSMC can function, such as development, and differentiation or vascular injury. During embryonic development, vSMCs retain a high proliferation rate and synthetic ability to secrete ECM components, that are able to influence the behaviour of neighbour cells. During differentiation, the selective activation of a subset of genes and cell markers determinates contractility functions associated with the maintenance of vessel structure. In this context, the repression of vSMC proliferative capability is important to confer stability to the vessel wall. In adult blood vessels, although vSMC mostly reside in the medial layer, they can also be found in specific regions of the vascular intimal layer characterised by intimal thickening (also known as diffuse intimal thickening areas)⁵¹. These groups of cells have been characterised by their monoclonal properties which might differ from medial polyclonal SMCs. In fact, contrarily to medial SMCs, the intimal population retains a higher proliferation index and active synthetic organelles for the production of cytokines and ECM components and particularly proteoglycans, which are responsible for interactions with apolipoproteins and intimal thickening during the advancement of atherosclerosis¹⁰⁰. vSMC proliferation functions can be modulated at this stage and an increase of their division rate is associated with the progression to pathological intimal thickening. This process is also accompanied by loss of α SMA positive cells, which might associate with their initial stage of phenotypical switching. In addition, sub-endothelial SMCs have also been found expressing macrophage typical markers, such as CD68, which might confirm early phenotypical transition into synthetic phenotype¹⁰¹. Together with expression of macrophage-associated genes, vSMCs can start acquiring macrophage-related function such as uptake of ox-LDL particles, therefore contributing to the foam-cell pool forming the necrotic core. This phenomenon, in combination with the formation of macrophage-derived foam cells, contributes to the perpetuation of inflammation, enlargement of the lipid core, and leads to apoptosis of cells within the plaque. While during the advancement of pathological intimal thickening vSMC proliferation is

reduced, during the progression to fibroatheroma this process is re-activated to counterbalance the injury and facilitate the healing and stabilisation of the plaque⁸⁵. During this phase, the fibrous cap is mostly formed by medial hyperproliferative α SMA/MHC positive vSMCs, recruited by migration to the inner core of the plaque in response to growth factors such as PDGF secreted by ECs and macrophages¹⁰². In human plaques, in addition to medial cells, intimal vSMCs contribute to this process¹⁰³. Moreover, multicolour lineage-tracing experiments investigating where proliferating cells localise in the plaque show that actively dividing vSMC localise in both the fibrous cap and the cells composing the necrotic core. Studies on mapping this small niche of hyperproliferating cells characterised them as selective "responders" capable of clonal expansion with unique properties^{104,102}, therefore indicating that a low number of vSMCs might be primed to phenotypically respond to pro-atherogenic stimuli¹⁰⁵. Single-cell analysis profiled the transcriptome of the different vSMC stages characterising vascular injury and neointima formation and show that the hyperproliferative fraction of "specialised" vSMC displays high expression of mesenchymal stem markers such as stem cell antigen 1¹⁰⁶. This cell population can reside in the healthy artery¹⁰⁷ as well as in injured vessels⁸⁰. In this regard, a sub-population of potential vSMC progenitor cell positive for stem cell antigen 1 have been found in the medial layer¹⁰⁸ as well as in the adventitial layer⁸³, contributing to the pool of specialised vSMC able to migrate in the intimal layer of athero-prone regions displaying pro-pathological osteoclast-like phenotype during vascular calcification⁸³.

1.4.2 vSMC migration.

The activation of migration has been described among the phenotypical changes that vSMC undergo in response to different hemodynamic conditions during vascular injury and the development of atherosclerotic plaque. vSMC localising in the intimal thick atherosclerotic-prone areas are thought to originate from migration of medial vSMC as result of adaptation mechanisms in response to changes in blood flow pressure and direction⁵¹. However, although evidence of vSMC migration *in vitro*

might suggest this hypothesis, it results to be difficult to prove using lineage-tracing techniques as many animal models do not develop similar stages of disease. Instead, the study of vSMC accumulation and migration during the final phases of the disease, where the initial pathological intimal thickening progresses towards the fibroatheroma stage, have provided more accurate evidence. In particular, vSMC were found to be part of the healing response to lesion development and to migrate from their medial location to the inner core of the plaque, forming the protective proteoglycan-rich fibrous cap^{109,110}. This has been characterised as a response to mechanical and local production of cytokines, such as the secretion of PDGF, interferon gamma and TGF beta, production of interleukins (such as IL1), activation of the WNT pathway and proteolysis of the ECM by metalloproteases^{111,86,112}. Instead, vulnerable and instable plaques are characterised by low number of non-foamy ACTA+ vSMCs and reduced proliferation and migration, phenomenon that are then replaced by apoptosis and vSMC loss⁵².

1.4.3 vSMC de-differentiation.

The switching to a less differentiated vSMC phenotype observed during atherosclerosis includes the acquisition by vSMC of a macrophage-like transcriptomic profile and macrophage-associated functions. Evidence that vSMC phenotypical modulation towards the macrophage-like signature starts during the pathological intimal thickening stage have been collected in human plaques and human cells exposed to lipids uptake^{69,98,113}, but also in animal models^{114,97,28}. In particular, vSMC have been described as capable of taking up cholesterol accumulated in the sub-endothelial space during the formation of pathological intimal thickening (lesion type II/III), which are visible as visible cytoplasmic lipids inclusions⁴⁰. The uptake of cholesterol and oxidised modified LDL particles contributes to the alteration of the vSMC phenotype which in turn increases the expression of macrophage-like genes and contributes to the formation of the foam-cell pool within the plaque¹¹⁴. The formation of vSMC-derived foam-cells initiates during the early phases of lesion formation^{115,37} and continues throughout the steps of atherogenesis, representing a

detrimental factor in atherosclerosis. In fact, dysregulated vSMC have been found within the cells populating the lipid core and at the border interface between the necrotic core and the fibrous cap in more advanced lesions. In particular, sub-endothelial vSMC were found to express both vSMC markers (such as ACTA2 and MYH11) and pro-inflammatory macrophage-like makers, CD68 and LGALS3¹⁰¹. Considering that a small fraction of macrophages can show high levels of plasticity and express SMC markers¹¹⁶, studies investigating the provenience of ACTA2⁺/CD68⁺ cells identified that 40% of this population has non-immune origin^{117,118}. However, in advanced plaques, it was observed that phenotypically modulated vSMCs are difficult to uniquely identify considering only these markers. The reason for this limitation is that synthetic vSMC within the plaque decrease the expression of their typical contractility markers (such as ACTA2, MYH11) in response to external stimuli, therefore rendering them difficult to identify²⁷. Studies performed in vSMC cultured *in vitro* contributed to the understanding of the molecular processes and driving chemical factors influencing foam cell formation and vSMC de-differentiation. In particular, the loading of vSMC with water-soluble cholesterol or ox-LDL particles induced the expression of macrophage-like genes, CD68 and LGALS3, while decreasing ACTA2, MYH11, TAGLIN and CNN1 expression^{98,113}. Moreover, the lipid laden vSMC shown increase in the expression of cholesterol transporters (ABCA1, ABCG1¹¹⁹) and release of pro-inflammatory and chemoattract cytokines (IL1, INF gamma, CXCL¹¹⁷). During the latest phases of plaque formation, the apoptosis of vSMC-derived foam cells contribute to the necrotic core's enlargement and the perpetuation of inflammation characterising vulnerable plaques⁵². Function, and dysfunction of the cells and matrix in the atherosclerotic plaque is governed by their transcriptome. In this thesis, I will focus on the role of the non-coding transcriptome. Hereafter I will introduce this type of RNA.

1.5 Non-Coding RNAs Classification, Biology and Function.

The concept that the human transcriptome might include regions which do not code for proteins was first hypothesised when the proportion of protein coding exons

found was found disproportionately low compared to the full transcriptome identified. In particular, by the end of 1990 a worldwide effort (Human Genome Project, HGP) was instigated to complete the sequence of the human euchromatin, 95% of which was completed and published in 2001¹²⁰. One of the unexpected observations from the first comprehensive sequencing was that only <1.5% of the entire genome represents coding portions, while the remainder is ascribable to non-coding areas¹²¹. In addition, results obtained by FANTOM¹²² and RIKEN ¹²³ studies, whose goal was to analyse the transcriptome of humans and mice respectively, showed that the large majority of genome is transcribed into transcripts. These findings were later confirmed by the ENCODE project, which estimated that the 93% of the human genome is transcribed into primary transcripts and that about 39% of these are protein coding genes¹²⁴ (similar observations were obtained in other mammals such as mice¹²⁵). The assumption that portions of non-coding DNA might have cellular function dates back decades¹²⁶. To date, different noncoding RNA molecule categories have been described as part of the noncoding transcriptome and the number of newly identified noncoding RNAs (NONCODE <http://www.noncode.org/>) has grown wildly. These noncoding molecules have been canonically divided into two big families: small noncoding RNAs whose length is <200nt (including microRNAs (miRNAs), transfer RNAs and small nucleolar RNAs) and long noncoding RNA (lncRNA) transcripts with length >200nt (including ribosomal RNAs, sense and antisense transcripts)¹²⁷. Similar to protein-coding genes, lncRNAs that are annotated in the human genome have also been included in the standard nomenclature and provided with a unique gene name symbol and name following the criteria of the HUGO Gene Nomenclature Committee (HGNC, www.genenames.org). To date, a total of 1914 microRNAs and 4515 long-noncoding transcripts have been annotated in the human genome with appropriate HGNC gene symbol¹²⁸ (Figure 1.5).

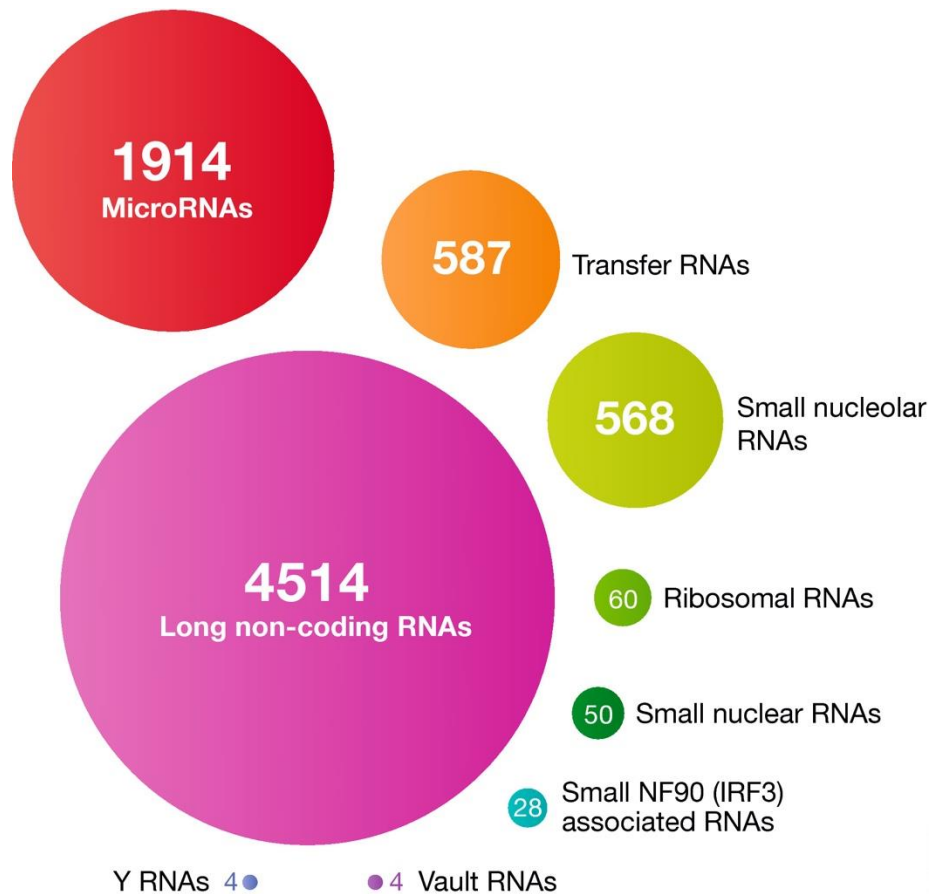


Figure 1.5 Schematic representation of the non-coding RNA classes.

This figure represents the proportion of the different types of non-coding molecules approved by the HUGO Gene Nomenclature Committee. These can be grouped into two major categories, long non-coding RNAs and small non-coding molecules (microRNAs, transfer RNAs, small nucleolar RNAs, ribosomal RNAs, small nuclear RNAs, small NF90 associated RNAs). Picture adapted from¹²⁸.

1.5.1 microRNAs (miRNAs) biogenesis and function in atherosclerosis.

1.5.1.1 miRNAs biogenesis.

This class of noncoding RNAs is perhaps the more studied and functionally characterised in different organisms. The discovery of miRNAs biology and function

started in the early 1980s, when the function of a transcript, lin-4 controlling phasis of development in *C. Elegans*, was studied for the first time. Years later, lin-4 and let-7 microRNAs (miRNAs) have been described in their role as transcriptional repressors of the RNA target molecule lin-14 targeted by RNA-RNA interaction¹²⁹. This discovery was followed by large-scale functional screening to address whether this class of noncoding RNA would have been functional or only belong to “junk” transcripts by loss-of-function experiments and phenotypical assays, which importantly contributed to the comprehensive characterisation of noncoding molecules^{130,131}. Since then, in silico high throughput analysis followed by functional characterisation has produced additional information on the determination of the total number of microRNAs in the human genome, currently estimated to be 2300¹³². The traditional model of processing and maturation of microRNAs consists in multiple steps performed first in the nucleus and then in the cytoplasm, where these molecules generally play their role. The mature form of microRNAs consists in a single-stranded 21-26 nucleotide (nt) long sequence which is produced from transcripts with a hairpin-shaped structure. The transcription of these molecules is mostly associated with Polymerase II, which transcribes a long primary transcript known as primary microRNA transcript (pri-miRNA) characterised by 5' cap and 3' tail sequence. These pri-miRNAs can overlap transcription units of coding genes and can generate from exons or the intronic portion of noncoding transcripts. In this regard, a class of hosted microRNAs has moreover been described as being generated from long noncoding transcript loci with similar transcription pattern of their hosting noncoding RNAs¹³³. The primary transcript is then rapidly converted into a pre-RNA transcript (60-70 nt long) by the nuclear enzyme Drosha¹³⁴, which together with the co-factor DGCR8 forms a complex referred to as Microprocessor able to specifically recognize and process the pri-miRNA in the nucleus¹³⁵. Following the Drosha-mediated processing, the pri-miRNA is then exported into the cytoplasm by the transport complex exportin to be processed into a double stranded sequence of 18-22 nt by the RNase Dicer. The small RNA duplex produced by the endonuclease Dicer is then loaded onto Argonaute protein (Ago) to form the RNA-induced silencing (RISC) complex where the two

miRNA strands are unwound¹³⁶. During the loading of the miRNA onto RISC, thermodynamic features and nucleotide sequence favour the selection of the guide strand. However, this process is not stringent in some cases the passenger strand can also be selected. Recent discoveries have also described alternative pathways for the processing of miRNAs, which reveal evolutionary flexibility characterising this process¹³⁷. AGO complex in turn favours the binding of the mRNA target by the microRNA whose main mechanism of action is the repression of gene expression at post-transcriptional level blocking the translation in the cytoplasm. In particular, of the mature microRNA sequence only a small region named “seed” sequence (6-8 nt) is deputy to complimentarily interact with the mRNA target. The seed sequence can recognise and silence multiple mRNAs through a mechanism which is probably mediated by multiple microRNAs (a single microRNA is unlikely able to induce gene silencing)¹³⁴. Following the binding, the miRNA-target complex favours the degradation of the mRNA target¹³⁸. However, recent studies have revealed the existence of more complex series of events where microRNA-mediated mRNA regulation can lead to different consequences. This is the case of specific features of the microRNA target influencing the efficiency of translation without altering the stability of mRNA¹³⁹. Importantly, considering that miRNA molecules can regulate multiple critical cellular and biological processes the regulation of their turnover it is a tightly modulated process. In this regard, one of the miRNA decay mechanisms recently described revealed the reverse outcome when a miRNA binds to one of its targets which is in turn able to induce its degradation¹⁴⁰

1.5.1.2 microRNA function in atherosclerosis.

Advances in experimental procedures and techniques have allowed functional studies of noncoding RNA molecules to identify them as key regulators of cellular and biological processes in cardiovascular diseases¹⁴¹. One of the first steps towards the identification of a functional noncoding RNA molecule is to measure its abundance in different cells and tissues. This approach can moreover be applied to evaluate the

involvement of noncoding molecules in the pathophysiology of disease by comparing their expression profile in physiological and pathological conditions. For the study of microRNA molecules, qRT-PCR specific probes have allowed the determination of the abundance of microRNA molecules in cells and tissues at physiological conditions but also to measure their transcriptional profile associated with pathophysiologic conditions¹⁴². Moreover, this technique has favoured the validation of microRNAs expression results obtained using small RNA sequencing. RNA sequencing has slowly replaced the use of microarrays and is based on the application of new generation sequencing to characterise the changes in expression associated to small noncoding RNA (< 300 nt) molecules in different cell populations and heterogeneous tissues on a genome-wide scale¹⁴³. Most recently, the development of small RNA sequencing applied at single cell resolution allowed the characterisation of microRNA expression in individual cells¹⁴⁴. RNA sequencing strategies were moreover useful tools to advance the detection and the study of potential microRNAs targets, which can also be identified by computational analysis using bioinformatic tools (such as miRBase¹⁴⁵), and their related transcriptional profiles following microRNAs loss- or gain-of-function studies. Functional approaches using luciferase assays and mutation on the microRNAs binding sites had moreover allowed the validation of putative microRNAs targets¹⁴⁶. In order to characterise the functional role of these molecules, different strategies and approaches have been developed to manipulate microRNA expression. The ablation of proteins involved in the biogenesis of microRNAs (such as Dicer or microprocessor complex subunit DGCR8 depletion) was one of the first studied technologies to block their expression^{147,148}. However, considering that this approach affects the processing and expression of multiple small noncoding RNAs, the produced phenotypes cannot be causally related to the manipulation of a single microRNA molecule. Genetic approaches have also been developed for the study of miRNAs. Although this strategy is more selective than the manipulation of the microRNA's processing machinery, it might be limiting considering that most miRNAs exist as families clustered together in the same genomic portion. Therefore, the deletion of the genomic portion where a specific microRNAs localises can impair the

expression and related function of neighbour miRNAs¹⁴⁹. To date, several other strategies have been developed to specifically affect the expression of miRNAs using nongenetic approaches. One example is represented by the use of “antagomirs”, “Antisense Oligonucleotides” or “microRNA sponge” designed to complementarily bind the miRNA target, therefore irreversibly inhibiting their function, used for both *in vitro* and *in vivo* applications^{150,151,152}. Similar techniques have been used to induce the overexpression of microRNAs. A valuable approach is to use chemically synthesised double-stranded mature microRNAs that mimic the Dicer-dependent miRNA product, referred to as “microRNA mimics”. However, although this strategy was very efficient in obtaining transient overexpression of the microRNA target for *in vitro* experiments, it does not represent an efficient approach to obtain long-term induction of microRNA expression in cell lines of *in vivo* experiments. In this case, the use of plasmids allowing the constitutive expression of microRNAs can be achieved using recombinant viral particles to overcome the low cell transfectability of primary cells or to obtain tissue-specific expression¹⁴⁹.

microRNAs are generally well conserved across species, although at a functional level they might act slightly differently due to evolutionary selection processes. This class of noncoding RNAs have been studied in a wide spectrum of physiological contexts, pluripotency, development, and differentiation. Different families of miRNAs have had their important role in the regulation of these processes in cells composing the vasculature described. Importantly, the expression of microRNAs is tightly controlled by specific regulatory networks which guarantee the correct level of expression of these molecules, influencing cellular gene expression and function. Unbalance of their expression levels, due to dysfunctional regulatory pathways, can be the driving factor during the development of cardiovascular disease and atherosclerosis. Diverse microRNAs, whose expression pattern changes during disease, have been described contributing to the pathophysiology of plaque formation regulating EC, immune cells and vSMC functions.

1.5.1.3 miRNA in Endothelial Cell (EC) activation.

Endothelial dysfunction is one of the initiating factors during atherosclerosis as previously described in the above paragraph 1.2.2. Several microRNAs have been described for their role during the initial stages of plaque development influencing EC activation. One example is represented by microRNA miR-217 found upregulated in ageing ECs. Microarrays and luciferase activity assays confirmed that miR-217 binds to the 3'-UTR of silent information regulator 1 mRNA. This protein is a deacetylase which is involved in the regulation of genes associated with longevity and metabolic efficiency¹⁵³. Therefore, gain of functions of this protein exert a protecting effect against ageing. Moreover, its beneficial effect against endothelial dysfunction and associated senescence has been shown¹⁵⁴. In particular, it was demonstrated that during atherogenesis the upregulation of miR-217 negatively regulates the expression of silent information regulator 1, therefore causing a premature senescent-like phenotype in ECs¹⁵⁵. Another microRNA involved in endothelial cell function and integrity is miR-126, involved in the EC repair and regeneration following vascular damage. In particular, this microRNA is one example in which roles associated with both strands, miR-126-3p and miR-126-5p have been described. Expression data shown a decrease of miR-126-5p during the stages of plaque formation caused by disturbed blood flow. Functional studies using luciferase assays to investigate potential targets of this microRNA identified DLK1 negative regulator of EC proliferation. Moreover, loss and gain of function studies mediated by antagomirs and mimics confirmed the phenotypic effect of miR-126-5p modulation on EC proliferation and repair through the modulation of DLK1 expression¹⁵⁶. At the same time, the counterpart of this miRNA, miR-126-3p was associated with athero-protection, decrease of inflammation and recruitment of progenitor cells for tissue repair and homeostasis¹⁵⁷. Another microRNA whose expression was found induced by shear stress is miR-21. Microarray followed by qRT-PCR shown an increase in miR-21 in EC exposed to prolonged shear stress. The modulation of this microRNA *in vitro* by overexpression or downregulation was observed to modulate PTEN expression, which was confirmed to be miR-21 target. In particular, the overexpression of miR-

21 decreased PTEN levels which in turn activated apoptosis and enhanced the production of NO in ECs, therefore representing a detrimental factor in the process of plaque formation¹⁵⁸.

1.5.1.4 miRNA in the dysregulation of cholesterol homeostasis.

The control of cellular and circulating cholesterol levels is essential for human health and dysfunction of cholesterol metabolism and the related genes and pathways can lead to hyperlipidaemia and atherosclerosis¹⁵⁹, as discussed in paragraph 1.2.2. MicroRNAs have been described being part of the important group of modulators which finely regulate this process. One example is miR-33a, located within the intronic region of SREBF gene encoding for a protein regulating cholesterol metabolism in the liver. Cholesterol loading of peritoneal macrophages showed a reduction of miR-33a and SREBF expression, which were reprimed by cholesterol depletion in the same cell model. Using prediction tools, putative binding sites for miR-33a were identified in the 3'UTR region of different genes such as ABCA1 and ABCG1 involved in the reverse cholesterol transport. These observations were then confirmed with loss and gain of function studies and luciferase assays which confirmed the specificity of target interaction with these proteins. In particular, the modulation of miR-33a levels affected expression of cholesterol efflux genes ABCA1 and ABCG1 in the opposite way, thereby repressing cholesterol efflux pathway which is then retained in foam cells¹⁶⁰. Another miRNA implicated in cholesterol metabolism and highly abundant in the liver is miR-122. Antisense oligonucleotides were used to understand the effect of miR-122 repression in the liver *in vivo*. The repression of miR-122 caused reduction of serum cholesterol levels mediated by the regulation of genes involved in lipid synthesis. Moreover, the repression of miR-122 was associated with enhanced inflammation and fibrosis by increasing the levels of pro-inflammatory myeloid cells indicating the anti-inflammatory potential of this microRNA¹⁶¹. The dysregulation of cholesterol metabolism leads to accumulation and consequent modification of lipid particles into ox-LDL causing fatty streak formation and plaque development. Studies investigating the effects of ox-LDL in monocyte to macrophage

activation using microarrays and qRT-PCR showed a decrease in miR-125a expression following exposure to the oxidised particles. Functional studies demonstrated an increase in ox-LDL uptake from macrophages following miR-125a downregulation which was associated with enhanced expression of scavenger receptor CD36 and release of pro-inflammatory cytokines (IL-6, TNF α). Using target prediction database and experimental validation the protein ORP9, oxysterol binding protein involved in the regulation of vesicles-mediated cholesterol transport, was found as direct target of miR-125a. Therefore, these data demonstrated that expression of miR-125a might be a beneficial factor during the first stages of macrophages uptake of cholesterol and its downregulation might lead to lipid accumulation and formation of toxic foam cells¹⁶².

1.5.1.5 miRNA in vSMC phenotypical switch.

The accumulation of vSMC in the sub-endothelial space and formation of neointima represents a critical process during the development of the plaque. MicroRNAs have been characterised to have important roles in this process and a valid example is represented by miR-22. Expression data shown a decrease in the expression of miR-22 in injured femoral arteries versus controls and *in vitro* assays demonstrated a downregulation in miR-22 expression following PDGF-BB stimulation, therefore indicating that this microRNA might be involved in vSMC phenotypic switching. In addition, the mimic-mediated upregulation of miR-22 induced the expression of vSMC genes (α SMA, MYH11) and oppositely regulated with miR-22 inhibitors. Functional assays allowed to identify EVI1 protein as target of miR-22. In particular, EVI1 was found downregulated following mimic-mediated overexpression of the microRNA and luciferase activity experiments confirmed the target specificity. EVI1 was described associated with the negative regulation of vSMC contractile phenotype. Moreover, chromatin precipitation assays shown enrichment of EVI1 with the promoter of vSMC genes (α SMA, MYH11, SFR) therefore confirming the involvement of miR-22 as negative regulator of vSMC contractile phenotype¹⁶³. Another microRNA aberrantly expressed in human atherosclerosis is miR-21, a

microRNA ubiquitously expressed in vSMCs, ECs, macrophages, and fibroblasts. In particular, miR-21 was found to be upregulated in diseased arterial sections versus healthy controls. Moreover, *in vitro* experiments in cultured vSMCs shown that hypoxia inducible factor 1 α induced expression of miR-21 whereas knock-down of this protein reduced the levels of the microRNA. Loss of function experiments have moreover shown that miR-21 knock-down reduced the vSMC proliferation and migration rates under PDGF-BB stimulation, known to induce vSMC division and motility. Functional data also revealed that miR-21 negatively affects the expression of TPM1, a regulator of vSMC cytoskeleton organisation. In particular, the miR-21 mediated modulation of TPM1 affected the levels of elongation and cellular shape of vSMC which possibly influence vSMC phenotypic switch in plaque formation¹⁶⁴. However, beneficial effects of this microRNA in stabilising advanced plaques were also described. A study demonstrated that the local delivery of miR-21 in more vulnerable plaques increases vSMC proliferation and fibrous cap formation therefore conferring stability to rupture-prone lesions¹⁶⁵. The MiR-221/222 family was also described for the regulatory role played in vascular cells and specifically for their opposite function observed in the modulation of vSMC and EC phenotype. Expression results in tissue from balloon-injured mouse arteries revealed a co-localisation of miR-221/222 with the medial layer of vSMC. Furthermore, comparison with wild type animals shown an increase miR-211/222 levels, indicating a correlation with disease stages. Functional loss- and gain-of-function experiments performed *in vitro* in vSMC shown that miR-122/222 overexpression positively correlated with an increase in vSMC proliferation rate, which was instead decreased by miR-122/222 knock-down. Migration of vSMC was moreover assessed following modulation of this microRNA cluster which resulted to have pro-migratory functions, therefore confirming the involvement of this microRNA cluster in the induction of vSMC phenotypic transition towards a dysregulated phenotype in vascular disease¹⁶⁶. It was demonstrated that the uptake of ox-LDL particles can also be mediated by dysregulated vSMC residing in the intimal layer of the vasculature, therefore contributing to the pool of foam cells forming the necrotic core of atherosclerotic lesions. The MiR-29 family has been

shown to be upregulated in atherosclerosis and *in vitro* loading of ox-LDL particles was shown to be the driving factor inducing the observed change in miR-29 expression. Moreover, functional data reported that the modulation of miR-29b isoform can affect the expression and function of methyltransferase DNMTs, important genes regulating gene expression by controlling DNA methylation patterns. In particular, the miR-29-dependent downregulation of DNMT was demonstrated to induce the expression of metalloproteases MMP2/MMP9 which in turn induces vSMC migration and pro-atherosclerotic phenotype¹⁶⁷.

1.5.2 Long noncoding RNAs (LncRNAs) biology and tools for their characterisation.

1.5.2.1 LncRNA biology.

This class of noncoding molecules, similarly to miRNAs, belongs to the >80% of the human non-coding transcriptome. In the late 1980s, the first human long noncoding RNA (lncRNA) molecule was discovered, H19, an lncRNA molecule involved in dosage compensation during the determination of sex which was initially described as mRNA¹⁶⁸. However, although the structure of this molecule resembled protein-coding mRNAs, the lack of translation was a unique characteristic that captured the attention of scientists for future investigations. This initial study was followed by the discovery of the locus Xist in the early 1990, where a lncRNA molecule is located, involved in the inactivation of X chromosome for the determination of sex in mammals¹⁶⁹. These pioneering research opened the way for the study of lncRNAs demonstrating the biological function and relevance of some of them in the regulation of critical cellular processes. Comparative studies of eukaryotic genome and transcriptome allowed the analysis of conserved coding and noncoding loci showing that the primary transcript of lncRNA is poorly conserved and that the 80% of these molecules have a primate origin¹⁷⁰. These observations raised opposite opinions on the levels of functionality of these transcripts, however studies taking into consideration multiple features such as structure, expression and function were

ultimately more applicable in the case of lncRNA molecules, which in fact proved to be characterised by conserved cell-temporal expression and functional profile¹⁷¹. The canonical classification of lncRNA as non-coding transcripts is based on observations that these transcripts were characterised by short open reading frame indicating the absence of coding potential. However, some lncRNAs have been described for their ability to undergo translation giving origin to short functional peptides also referred to as micropeptides¹⁷². Sequencing studies on ribosome-associated RNA, revealed the presence of reads from lncRNA regions which can be associated with 80S ribosomes, therefore indicating the possibility of their translation into proteins⁴³¹. However, reports have shown that the association of lincRNAs to ribosome subunits is not itself enough to prove their translation to proteins. In particular, the possibility to profile the ribosome occupancy at the 3' Untranslated Region of lincRNA transcripts might give indications on the processing of these noncoding molecules compared to protein-coding genes⁴³². It has been shown that, contrarily to protein coding genes, in the case of lincRNAs the ribosome occupancy profile does not change at the 3' Untranslated Region due to the lack of stop codon. The use of mass spectrometry for low-molecular weight proteins, identified the production of micropeptides shorter in length (>9-10 amino acids) than the known proteins encoded by noncanonical regions upstream the coding sequence located in the 5' untranslated regions of mRNAs⁴³³. In addition to this, the combination of the above techniques with CRISPR-Cas9 technology and single-cell transcriptomics allowed the discovery of uncharacterised functional micropeptides encoded by lncRNA loci⁴³⁴. A valid example representing this category recently described is the lncRNA-00961, endothelial enriched lncRNA, whose locus is also able to encode for SPAAR, a functionally active micro-peptide. *In vitro* and *in vivo* studies have described as the lncRNA and SPAR can have independent effects in the regulation of cell function during differentiation including EC adhesion, tube formation and migration¹⁷³. In this regard, SPAR-encoding lncRNA was found downregulated upon skeletal muscle injury while the downregulation of the encoded micro-peptide SPAR was found associated with enhanced muscle regeneration through modulation of mTORC1¹⁷⁴. Similarly, Myoregulin, a micro-

peptide encoded by a lncRNA was identified interacting with SERCA impeding Ca^{2+} to be taken up by the endoplasmic reticulum, therefore leading to dysfunctional contraction. The genetic deletion of Myoregulin improves muscle contraction and performance¹⁷⁵. Moreover, DWORF was described as a short muscular micro-peptide with contractility-associated roles whose expression enhances calcium-dependent contractility rescuing dysfunctions in a cardiomyopathy mouse model opening possibilities for gene-therapy approaches¹⁷⁶. Relative to the features of lncRNA transcripts, the majority of these molecules are transcribed by RNA polymerase II and are processed similarly to protein-coding genes with 5' cap and some of them polyadenylated at their 3' end, or have both, polyadenylated or non-polyadenylated, isoforms¹⁷⁷. The structure of lncRNA can include multiple exon/intron regions which are then processed by splicing, giving origin to several isoforms¹⁷⁸. Generally, lncRNA chromatin is characterised by low levels of histone H3K4 methylation, associated with a low transcription rate, and high abundance of acetylation at their promoter region which is considered a feature of active promoter or enhancer-associated region¹⁷⁹. The secondary structure, referred to as the spatial folding acquired by the lncRNA in its functional conformation, influences its stability and associated cellular function and it was demonstrated that this represents the primary level of conservation across species¹⁸⁰. Advancements in deep RNA sequencing strategies have allowed researchers to obtain important information on the genomic location of lncRNAs with respect to protein-coding genes allowing their functional classification into intragenic, which overlap with protein coding genes or intergenic lncRNAs (lincRNAs), whose location does not overlap annotated protein coding genes. LincRNAs are usually shorter in length than protein-coding genes, show high conservation levels compared to other categories, display mostly nuclear localisation and are believed to regulate functions common to different cellular types and tissues, such as development and differentiation¹⁸¹. One example of lincRNA is represented by the highly conserved lincRNA-21, whose expression is modulated by p53 and whose role is associated with the repression of p53-dependent genes¹⁸². LincRNAs can also be involved in the direct regulation of gene expression and some display enhancer-

associated signatures. The second category of long intragenic RNAs overlapping with protein-coding genes, includes different biotypes that can be divided into: antisense, bidirectional, intronic and overlapping sense lncRNAs (Figure 1.6).

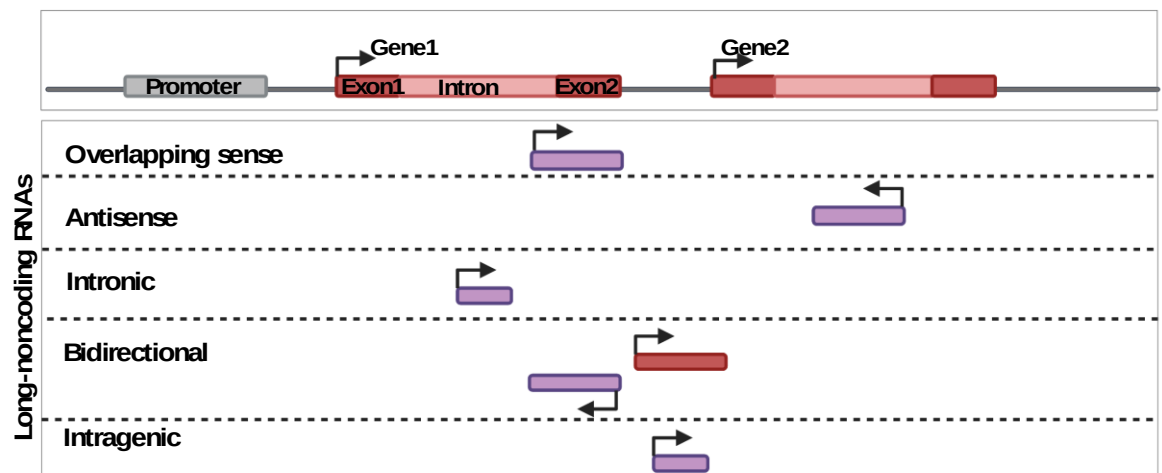


Figure 1.6 Schematic representation of lncRNA localisation.

lncRNAs can be grouped into categories based on their genomic location and sense of transcription in relation to protein coding genes. In this picture lncRNAs (purple boxes) can be located overlapping exons of protein coding genes (dark red boxes), or introns of protein coding genes (light red boxes). Intergenic lncRNAs can also have bidirectional orientation when the sequence of the lncRNA is in proximity (< 1000bp) and in the opposite strand of a protein-coding gene, while non-overlapping lncRNAs can also be localised in the genomic portion between two genes, intragenic lncRNAs.

- Natural antisense transcripts are lncRNA transcripts whose transcription has an opposite orientation to the overlapping protein-coding gene. These transcripts are mostly enriched at the 5' and 3' ends of their overlapping sense genes. Related to their functional role, antisense lncRNAs can regulate their own sense genes in *cis*, or they can act in *trans* displaying regulatory functions of non-paired genes with different genomic locations. Examples are the Xist/Tsix involved in the mechanisms of X chromosome inactivation, SINEUP lncRNAs able to activate the transcription of overlapping sense transcripts

using lncRNA-mRNA interaction or the most recent lncRNA HMGA2-AS1 regulating the sense transcript HMGA1 in cancer^{183–185}.

- Bidirectional lncRNAs have been described as transcripts whose promoter can transcribe either in the sense or as antisense orientation which are generally located in the portion of the genome overlapping the 3' end of their paired genes. However, considering that they mostly do not extend to the 5' end, the identification of their transcriptional start site (TSS) can be difficult to achieve leading to underestimation of their number¹⁸⁶. An example of this category is the bidirectional lncRNA Catsper1au acting as regulator of the paired transcript Catsper1 during spermatogenesis¹⁸⁷.
- Intronic lncRNAs can harbour within intronic regions of annotated genes¹⁸⁸. Examples of this group are ANRASSF1 which originated from the intron of the protein coding gene RASSF1 involved in the regulation of cell proliferation during cancer. ANRASSF1 was shown to be the modulator of RASSF1 expression where increase in the levels of the lncRNA decreases the levels of the protein¹⁸⁹. This category includes circular intronic RNAs that can acquire folded circular structures more stable than linear lncRNAs with mostly cytoplasmic location, such as the human circular CDR1 acting as negative modulator of miR-7 during brain development¹⁹⁰.
- Overlapping sense lncRNAs includes a group of transcripts mostly enriched in the 5' end of coding genes, encoded in the same direction of their paired genes. They can be recognised by the acronymous -OT at the end of their annotated name, which is generally the name of the overlapping sense gene¹⁹¹. This is the case of SOX-OT, a sense lncRNA transcript overlapping the gene encoding for the pluripotency regulator factor SOX2. SOX-OT was found to be dynamically modulated during embryogenesis and its dysregulation was associated with cancer. In particular, the overlapping sense transcript was

described having a regulatory role modulating the associated SOX2 transcript¹⁹². Similar is the case of the lncRNA SRA1 displaying sense orientation to the transcript producing SRAP protein involved in the modulation of the transcription of steroid receptors, therefore controlling important cellular functions. In particular, it was shown that the SRA gene can contemporaneously encode for coding-protein gene SRAP and the noncoding transcript counterpart SRA1, which in turn is involved in the modulation of SRAP protein expression¹⁹³.

Progress in the tools available for investigation have allowed for the evaluation of cellular expression and the compartmentalisation of lncRNA transcripts, which has revealed that the subcellular localisation of these molecules is strictly connected with their functional role. Subcellular fractionation techniques coupled with *in situ* Hybridisation (ISH) and Fluorescent *In situ* Hybridisation (FISH) have allowed scientists to localise lncRNA expression in cell and tissues. In this regard, studies have demonstrated that a significant fraction of lncRNA molecules is retained in the nucleus¹⁷⁸ whereas some of them can be exported in the cytoplasm and can shuttle between the two cellular compartments. In particular, this possibility was associated with the presence or absence of Alu-derived sequences in the sequence of lncRNA, which increase the nuclear accumulation disfavours the exportation to the cytoplasm¹⁹⁴. The study of lncRNA cellular localisation have allowed to investigate their associated functions that can be classified into different groups, which are summarised in Figure 1.7.

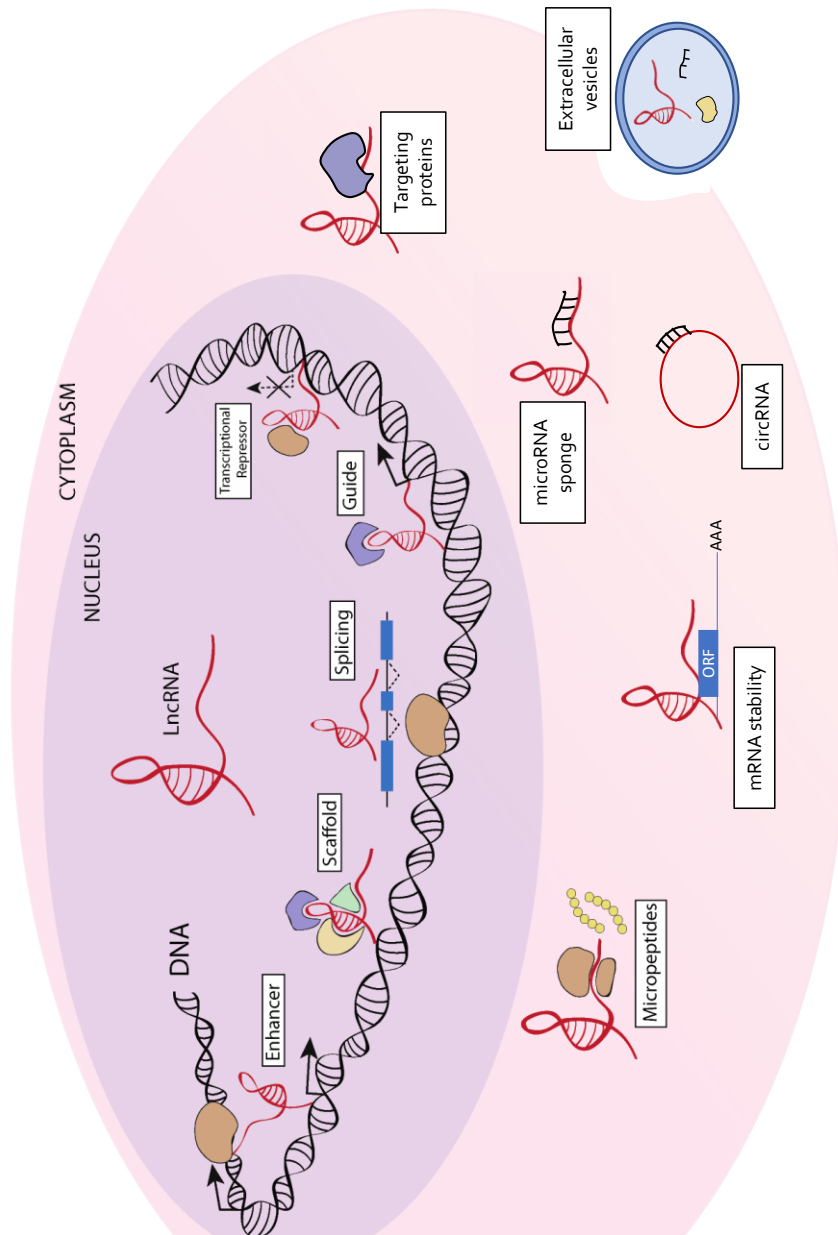


Figure 1.7 Cartoon representing the main cellular function of lncRNAs.

Figure 1.7 Cartoon representing the main cellular function of lncRNAs.

Long non coding RNAs (lncRNAs) have been described as molecules involved in the regulation of gene expression and function at the transcriptional and post-transcriptional level. lncRNAs can interact with DNA, RNA and proteins and based on their subcellular localisation play different roles. Nuclear lncRNAs can regulate gene expression acting as an enhancer of neighbour genes or as a scaffold for the assembling of transcriptional factors. They can moreover recruit and guide chromatin modellers to their DNA target sites or influence the splicing of mRNAs. Some lncRNA retain the ability to be exported in the cytoplasm where they can influence mRNA stability, interact with protein complexes, encode for functional micropeptides or regulate the function of microRNAs. In this case, cytoplasmic lncRNA can inhibit the binding of the microRNA to its target by acting as a molecular sponge or by competing for the same target site. Moreover, some lncRNA have been found secreted in the systemic circulation being encapsulated into extracellular vesicles for cell-cell communication. Figure adapted from Vacante F. et al *Vascular Pharmacology* 2019

1.5.2.2 lncRNA function(s).

The group of recruiter and scaffold lncRNAs include lncRNA with functions associated with their ability to create complexes and interact with proteins regulating chromatin states and gene transcription. An example is the lncRNA involved with epigenetic regulation acting as guide for Polycomb repressive complex PCR2. Protein-interaction studies have allowed researchers to identify some lncRNAs which directly interact with the subunit EZH2 of PCR2, therefore contributing to epigenetic control of gene expression¹⁹⁵, such as HOTAIR required for the H3 lysine-27 trimethylation PCR2-mediated¹⁹⁶. In addition, the lncRNA Air interacts with H3K9 methyltransferase G9a guiding its recruitment to the Slc22ac gene, therefore favouring its allele-specific silencing. Reduction in Air function translates into decreased methylation-mediated repression of the Slc22ac gene¹⁹⁷. This category of noncoding transcripts can also coordinate the interaction of proteins facilitating long-range chromatin loops. Examples are HOTTIP and Mistral which have been described to be encoded from the same locus HOXA, regulating a cluster of genes localised distally with respect to HOXA location. Chromatin-looping assays demonstrated that HOTTIP and Mistral can favour the interaction of epigenetic factor MLL with HOXA genes therefore favouring long-range gene activation¹⁹⁸. This is also the case of enhancer-associated lncRNAs which are specifically involved in chromatin-looping complexes mediating gene activation, such as the lncRNA LEENE whose role is related with the activation of endothelial nitric oxide synthase by facilitating the recruitment of RNA polymerase II to the nitric oxide synthase gene¹⁹⁹.

Decoy and coregulator lncRNAs include transcripts that can directly interact with transcription factors acting as decoys for their recruitment. One example is PANDA, induced by p53-dependent activation, which interacts with the transcription factor NF- κ B to reduce the expression of apoptosis-related genes²⁰⁰. In addition, NRON, a cytoplasmic lncRNA, was found to negatively regulate the function of the transcription factor NFAT by sequestering it in the cytoplasmic compartment of T cells, therefore inhibiting nuclear functions¹³¹. The negative regulation of gene

function and expression can also be mediated by repression of polymerase II activity mediated by lncRNAs, such as a transcript encoded by dihydrofolate reductase which reduced the expression of the protein by interfering with the general transcription factor IIB at the promoter level inducing promoter-specific gene silencing²⁰¹. mRNA processing lncRNA are known to influence the processing, translation, and stability of translated mRNAs. One function can be mediating the splicing process of mRNA molecules, such as MALAT1, which localises with splicing factors in specific nuclear compartments. In particular, when polymerase II-dependent transcription is active, MALAT1 can modulate the recruitment of slicing factors at the sites of transcription and the modulation of its expression might induce alternative splicing processes²⁰².

In the cytoplasm, lncRNA have been also described for their role in mediating mRNA stability and degradation. One example is a group of lncRNAs named half-STAU1-binding site RNAs. STAU1 is a protein involved in the mRNA decay pathway which binds Alu elements in the lncRNAs, which in turn transactivates the STAU1-mediated decay of mRNA target²⁰³. Furthermore, lncRNA can mediate mRNA stability by negatively influencing miRNA-mediated repression of mRNA target. This is the case of BACE-AS, antisense lncRNA to Alzheimer-associated beta-secretase-1 protein. In particular, BACE-AS binding to the transcriptional repressor miR-485-5p is able to promote the increase in the mRNA stability and consecutive expression of Alzheimer-associated beta-secretase-1 protein with feed-forward mechanism²⁰⁴. Recently, the lncRNA-microRNA axes have been elucidated more in detail and some of the lncRNAs have identified having the ability to bind microRNAs through interaction with Ago and the existence of multiple conserved microRNA recognition sequences⁴³⁵. In particular, studies have shown that lncRNAs can act as competitive endogenous RNAs and positive regulators of gene expression by competitively sequestering microRNAs from the interaction with their targets⁴³⁶. However, even though innovative prediction tools might successfully identify possible crosstalk interactions between lncRNAs and microRNAs, it is important to consider what is the proportion of these networks that are physiologically relevant. Strategies to assess their functional role

include the use of high-throughput sequencing of crosslinked and immunoprecipitated RNAs to identify microRNAs recognition sequences associated with RISC complex⁴³⁷. Moreover, multiple factors influence the effectiveness of these interactions, such as the localisation of lncRNA or the accessibility of the sponge lncRNAs to its target microRNA, which is dependent on secondary structures and availability of the microRNA in the cytoplasm. Therefore, even though a lncRNA could be predicted to bind multiple copies of a microRNA, this interaction needs to be experimentally validated⁴³⁵. A valuable example of experimentally validated competing endogenous lncRNA is represented by the linc-MD1, which regulates muscle differentiation. In particular, it was shown that the lncRNA MD1 sponges miR-133 to de-repress the mRNA of transcription factors, MAML1 and MEF2C, involved in the transcription of muscle-specific gene expression²⁰⁵.

1.5.2.3 Tools to characterise lncRNAs: a “pipeline” for the study of lncRNA.

With the progressive refinement of tools to identify, structurally characterise and functionally study lncRNA molecules, the number of well described lncRNAs has significantly increased in the past decades. Considering the bioinformatic and experimental approaches developed to study these noncoding molecules, the steps that bring us from the identification to the functional characterisation of lncRNAs can be distinguished as follows:

1.5.2.3.1 Identification of lncRNA candidate.

The first step in the study of lncRNAs is identification, namely, to detect their expression profile of starting from cell population or tissue samples. Early stages of research involved the use of cloning and first-generation sequencing techniques for the discovery of lncRNAs²⁰⁶ or microarrays (followed by qRT-PCR and northern blot validation) which were based on the complementary binding of pre-designed fluorescent probes trapped into arrays with the cDNA of interest²⁰⁷. However, this method requires knowledge of the sequences to be identified, therefore limiting new

transcript discovery. These methods were progressively replaced by more accurate, sensitive and effective strategies for large-scale identification of noncoding molecules, such as high-throughput RNA sequencing²⁰⁸. In particular, next generation sequencing (such as the Illumina platform) gives the possibility to deep-sequence multiple cell populations or tissues, obtaining detailed information on the abundance of these transcripts including lowly expressed lncRNAs²⁰⁹. Moreover, single cell sequencing technology (e.g. 10X genomics) has allowed the detection of lncRNAs at single cell resolution, particularly important in heterogeneous samples²¹⁰. However, new generation sequencing approaches includes the preparation of cDNA libraries of 100-120pb whose sequence, obtained with sequencing, will be then aligned to the reference genome. Due to lncRNA being poorly annotated, this highly sensitive strategy results in limited information and sometimes is not effective. In addition, considering the low abundances, in some cases lncRNA expression results are below the threshold detected by single cell sequencing, which therefore can only be used to increase sequence coverage and not for the investigation of differential expression profiles. To overcome these difficulties, the development of new methods to sequence long transcripts (such as Oxford Nanopore MinION) has allowed the discovery of unannotated and the more detailed sequence characterisation of previously annotated lncRNAs²¹¹.

1.5.2.3.2 Characterisation of lncRNA expression and localisation.

Following the identification of the lncRNA of interest using previous microarray strategies or newly described high-throughput sequencing technologies, the validation of the results obtained is usually performed by qRT-PCR (using Sybr Green or TaqMan technologies). This strategy uses online available software such as, Ensembl²¹², RefSeq²¹³ or NCBI-PrimerBlast <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> that can be used for the design of specific primers or probes to amplify lncRNA transcripts. This allows to confirm or disconfirm any change in the lncRNA transcriptional profile observed when analysing different cell populations/tissues and different conditions (healthy/disease or

control/treated or wild type/genetically modified samples). Considering that the function of lncRNA transcripts is related to their cellular localisation, to detect the expression of the lncRNA of interest in the cellular compartments, subcellular fractionation can be performed. This strategy can also be applied to compare the localisation profiles of lncRNAs in cells exposed to different conditions or at different stages of differentiation. The characterisation of lncRNA subcellular localisation obtained by fractionation is usually confirmed by the application of other histologic strategies such as *in-situ* Hybridization, which allows the detection of lncRNAs in fixed tissues, and/or FISH to identify the localisation of lncRNA transcripts in fixed cells²¹⁴. Moreover, this strategy can also be used for the identification of the lncRNA copy number in cells. Considering the high tissue-specificity and the lack of complete annotation of lncRNA structure and transcripts, the knowledge of lncRNA transcriptional isoforms is essential to achieve the comprehensive knowledge required for further functional studies. Rapid amplification of cDNA ends (RACE) combined with sequencing can be applied to obtain information on transcripts 5' and 3' ends. Whereas cap analysis gene expression can be used for the identification of polyadenylated lncRNAs²¹⁵. However, RACE requires the knowledge of the transcripts end sequences which in the case of very complex lncRNA loci might represent a limitation. Moreover, considering that some lncRNA can include non-polyadenylated transcripts, the second strategy might result in incomplete identification of lncRNA molecules. To overcome these limits, the development of long-reads sequencing allows the full-read determination of lncRNA transcript sequences, the detection of transcriptional start sites and the identification of 3' ends.

1.5.2.3.3 Study of lncRNA function.

Identification of the functional role of lncRNAs is based on the evaluation of the functional properties of lncRNAs and includes *in vitro* and *in vivo* approaches. There is no specificity on which of the two approaches to use first, however most studies of conserved lncRNAs include both approaches. The possibility to explore molecular and cellular functions *in vivo* represents a very powerful opportunity, particularly in the

case of exploring future therapeutical approaches in translational medicine. However, considering that a large proportion of lncRNA is not conserved and that animal models sometimes do not resemble the response mechanisms activated in humans, it is sometime difficult to find confirmation and the interpretation of the data requires careful analysis. Considering that lncRNA expression profiles can be affected in specific physiological or pathological conditions, the modulation of lncRNA expression represents a unique opportunity to study the functional role of these molecules and the effect of their manipulation on cellular phenotypes. To this end, *in vivo* and *in vitro* approaches can be used:

- Loss of function strategies allow the complete (knock-out) or partial (knock-down) depletion of the lncRNA of interest to be achieved in a transient or constitutive way. Different approaches have been described to achieve transient knock-down of lncRNA molecules, such as the use of RNA interference²¹⁶ in the case of cytoplasmic lncRNAs (siRNAs) or modified antisense oligonucleotides (such as GampeR) to efficiently knock-down nuclear lncRNAs. While transcription activator-like effector nucleases based on zinc-finger nucleases (TALEN)²¹⁷ and clustered regulatory interspaced short palindromic repeat (CRISPR/Cas9)²¹⁸ approaches resulted in efficient tools in the case of constitutive depletion of the locus of interest and generation of genetically modified animals. Of course, both approaches require careful consideration of the pros and cons of their use and related target-off effects.
- Gain-of-function approaches have been used to overexpress or activate the lncRNA of interest. In this case, the overexpression can be achieved by ectopic delivery or endogenous activation of the locus. In the first case, recombinant viral vectors (Adenoviruses^{219,220}, Lentiviruses, AAVs²²¹) expressing the transcript of interest can be used to infect cells cultured *in vitro* or delivered into the circulatory system or injected locally in mouse models *in vivo*. On the other hand, endogenous activation can be achieved using CRISPR/Cas9 approaches to activate the transcription the lncRNA locus of interest²²². In this

case, it is also important to consider all the features of both technologies, such as size limits and toxicity related to the use of recombinant vectors or the conformational chromatin state and activation levels of lncRNA promoters which can negatively affect CRISPR-mediated overexpression strategies.

Collectively, these strategies represent the base for lncRNA manipulation that can be chosen for further relevant functional assays *in vitro* and animal models to explore the consequences of lncRNA dysregulation.

1.5.2.3.4 Identification of lncRNA interactors.

Characterisation of molecular lncRNA interactors takes into consideration information obtained through previous investigations of the expression, localisation, and expression-associated phenotypes, allowing researchers to formulate a more robust hypothesis on possible lncRNA molecular partners. The use of online bioinformatic tools (such as <http://starbase.sysu.edu.cn/> , <http://rna.sysu.edu.cn/chipbase/> , <https://lncipedia.org>) for the prediction of molecular interactors might help scientists to refine hypotheses and choose adequate validation strategies. Considering the multiplicity of interactions that lncRNA molecules can display, several experimental procedures have been developed for the study of RNA-DNA, RNA-RNA and RNA-proteins complexes. Generally, methods for the identification of these complexes includes the crosslinking of lncRNA-interaction bounds followed by stringent washes to eliminate unspecific interactions. The pull-down of lncRNA-protein complexes achieved with complementary probes designed to capture the lncRNA of interest, followed by mass spectrometry resulted in a very efficient method for the study of proteic interactors (such as crosslinking and immunoprecipitation²²³). Derivates of this strategy are ChIRP-MS and RAP-MS, characterised by a step of RNA capture and purification associated with mass spectrometry^{224,225}. Methods to identify and study possible lncRNA-DNA interactions include ChIRP, RAP and CHART-Seq, based on the capture and cross-linking of RNA of interest from a cell extract followed by deep sequencing

of the enriched DNA sequencing therefore suggesting possible interactions with genomic DNA²²⁶. Furthermore, lncRNAs have also been described as able to form RNA-RNA interactions that can potentially be mediated by proteins. These interactions can be mediated by proteins or not and specific strategies have been developed to explore both possibilities. One efficient method validated for the high-throughput characterisation of lncRNA-RNA sites of interaction is represented by crosslinking ligation and sequencing of hybrids (CLASH). This technique is based on the crosslink of a bait protein to stabilize any RNA interactions followed by linking of the RNA extremities and preparation of cDNA library for deep sequencing^{227,228}. While examples of strategies for the identification of direct RNA-RNA interactions are PARIS or SPLASH, which allow for transcriptome-wide RNA interactions through *in vivo* cross-linking and deep sequencing^{229,230}.

1.5.3 Long noncoding RNAs in cardiovascular disease, including atherosclerosis.

Several lncRNAs have been found involved in the cellular interactions characterising atherosclerotic pathophysiology. Considering the high tissue- and cellular-specificity, lncRNAs have been studied in relation to specific cellular contexts which in turn contribute to the pathology of disease.

1.5.3.1 Endothelial cell dysfunction.

Different studies have highlighted the role of lncRNAs in the regulation of EC function, whose de-regulation is observed during the stages of plaque development. One example is represented by the lncRNA SNHG12, which was found highly expressed in the endothelium and progressively downregulated during lesion progression in mouse, human and pig samples. To study SNHG12 function, GapmeR-mediated loss of function experiment performed in atherosclerotic mice have shown accelerated atherosclerosis, while overexpression of this lncRNA leads to protection of intimal damage. To understand the molecular mechanism of actions, the authors have

performed pull down and mass spectrometry experiments and identified SNHG12 interaction with DNA damage response kinase DNA-PK. Therefore, a decrease in the lncRNA levels can lead to a dysregulation of the DNA-associated pathways which in turn lead to DNA damage and EC senescence²³¹. Another EC-expressed lncRNA is HOTAIR, whose expression was found decreased in EC isolated from atherosclerotic lesions versus healthy vessel walls. Functional assays have described HOTAIR as a positive regulator of EC proliferation and migration, while suppressing apoptosis. Investigation on the regulation of HOTAIR expression revealed that the thymic stromal lipoprotein, with a known protective role in atherosclerosis, positively regulates the expression of the lncRNA. However, during atherosclerosis, the downregulation of the protein in turn reduces HOTAIR expression therefore contributing to EC dysfunction²³². The smooth-muscle and Endothelial cell-enriched lncRNA SENCR was identified as enriched in the cytoplasm of ECs, overlapping the Friend Leukaemia Integration virus 1 gene, a gene involved in EC differentiation²³³. In a model of differentiation of ECs from human embryonic stem cells, SENCR expression was associated with the induction of early mesodermal and endothelial commitment. With regard to the functional role, SENCR induced proliferation, migration and angiogenesis²³⁴. Moreover, loss of function studies revealed that SENCR knock-down increased EC permeability disrupting EC membrane stability. Mechanistically, pull-down and mass spectrometry experiments have shown that this lncRNA can physically interacts with cytoskeletal associated protein and can promote EC junction and vascular integrity. These processes result instead compromised in the case of SENCR knock-down²³⁵. The metastasis-associated lung adenocarcinoma transcript, MALAT1 is a well expressed lncRNA in ECs, SMCs, immunity and hematopoietic cells, whose expression was found upregulated in dysregulated EC as response to hypoxic conditions. *In vitro* knock-out of MALAT increased EC migration and basal sprouting, while the cell proliferation rate subsequently decreased therefore impairing CE functions. Moreover, loss of MALAT *in vivo* negatively affected vascularization and capillary density after limb ischemia, therefore affecting vessel growth²³⁶. MALAT1 expression was moreover increased following EC exposure to

high glucose levels, together with increase of pro-inflammatory glucose-activated genes. Further functional studies shown that the knock-out of this lncRNA prevented gene dysregulation induced by high glucose stimuli, therefore suggesting a possible involvement of MALAT1 in regulating hyperglycaemia-dependent pathways²³⁷. However, this lncRNA was also described for the potential beneficial effects associated to its expression in hematopoietic cells. In particular, a study demonstrated that the loss of MALAT1 increased plaque formation in an atherosclerosis mouse model, and that the transplantation of bone marrow from MALAT1-deficient mice enhanced lesion formation. Further *in vitro* studies in cells isolated from MALAT1 knock-out mice showed increased adhesion and expression of pro-inflammatory genes, therefore suggesting anti-inflammatory and atherosclerosis-protective role played by this lncRNA²³⁸. A schematic representation of lncRNA involvement in atherosclerosis can be found in Figure 1.8.

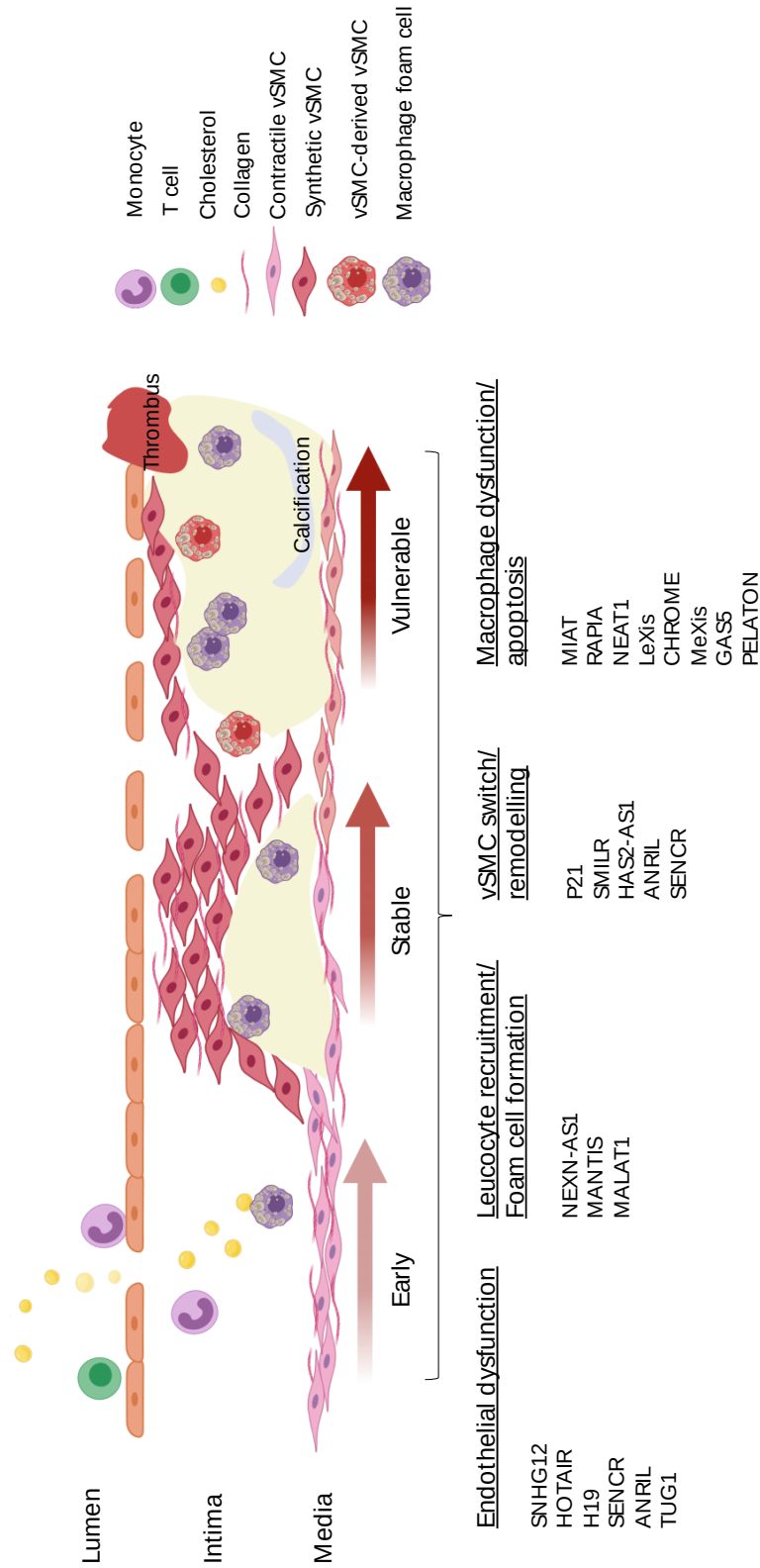


Figure 1.8 The involvement of lncRNAs in atherosclerosis.

Figure 1.8 The involvement of lncRNAs in atherosclerosis.

In the past few years different lncRNAs have been described in their function during the development of human and mouse plaque. This picture summarises main processes characterising 3 of the key phases during the development of the lesions: i) early plaque characterised by endothelial dysfunction and leucocytes recruitment, ii) stable plaque characterized by formation of foam cells and vSMC phenotypical switch, iii) vulnerable lesions where macrophage apoptosis, calcification erosion of fibrous cap are frequent and can lead to thrombus formation and plaque rupture. Each of these processes is tightly modulated by molecular networks that includes lncRNA molecules. Examples of these are reported in this pictures in association to the main where they play associated function.

1.5.3.2 SMC phenotypical switch.

The phenotypic transition between contractile and synthetic vSMC has also been related to lncRNA dysregulation and several lncRNA molecules have been characterised for their role during this process. ANRIL is a lncRNA encoded by a locus described as genetic risk factor for coronary artery diseases (CAD) 9p21.3 and its expression was found increased in patients affected by CAD²³⁹. A study investigating the differences between risk-associated vSMC, derived by differentiation of induced pluripotent stem cells isolated from individuals displaying the risk haplotype and stem cells-derived vSMC from non-risk patients, identified the detrimental effect of ANRIL expression. In particular, the genetic deletion of the risk-associated haplotype conferred vSMC protection from the risk-related phenotype. ANRIL expressing vSMCs, however, were characterised by a risk-associated phenotype²⁴⁰. Moreover, ANRIL expression was positively regulated in ox-LDL treated vSMCs. Loss of function experiments revealed that ANRIL knock-down decreased the expression of ox-LDL-induced ROS and alleviated pro-inflammatory phenotype in vSMC. Mechanistically, it was shown that this lncRNA might act as a molecular scaffold for histone modification proteins involved in the regulation of the expression of ROS-associated genes²³⁹. Interestingly, this ANRIL belongs to the group of lncRNAs with multiple transcripts, among which one can form circular RNAs referred to as circ-ANRIL. Studies investigating the role of this circular transcript highlighted the duality of ANRIL function in atherosclerosis. Circ-ANRIL was found downregulated in individual carriers of risk-haplotype versus non-risk patients. Functional experiments showed that circ-ANRIL binds to the ribosomal assembly factor Pescadillo, therefore influencing the processing of pre-rRNAs which in turn activates p-53 dependent apoptosis and anti-proliferative pathways in vSMCs, therefore conferring atheroprotection²⁴¹. In addition, ANRIL was also found downregulated in ageing vSMC and its overexpression promoted viability and inhibition of senescence in vSMCs cultured *in vitro*. Functional studies by luciferase assay have moreover shown that ANRIL might be able to bind and repress the activity of miR-181a whose primarily

target is *SIRT1* gene, therefore alleviating vSMC cycle arrest mediated by p53 pathway²⁴². Another example of smooth muscle enriched lncRNA with functions related to the control of vSMC plasticity in disease, is represented by SMILR. This lncRNA was initially identified in phenotypically modulated vSMC exposed to pathogenic PDGF-BB and IL-1 cytokines, where it was found overexpressed compared to control cells. Moreover, RNA sequencing results from stable and unstable human plaque regions confirmed the upregulation of SMILR in vulnerable advanced plaques versus stable regions²⁴³. Functional assays revealed that SMILR regulates vSMC proliferation *in vitro*, and, that the loss of SMILR attenuates PDGF-BB/IL1-induced vSMC proliferation, whereas lentiviral-mediated overexpression of SMILR revealed opposite effect by increasing vSMC proliferation. Moreover, SMILR knock-down leads to a significant decrease of cellular proliferation in *ex vivo* model of vein grafting. Mechanistically, pull down experiments revealed that, through direct interaction with centromere protein F and RNA binding protein STAUFEN1, SMILR regulates the late phases of mitotic cell cycle inducing pro-proliferative dysregulated vSMCs phenotype²⁴⁴. The lncRNA-p21 has also been described among the lncRNAs associated with CAD-associated risk²⁴⁵. Molecular studies on the regulation of lncRNA-p21 demonstrated that the lncRNA belongs to the transcriptional targets regulated by p53, and that it mediates the p53 dependent gene repression. In fact, inhibition of lncRNA-p21 resulted to decrease the expression of p53 modulated genes¹⁸². In the pathological context, lncRNA-p21 was found downregulated in plaques isolated from atherosclerotic mice versus controls. Functional experiments investigating the role of the lncRNA-p21 in mouse and human vSMCs revealed that loss of function increased vSMC proliferation and viability, while the knock-down of the lncRNA decreased cell apoptosis²⁴⁶. Mechanistically, lncRNA-p21 was found binding MDM2, a repressor of p53, therefore enabling p53 to interact with the promoter of its target genes. Collectively, these results suggest that the expression of lncRNA-p21 might exert a protective anti-proliferative role, which might be beneficial during the progression of early to late atherosclerotic plaques. The lncRNA NEAT1 was moreover described as influencing the phenotypical transdifferentiation

of vSMCs. Expression results found this lncRNA increased in balloon-mediated vascular injury model in mice and in atherosclerotic plaques versus healthy controls. Moreover, the modulation of NEAT1 dysregulated vSMC function, and in particular, NEAT1 silencing increased vSMC contractility genes and decreased proliferation and migration, while its overexpression induced opposite phenotypes. *In vivo*, the loss of NEAT associated with decreased neointimal formation and attenuated vSMC proliferation. Further mechanistic experiments shown that NEAT1 is able to induce the silencing of vSMC typical genes by sequestering a chromatin modifier, therefore promoting vSMC's switch to less contractile state²⁴⁷. The silencing of NEAT1 was moreover found having a beneficial effect in human macrophages exposed to ox-LDL stimuli. In particular, NEAT1 knock-down reduced the expression of scavenger receptor CD36, the cholesterol uptake and macrophage apoptosis. Moreover, NEAT1-depleted macrophages showed an attenuated pro-inflammatory phenotype with reduction of IL-6, TNF and cyclooxygenases expression²⁴⁸. In addition, bioinformatic analysis and validation assays revealed miR-342-3p as NEAT1's downstream molecular target. In particular, the expression of miR-342-3p was associated with reduced inflammatory response of ox-LDL modulated macrophages and therefore NEAT1-mediated inhibition of this microRNA might increase pro-pathological associated pathways in macrophages²⁴⁹.

1.5.3.3 Macrophage activation and cholesterol metabolism.

Several lncRNAs have been characterised for their molecular role during important processes contributing to plaque formation and development, such as the formation of fatty streak, inflammation, and enlargement of necrotic core. As previously described in this chapter, the accumulation of cholesterol, and the dysfunctional macrophage pathways regulating the metabolism and clearance of lipids constitute driver factors to the activation of pro-pathological processes in lesions. Expression studies shown that CHROME lncRNA was upregulated in plasma from atherosclerotic patients versus controls, and functional results revealed that CHROME expression can

be modulated by the sterol-activated liver X receptor transcription factor which controls cellular and systemic cholesterol metabolism and homeostasis. Loss- and gain-of-function studies demonstrated that the modulation of CHROME regulates cholesterol efflux and HDL biogenesis through a mechanism of action that is mediated by the regulation of a set of microRNAs. In particular, among the target of microRNAs modulated by CHROME, the efflux transporter ABCA1 was dysregulated which therefore impaired cholesterol metabolism and macrophage function²⁵⁰. Another example of lncRNA modulating macrophage function is the myocardial infarction associated lncRNA MIAT was found upregulated in atherosclerotic rats versus control animals²⁵¹. Moreover, MIAT was identified secreted and upregulated in the serum of symptomatic vulnerable plaque patients versus the levels observed in the circulation in healthy controls^{252,253}. Expression of MIAT was also found upregulated in macrophages isolated from the necrotic core of atherosclerotic mice. Functional studies shown that the loss of MIAT attenuated atherosclerosis *in vivo* and promoted macrophage clearance of apoptotic cells *in vitro*. Moreover, the mechanism of action was interrogated and revealed that MIAT can act as a molecular sponge negatively affecting the role of miR-149-5p, therefore positively regulating the expression of the anti-phagocytic molecule CD47²⁵⁴. Stable and unstable RNA sequencing results identified PELATON, a plaque macrophage enriched lncRNA whose expression was found upregulated in advanced versus stable plaques. Histologic examination of PELATON distribution in plaques localised its expression in association with macrophages in more unstable regions of the plaque, such as the shoulders and plaque core. Functional assays have also shown that the loss of PELATON reduces macrophage lipid uptake and ROS production, therefore attenuating pro-inflammatory associated phenotype²⁵⁵. MeXis is a lncRNA whose expression is regulated by the sterol-activated nuclear receptor regulating the expression of cholesterol metabolism associated genes. MeXis was described as transcriptional regulator for the expression of *ABCA1* transporter implicated in the cholesterol efflux. Loss-of-function results indicated that MeXis knock-out impaired *ABCA1* expression and cellular responses to cholesterol overload, leading to

accelerated atherosclerosis *in vivo*. Mechanistically, MeXis was found playing a role as co-activator of the sterol-activated nuclear receptor pathways and therefore controlling macrophage gene expression and associated function²⁵⁶. Similarly to other lncRNAs, the lncRNA growth arrest-specific 5, GAS5, was found associated with a locus with high risk locus for the development of CAD²⁵⁷. Expression analysis revealed that this lncRNA was upregulated in atherosclerotic mice versus controls. In particular, *in vitro* assays shown increased levels of GAS5 following macrophage stimulation with ox-LDL. Functionally, the overexpression of this lncRNA enhanced the production and secretion of pro-inflammatory IL-6, IL-1, TNF and metalloproteases, while GAS5 inhibition decreased them, therefore generating an opposite phenotype. Moreover, the modulation of GAS5 expression revealed that this lncRNA can regulate the expression of *ABCA1* and therefore negatively affect cholesterol metabolism in macrophages. Further *in vivo* experiments also confirmed the increase in atherosclerosis and cholesterol dysregulation of GAS5-depleted atherosclerotic mice²⁵⁸. It was identified that GAS5 acted as sponge for miR-221, involved in the pathophysiology of atherosclerosis. In particular, miR-221 overexpression antagonizes the effect observed following GAS5 overexpression, therefore reducing macrophages inflammatory response²⁵⁹. Collectively, these studies suggest a potential role for GAS5 expression as pro-pathogenic factor which in turn inhibits the anti-inflammatory role mediated by miR-221 during vascular injury and atherosclerosis.

1.5.4 lncRNA-microRNA crosstalk: The importance of lncRNA Host Genes.

The study and characterisation of different groups of noncoding RNA molecules have unravelled some important associations and interactions between noncoding RNAs belonging to different groups, and in particular the regulatory networks that include long noncoding RNAs and microRNAs. These two distinct noncoding molecules have been described as co-operating for the regulation of the same molecular networks by playing synergistic roles or having antagonistic roles by having opposite functions. An increasing amount of evidence on and studies of lncRNA-microRNA crosstalk

suggest the existence of another level of gene regulation, different from noncoding-protein-coding genes. Moreover, these newly described interactions have been characterised in their duality of regulation with lncRNA being able to modulate the expression and functions of microRNAs, such as acting as a molecular sponge for miRNAs and preventing interaction with their targets (lncRNA sponge) or competing for the binding of the same molecule (competing lncRNAs) and vice-versa, with miRNAs triggering lncRNA decay. Valuable examples of each of the above-mentioned regulatory networks have also been implicated in the regulation of atherosclerosis. The taurine upregulated gene 1 (*TUG1*) has been described upregulated in atherosclerosis, while its knock-down decreased plaque formation in atherosclerotic mice. In particular, *TUG1* overexpression induced macrophage inflammation and reduced apoptosis by acting as a sponge for miR-133a, therefore upregulating the microRNA target, fibroblasts growth factor 1²⁶⁰. One example of competing endogenous lncRNA is instead represented by the lncRNA *TGFB2-OT1* overlapping the transforming growth factor beta 2 transcript. This lncRNA was found overexpressed in response to ox-LDL stimulation, one of the main drivers of atherosclerosis. Moreover, *TGFB2-OT1* has been described having a role in the regulation of EC autophagy in vascular disease by competing for the regulation of autophagy-related protein 13, also regulated by miR-4459²⁶¹. One case in which miRNAs regulate the expression of lncRNA is represented by *MALAT1* and miR-9. The dysregulation in the levels of *MALAT1* has been involved in different cellular processes leading to atherosclerosis. In particular it was found that miR-9 can directly bind *MALAT1* inducing its decay with AGO2-dependent pathway²⁶².

1.5.4.1 MicroRNA Host Genes (HG) lncRNA.

As well as acting as regulators of microRNA functions, long noncoding RNA loci can generate small RNA molecules. This class of lncRNAs has been described to display within their genomic locus the precursor of small nucleolar RNAs or microRNAs. In the past, the function of this group of molecules was functionally unknown and studies have investigated whether lncRNAs were merely acting as precursors for

small RNA transcripts or have independent roles. Currently, an increasing number of lncRNA host genes is characterised and annotated for their functional role which can be dependent or independent from their hosted small RNAs. Based on the HUGO nomenclature, lncRNA hosting small nucleolar RNAs are also referred to as SNHG, whereas in the case the small RNA is a microRNA the host gene is named with the format [miR gene name]HG¹²⁸. A curated list of annotated miRHG and SNHG can be found at the <https://www.genenames.org/data/genegroup/#!/group/1690> and <https://www.genenames.org/data/genegroup/#!/group/1838> pages of the HGNC archive. Regarding their structural organisation, lncRNA HGs can include hosted transcripts in their intronic or exonic sequences. This observation is in line with previous findings demonstrating that only 28% of microRNAs are transcribed from independent loci, while most of them are hosted by coding or noncoding genes, also referred to as intragenic miRNAs (FIGURE 1.9).

1.5.4.2 Biogenesis of lncRNA-microRNA host gene complexes.

Studies investigating the complex biogenesis of hosted miRNAs have grouped the processes leading to their production from corresponding MIRHG loci into two main categories, the “synergic model” and the “competitive model”. In the first model, the splicing of the MIRHG favours the biogenesis of the hosted miRNA, therefore leading to the co-production of the MIRHG transcript and microRNA molecule. This model mostly characterises the biogenesis of intronic miRNAs. One example is the lncRNA NEAT1 whose expression is finely regulated by splicing mechanisms to balance the transcription of the hosted miR-612²⁶³. While the competitive model describes the condition where the MIRHG splicing complexes compete with miRNA processing factors, resulting in the production of one of the two molecules. This is mostly observed in cases where the microRNA is localised in the intron-exon junction. In specific circumstances, alternative splicing can favour the synthesis of the MIRHG transcript over the production of the pre-miRNA, as with MCM7HG and the hosted miR-106-25. In particular, the splicing favours the production of MCM7 transcript inhibiting the generation of pre-microRNA. While inhibition of splicing increases miR-

106-25 production, and knock-down of Drosha, a key protein for the processing of microRNAs, increases the production of MCM7²⁶⁴. It was observed that most hosted miRNAs display a similar expression pattern than their lncRNA HGs. However, some studies have shown different expression profiles which can be the result of the existence of multiple promoters belonging to the same locus. This is the case of the miR-483-3p, hosted in the insulin-growth factor 2 locus, which was found co-expressed with the host gene transcript, however host gene-independent expression was also observed being driven by a separate promoter transcribing for miR-483-3p²⁶⁵. In relation to the function of lncRNA host genes and their associated microRNAs, an increasing number of studies identified independent functions of lncRNA from the co-located microRNAs. A valuable example is the muscle-specific lncRNA MD1, host gene for miR-133b. The lncRNA MDM1 was demonstrated to control the expression of miR-133 by acting as a molecular sponge during muscle differentiation. In addition, independent transcription of lncRNA MDM1 was observed being regulated through a HUR-mediated mechanism. HUR is a protein modulator of pri-miRNA and lncRNA transcripts stability which is negatively regulated by miR-133. However, lncRNA-MDM1 can independently regulate HUR protein levels with a feedforward mechanism by recruiting its repressor, miR-133²⁶⁶. MIR205HG is another example which can be included in the group of lncRNA host genes with separate functions from their hosted microRNAs. MIR205HG (also referred to as LEADeR) hosts miR-205 in exon-intron boundaries. It was demonstrated as this locus can generate pre-miR-205 contemporaneously to the nuclear MIR205HG, which is implicated in the regulation of prostate epithelial cells identity genes independently from miR-205 function²⁶⁷. Moreover, MIR100HG locus, host gene for miR-100, was shown to transcribe for a functionally active and stable lncRNA MIR100 with independent function from the co-located miR-100 in the regulation of cell cycle progression and proliferation²⁶⁸. Further meta-analyses performed in a wide panel of tissues and cell types, have shown that lncRNAHG can also play a super-enhancer role by modulating the expression of co-located microRNAs. In particular, enhancer-associated lncRNA host genes were described as able to recruit microRNA processing

proteins Drosha/DGCR8 to favour microRNA transcription. In addition, genetic modulation of super-enhancer expression leads to a decrease in the levels of co-located microRNAs, therefore creating a super-enhancer-miRNA network where lncRNAHG constitutes another level of microRNA transcriptional regulation²⁶⁹. Collectively, these studies highlight structural complexity of lncRNAHG-miRNA interactions which will require more investigations and additional work to be fully understood and characterised. Nevertheless, these discoveries reveal the existence of an additional level of regulation of gene expression represented by tightly controlled lncRNA-microRNA axes, which can influence important cellular processes. Additionally, considering the high level of complexity of these networks and the fine regulation required for their proper functioning, the dysregulation of their interaction can contribute to the pathogenesis of disease.

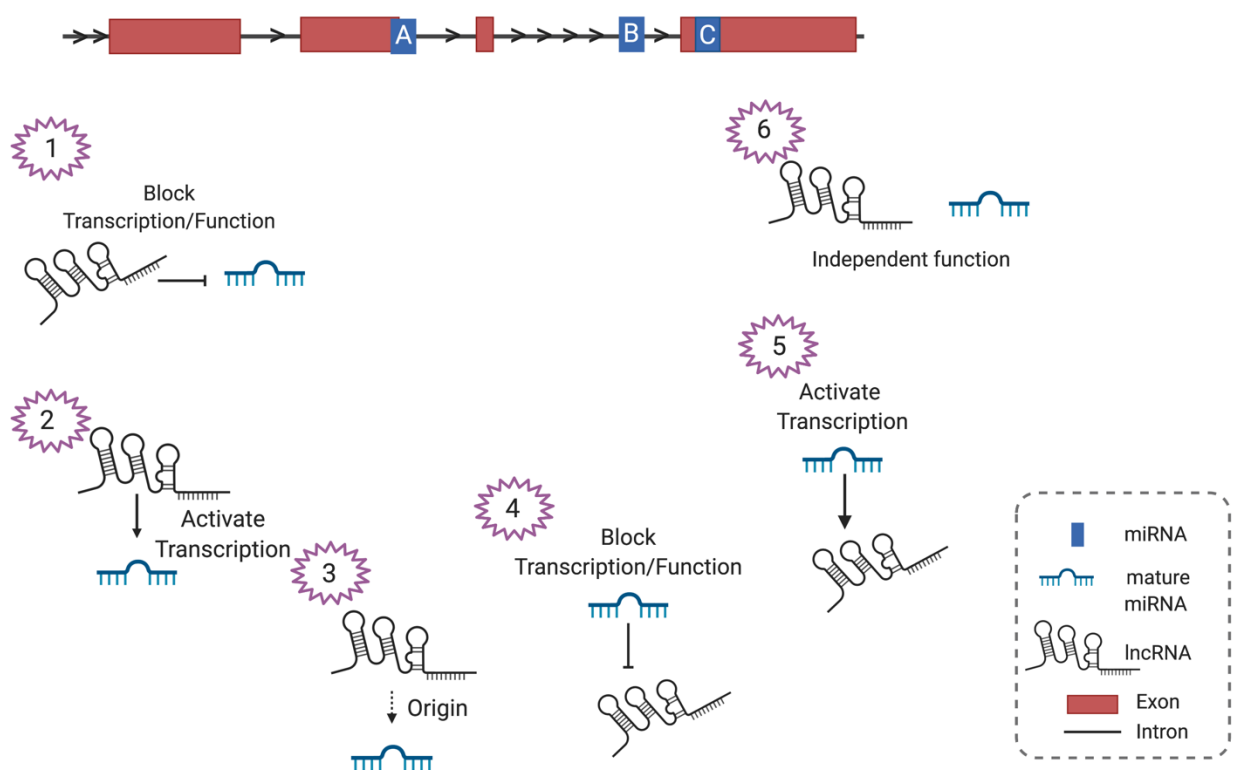


Figure 1.9 lncRNA host genes of microRNAs structure and function.

Summary of the main known interactions between long noncoding RNAs (lncRNAs) and microRNAs (miRNAs). MiRNAs can be co-located with lncRNAs which in turn are referred to as lncRNA host genes. MiRNAs can localise in intron-exon boundaries (A

in the picture), can be intronic (B) or overlap lncRNA exons (C). Regardless the functions and interactions of these two noncoding RNAs, lncRNA can activate block the transcription of hosted miRNAs by competing with transcription factor complexes or acting as molecular sponges (1 in the picture), otherwise they can promote the transcription of co-located microRNAs acting as enhancers or interacting with transcription factors (2). lncRNA can also not have a function per se, therefore including pri-miRNAs among their transcriptional isoforms (3 in the picture). Likewise, miRNAs can modulate lncRNA host genes with feedforward network by negatively blocking their transcription factors (4). Contrarily, they can induce lncRNA host gene transcription or act in synergy to promote or block connected pathways (5). Finally, lncRNA host genes can be stable and display different roles than their hosted microRNAs which in turn are involved in the regulation of different regulatory networks (6).

1.6 *CARMN* axis: miR-143/145 and lncRNA *CARMN* in vSMCs.

The two microRNAs miR-143 and miR-145 have been extensively described as “pillar” regulators of vSMC function. Importantly, several studies have also described their critical role in a wide spectrum of pathological contexts such as cardiovascular diseases (such as atherosclerosis²⁷⁰, pulmonary arterial hypertension^{271,272}) as well as cancer²⁷³, metabolic disorders²⁷⁴ and moto-neuron disease²⁷⁵. More recently, *CARMN* host gene for miR-143/145 cluster was characterised as having a functional role as well as originating the microRNA stem-loop.

1.6.1 MiR-143 and miR-145 in vSMC physiology and atherosclerosis.

Initial molecular studies on the genomic organisation of these two microRNAs, shown that they are included in the same bicistronic unit (located in the mouse chromosome 18) and can be co-transcribed in the same pri-microRNA, therefore sharing common regulatory elements²⁷⁶. Moreover, functional studies have investigated the abundances of the guide and passenger strands and have identified as miR-143-3p

and miR-145-5p are the most expressed strands with potential function (referred to as miR-143 and miR-145 in this section). Comparison of genomic sequences on the region upstream of the cluster revealed a highly conserved region between mouse and human (located in the human chromosome 5). In this regard, two studies performed in vSMC have identified the existence of two possible promoters regulating miR-143/145 expression levels^{276,271}. Different studies have investigated miR-143/145 expression levels during mouse embryonic and post-natal development. In particular, microarray experiments in a tissue panel from various developmental stages and lacZ reporter mice have shown that miR-143/145 are widely expressed during early stages of cardiogenesis, but that during late stage of development preferentially localise with vascular (aorta, pulmonary vessel, coronary vessels) and visceral SMC^{270,276}. These results were also confirmed by qRT-PCR and In situ Hybridisation in tissue sections²⁷⁷. Postnatally, however, miR-143/145 were undetectable in cardiomyocytes. Similar same expression profile was also observed in analysis of microarrays performed in primary cardiomyocytes differentiating into smooth muscle lineage following stimulation with Myocardin-related transcript factors where miR-143/145 were the most strongly upregulated. These results were also confirmed by analysis within the genomic portion upstream of the cluster, where it was found to have a binding site for serum response factor/Myocardin regulatory complex²⁷⁸. To further understand if changes in the expression of miR-143/145 were involved in cardiovascular disease *in vivo*, the expression of the cluster was assessed in diseased versus healthy condition. In particular, microRNAs miR-143/145 were found to be downregulated in injured carotid arteries and atherosclerotic lesions versus controls. Moreover, biopsies from patients suffering from aneurysm showed significant downregulation of miR-143/145 versus controls^{276,279}. However, studies profiling the expression of microRNAs in human plaque found upregulation of miR-143 and miR 145 in plaques from symptomatic patients experiencing stroke versus asymptomatic patients^{280,281}. Contrarily, other two independent studies observed no changes in the levels of both microRNAs in symptomatic versus asymptomatic carotid plaques^{282,283}. Loss of function experiments performed in mice have shown that vSMC

composing the aorta from miR-143/145 knock-out mice were significantly smaller, with reduced contractility fibres and an increase in rough endoplasmic reticulum. Functional experiments demonstrated reduced contractility of femoral artery explants and less responsiveness of vSMC to contractility stimuli such as Angiotensin II exposure, therefore demonstrating that morphological alteration of vSMC depleted for miR-143/145 led to functional dysregulation²⁷⁰. MiR-143/145 KO vSMC showed increased migration when stimulated with PDGF-BB *in vitro* and decreased expression of vSMC typical genes (such as Alpha Actin and Myosin Heavy Chain 11) versus wild type (WT) cells²⁷⁹. Bioinformatic analysis and experimental validation identified targets regulated by miR-143/145, among which were transcription factors KLF4/5, actin polymerisation proteins (SGRAP1/2), proteins involved in cell migration (ADD3), and the co-activator of SRF (MRTF)²⁷⁸. Moreover, *in vitro* functional assays showed an overexpression of miR-145 is able to suppress the PDGF-BB mediated vSMC viability, while the antimir-mediated knock-down in miR-145 did not induce the opposite effect²⁷⁶. Further experiments investigating the consequence of vSMC ability to uptake cholesterol have demonstrated that the expression of this cluster is significantly dysregulated in cholesterol-loaded vSMC versus control cells, therefore suggesting the involvement of miR-143/145 in the process of vSMC-derived foam cell formation¹¹³. In addition, *in vivo* observations revealed the spontaneous development of neointima and increased neointimal accumulation of vSMC in KO versus WT animals, which might predispose animals to the development of vascular disease versus wild type control²⁷⁰. In addition, the adenoviral-mediated overexpression of miR-143 and miR-145 proved beneficial in a balloon-injury model of vascular disease by reducing neointimal formation versus control mice²⁷⁷. While, in the case of arterial injury obtained using a carotid artery ligation model, the loss of this cluster reduced the neointima formation versus wild type mice, therefore suggesting that the depletion of miR-143/145 might protect against vascular remodelling²⁷⁸. Moreover, atherosclerotic mice depleted of the microRNA cluster displayed attenuated lesion progression versus wild type animals, although a significant number of vSMC within the plaque were found switched to their synthetic

phenotype²⁸⁴, therefore highlighting the complexity of this locus. Nevertheless, another factor enhancing the complexity of this cluster is represented by the different roles that these two microRNAs can play in diverse vascular contexts. MiR-145 and miR-143 have been found upregulated in the pathological vascular remodelling characterising hereditary and idiopathic pulmonary arterial hypertension and hypoxia-induced model of disease. Nevertheless, loss-of-function studies revealed that in this pathological context the loss of miR-143/145 leads to protection from the development of disease and reduction of vascular remodelling^{271,272}.

1.6.2 The lncRNA *CARMN* in cardiovascular biology and disease.

CARMN is a long intergenic RNA belonging to the group of host genes which includes two microRNAs embedded in the same locus, miR-143 and miR-145, localised respectively in one exon and one intron. The structural organisation of *CARMN* comprises multiple spliced transcriptional isoforms (the most updated annotation includes 19 different transcripts) with different transcriptional start sites (Figure 1.6). *CARMN* is the HUGO gene name indicating “Cardiac Mesoderm Enhancer-associated lncRNA”, as it was first described in cardiomyocyte development. In a study profiling the lncRNA transcriptome during the differentiation of cardiac progenitor cells into adult cardiomyocytes, *CARMN* was found significantly upregulated in differentiating foetal cardiac progenitor cells, while miR-143/145 were regulated in the opposite direction. As part of the characterisation of this locus, bioinformatical analysis was used to investigate the coding score and the existence of possible peptides originating from this locus. However, neither reported coding potential or the presence of a complete open reading frame. Moreover, analysis of the genomic portion including *CARMN* and miR-143/145 identified levels of conservation in vertebrates. Localisation experiments performed in cardiomyocytes indicated that *CARMN* has a mostly nuclear localisation, therefore hypotheses on the potential mechanism of action were focused on the function played by nuclear noncoding molecules. Chromatin immunoprecipitation analysis of online data showed a typical

signature of super-enhancer locus with enrichment of H3K27Ac chromatin mark which was also confirmed by experimental validation. Moreover, loss of function studies showed that human and mouse *CARMN*-depleted progenitor cells were blocked in their differentiation program by dysregulation of differentiation markers. In this study, the expression of microRNAs was not affected by the modulation of *CARMN* levels²⁸⁵. A second study instead explored the role of *CARMN* during cardiogenesis. During the differentiation of cardiac progenitor cells, cells can differentiate into mature cardiomyocytes or produce smooth muscle cells. *CARMN* transcripts, in association with miR-143/145 expression, were found up-regulated in during the commitment towards smooth muscle cell lineage, whereas they were downregulated when cells were induced towards a cardiomyocyte fate. To determine whether *CARMN* was involved in the vSMC lineage commitment, differentiating progenitor cells were depleted for *CARMN* and the results obtained shown a reduced vSMC differentiation together with downregulation of miR-143/145²⁸⁶.

Collectively, these results have highlighted the importance of a highly conserved region which transcribes a microRNA stem loop, generating the smooth muscle specific miR-143/145, and a long intergenic host gene RNA, *CARMN*. Several studies have characterised miR-143/145 in the determination of vSMC phenotype in physiologic conditions and their critical role in the maintenance of vSMC contractile phenotype during vascular injury and atherosclerosis. However, some contradicting results obtained from different *in vivo* experiments where miR-143/145 expression was found associated with beneficial or detrimental effect in the progression of the plaque reveal the existence of complex regulatory networks which still require more studies to be fully understood. More recently, the discovery of the host gene *CARMN*, located upstream to the microRNA locus, revealed an additional level of complexity. The presence of multiple transcriptional isoforms deriving from different transcriptional start sites might suggest the existence of multiple factors influencing the expression of *CARMN*. Host genes have been described having similar expression patterns or opposite profiles than their hosted small RNAs, which is sometime

determined by the location of the hosted molecules within their paired hosted gene loci. In the case of human *CARMN*, it includes one exonic microRNA (miR-143) and one intronic microRNA (miR-145), which instead in mouse are both embedded in the intronic region of *CARMN* locus. Loss of function experiments aiming at the identification of the molecular interaction of *CARMN*/miR-143/145 (*CARMN* axis) provided opposite results, with microRNAs being modulated by *CARMN* knock-down as well as showing unchanged expression levels. These observations therefore suggest the need for more work and additional tools to be able to dissect the molecular network regulating this complex locus.

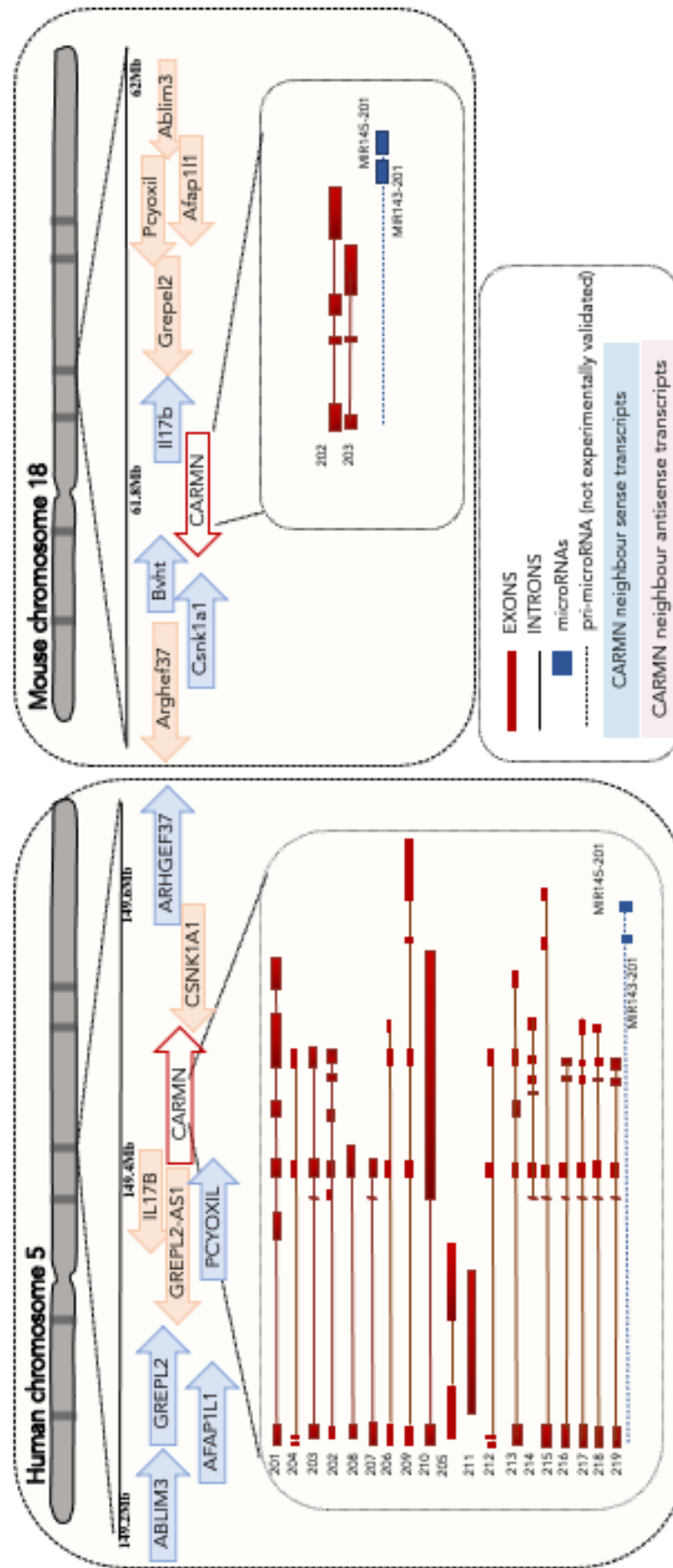


Figure 1.10 Schematic representation of human and mouse CARMN locus.

The CARMN locus has been described as conserved in human and mouse where it shows similar genomic organisation. The locus is located in human chromosome 5 and mouse 18. The human CARMN includes for 19 CARMN transcriptional isoforms (in dark red, 201 to 219) and pre-microRNA stem loop transcribing for miR-143 and miR-145 (in dark blue). Considering that it is not clear where the primary transcript for miR-143/145 starts, it is represented in the picture as a dotted line in blue. In mice, CARMN is transcribed in reverse orientation and only two transcripts are currently annotated as lncRNA (in dark red, 201 and 202). Similar to its human counterpart, the mouse locus includes the pre-miR143/145 (in dark blue), whose possible primary transcript is represented with a blue dotted line. Light blue and orange arrows represent CARMN neighbour genes. The arrows indicate the sense of transcription of each gene. Scale in Mega Bases (Mb).

Figure 1.10 Schematic representation of human and mouse CARMN locus.

1.7 Hypothesis and Aims.

Previous studies have identified the possibility for lncRNA host genes to play functionally important regulatory roles independently from their hosted microRNAs, as well as mediating cell phenotypes through the regulation of the co-located noncoding molecules. MiR-143/145 are highly expressed in differentiated vSMCs, where they exhibit a regulatory function to maintain cell contractility. Moreover, the expression levels of this SMC-specific microRNA cluster have been found downregulated during the vSMC phenotypical switch to pro-migratory and de-differentiated state in atherosclerosis and other cardiovascular disease. However, the effect of miR-143/145 downregulation caused by pro-atherogenic stimuli has not been studied or characterised functionally during the progression from early to late stages of plaque development. The possibility for CARMN and miR-143/145 to be part of the same regulatory network have been hypothesised in previous studies. Nevertheless, the molecular relationship that governs the expression and function of CARMN axis, which includes CARMN lncRNA and the miR-143/145 cluster, as well as the possibility for the two noncoding RNA to have independent roles has not been explored. The hypothesis of this PhD project can be summarised with the statement below and graphically represented below in Figure 1.11:

The modulation of the expression of CARMN regulates vSMC proliferation, migration, and de-differentiation phenotypes during the progression of early to advanced atherosclerosis, with a molecular mechanism of action that is in part mediated by the regulation of miR-143/145.

The aims of this project are:

- 1) To characterise the expression of *CARMN* and miR-143/145 during vSMC phenotypical transition *in vitro*, and to investigate the consequence of the modulation of *CARMN* in vSMC phenotypes.
- 2) To test at the molecular level whether *CARMN* has a functional effect independent of miR-143/145 regulation.
- 3) To characterise the expression of *CARMN* axis during the process of transition from early to advanced plaque in human and mouse *in vivo*.
- 4) To explore the effect of genetic loss of *CARMN* *in vivo* in a mouse model of atherosclerosis.

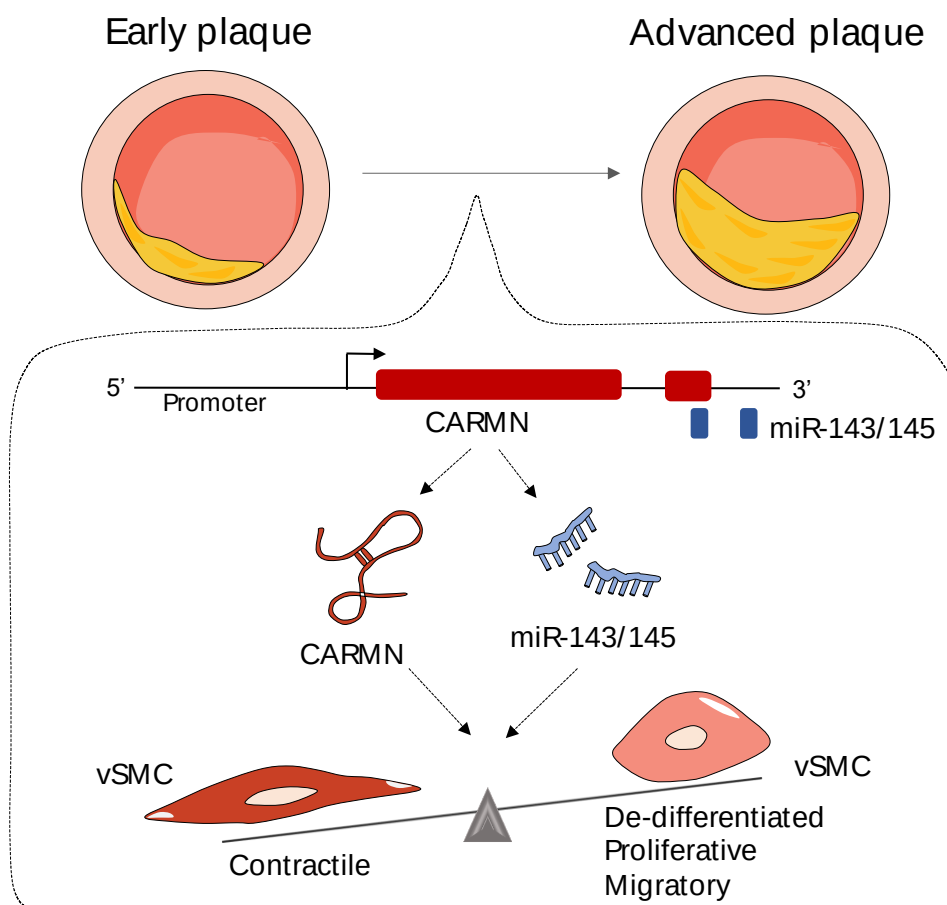


Figure 1.11 Graphical representation of the hypothesis of this project.

Chapter 2 Materials and Methods

2.1 General laboratory training.

All the reagents used in this study respected the highest commercially available standard. Hazardous chemicals were handled stored and disposed following good laboratory practices guidelines in line with Control of Substances Hazardous to Health (COSHH) regulations. Personal Protective Equipment (PPE) (laboratory coats, nitrile powder-free gloves, face masks, eye protectors and fume hoods) were used when appropriate and advised from Health and Safety guidelines. All the experiments were performed in compliance with Risk Assessment rules based on Health and Safety legislation and after appropriate laboratory training.

2.2 General cell culture techniques.

All cell types used in this research were cultured in dedicated tissue and cell culture room cleaned and maintained as per laboratory guidelines. Cells were cultured in humidified atmosphere at 37°C in 5% CO₂. All types of cell treatments were performed in standard safety class II vertical laminar flow cabinet under sterile conditions previously cleaned with 1% Virkon and 70% Ethanol. Reagents and tools required to treat and to culture cells were cleaned with 1% Virkon and 70% ethanol before and after use.

2.3 Culture of Human Coronary Artery Smooth Muscle Cell (hCASMCs).

hCASMCs were cultured in Smooth Muscle Cells Growth Medium 2 (Promo Cell) supplemented with 10% foetal bovine serum (FBS) (Life Technologies, Paisley, UK), Supplement (Promo Cell), 50 µg/mL penicillin and 50 µg/mL streptomycin (Gibco, Paisley, UK) and L-Glutamin (Gibco, Paisley, UK). The medium including all the supplements and growth factors above described is referred to as “complete medium”. HCASMCs used in this thesis derive from multiple batches isolated from

one donor (healthy male patient of 30 years old) and were purchased from Lonza (ID CC-2583) (Basel, Switzerland). The cells were used until passage 6. Cells were bulked into T75 or T150 cm² vented-cap cell culture flasks (Corning, Poole, UK) and complete media was replenished every 2-3 days as required. Cells were passaged at 80% confluency to avoid overgrowth following the below steps: 1) cell medium was removed, and cells washed once with PBS 3mL of Trypsin (Gibco, diluted to 1% in PBS) were added to the flask and the cells were incubated at 37°C for 2-3 minutes. At the end of the incubation, 7 mL of complete medium was added to neutralise the trypsin. Cells were then centrifuged (1200g, 5 minutes), supernatant was discarded, and the pellet resuspended in appropriate fresh culture medium to distribute them among flasks. The experiments performed in this thesis were performed independently as indicated in each figure legend. Each independent experiment was performed in technical replicates.

2.4 Transfection of hCASMCS.

hCASMCS were plated at a confluency of 1 to 2 x10⁵ cells/well (depending on the treatment to which cells were submitted) in 6-well plate. Following 24 hours from the plating, cells were transfected with GapmeR or mimic reagents or a mix of the two reagents together. GapmeR are single stranded Antisense Oligonucleotides (ASOs) designed in a complimentary way to the target sequence. Their binding to the target RNA will favour the recruitment and degradation mediated by RNase-H enzyme thus blocking the mRNA translation. As RNase-H is found in nucleus and cytoplasm, they resulted to be very efficient tools to induce the knock-down of nucleic lncRNAs. Custom GapmeRs were purchased from Exiqon (Qiagen). A schematic representation of their location and their sequences can be found in Figure 2.1 and Table 2.1, respectively. Mimics were provided through collaboration from miRagen Therapeutics as double-stranded RNA molecules (guide strand and passenger stand) designed to mimic endogenous miR-143/145 cluster. In similarity to native microRNAs, these molecules are processed by Argonaute (Ago) to then bind

mRNA targets. Dicer-Substrate Short Interfering RNAs (DsiRNAs) are 27bp double-stranded RNAs capable of interfering with mRNA translation of desired target.

Transfection mix was prepared in a total volume of 1 ml of transfection medium per well firstly divided into 2X 500µl mix. One 500 µl mix contained the transfection reagents plus Opti-MEM medium and the second 500 µl mix contained Lipofectamine RNAiMax (Invitrogen, Cat.13778-150) and Opti-MEM medium (Gibco). The two mixes were combined for 20 minutes as manufacturer's instructions. The conditions considered were the following: cells transfected with 40 µM of GapmeR targeting *CARMN* (Gap*CARMN*) or GapmeR Control (GapCTR). Cells transfected with 5µM mimic Control (mimicCTR) or 5 µM of mimic 143/145 (mimic143/145). Cells co-transfected with GapmeR and mimic reagents: Gap*CARMN* (40 nM) + mimicCTR 5nM (Gap+mimicCTR) or Gap*CARMN* (40 nM) + mimic154/145 5nM (Gap+mimic143/145). In the case of CSNK1a1 knock-down, the conditions considered were cells transfected with negative control 40µM or 80µM (siNC) and cells transfected with dsRNA targeting CSNK1a1 at 40µM or 80µM (siCSNK1a1). In each GapmeR, mimic or dsiRNA). Cells were washed once with Opti-MEM medium before incubation with the transfection medium (1ml total per well) for 6 hours. After 6 hours, 1 ml of complete 10%FBS Smooth Muscle Cell Growth Medium 2 (Promo Cell) was added. 24 hours following transfection, medium was replaced with fresh 0.2% FBS medium, referred to as "basal medium", to synchronise cells to their quiescent phase for 48 hours before to start the following treatments.

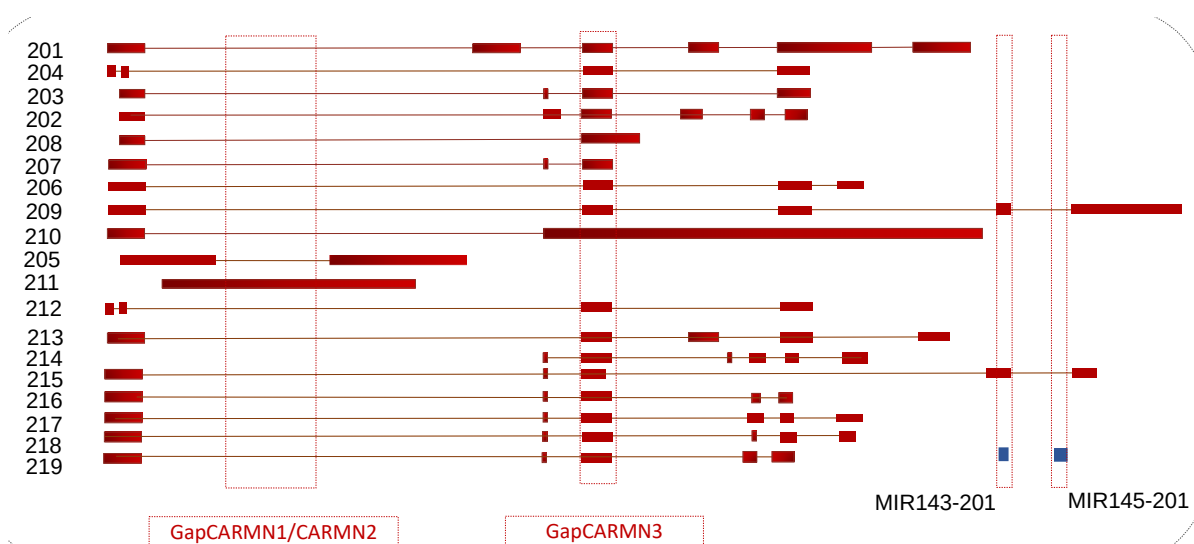


Figure 2.1 Schematic representation of GapmeR design localisation in human *CARMN* locus.

CARMN lncRNA localises in the human chromosome 5 where also miRNAs, miR-143 and miR-145 localise. In this picture, *CARMN* transcripts are represented in red with multi-exonic structure (red boxes) and their relative introns (red lines). Pre-miR143/145 are instead represented by blue boxes. Moreover, the overlapping region of miR-143/145 with *CARMN* transcripts is highlighted in yellow. In the picture, the region binding by GapmeRs, GapCARMN1, GapCARMN2, GapCARMN3 is indicated by the red box (in the case of intronic GapmeRs) and grey box (in the case of exon GapmeR). The used annotation is based on Ensembl database (GRCh38/hg38 version).

Name	Sequence
Gapmer Negative Control	A*A*C*A*C*G*T*C*T*A*T*A*C*G*C
Gapmer <i>CARMN</i>	A*T*A*G*G*T*G*T*C*A*G*G*T*G*T*C
Gapmer <i>CARMN2</i>	T*T*G*A*G*G*T*A*G*C* T*A*A*G*A*G
Gapmer <i>CARMN3</i>	T*C*T*G*T*G*A*A*A*G*G*T*G*A*T*G

Table 2.1 Sequences of GapmeR Antisense Oligonucleotides targeting *CARMN* or negative scramble control GapmeR used in the study.

2.5 Cell proliferation assay.

HCASMCs were plated at a density of 1×10^5 cells/well into 6-well plates in 2mL of complete medium. 24 hours after plating, cells were transfected with GapmeRs, Mimics or DisRNA reagents as described below. 24 hours post transfection, medium was replaced by starvation medium (or basal medium) supplemented only with 0.2% of FBS for 48 hours. At 48 post starvation, cells were treated with 0.2% medium supplemented with human Platelet Derived Growth Factor (PDGF)-BB (R&D System) 20ng/mL or basal conditions (0.2% FBS) used as negative control. In all the conditions (treated and untreated) cells were simultaneously supplemented with 5-ethynyl-2'-deoxyuridine (EdU) in a concentration of 10 μ M, according with manufacturer's instructions. 48 hours after the treatment, cells were harvested and fixed with 70% ethanol for Flow Cytometry cell Sorting (FACS) analysis or harvested in QIAzol lysis reagent (Qiagen, Hilden, Germany) as described in RNA extraction paragraph. EdU incorporation was detected using Click-iT EdU with Alexa 488 antibody (Life Technologies, C-10336) following manufacturer's instructions and analysed by Fortessa Analytic Flow Cytometry with FACS-Diva software. Unstained Cells, lacking EdU and antibody, were used as negative control to set the gates. 10.000 cells were collected to each sample and data analysis was performed using FlowJo analysis software (Tree Star, Ashland, USA).

2.5 Scratch Assay.

HCASMCs were plated at a density of 1.8×10^5 cells/well into 6-well plates in 2mL of complete medium. 24 hours after plating, cells were transfected with GapmeRs, Mimics or DisRNA reagents as described below. 24 hours post transfection, medium was replaced by starvation medium supplemented only with 0.2% of FBS for 48 hours. At 48 post starvation, cells were scratched with a sterile pipet (~200 μ m-wide wounds) and replaced with fresh 0.2% medium. At 10 hours following the scratch cells were harvested for quantification of the relative migration distances. Pictures were captured at 10X magnification at 0- and 10-hours post-scratch and the migration distances were analysed by using ImageJ software.

2.7 Cholesterol loading assay.

HCASMCs were plated at a density of 2×10^5 cells/well into 6-well plates in 2mL of complete medium. 24 hours after plating, cells were transfected with GapmeRs or Mimics reagents as described in section 2.4. 24 hours post transfection, cells were treated with water soluble cholesterol-methyl- β -cyclodextrin (CMBCD) (Sigma C4951) at a final concentration of $10 \mu\text{g/mL}$ in 0.2% Bovine Serum Albumin (BSA) in 0.2% FBS medium for 72 hours or in 0.2% BSA in 0.2% FBS medium for the untreated cells. Cells were then harvested for downstream RNA analysis.

2.8 Oil Red O (ORO) staining.

Cells were plated on coverslips (pre-cleaned with ethanol and H₂O) in 6-well plate. At the end of the treatment, the medium was removed and washed twice in PBS. Cells were then fixed in 4% paraformaldehyde (PFA) for 1 hour at RT. After rinsing coverslips in tap H₂O, 1-2 ml of 60% isopropanol was added to each well for 5 minutes. Isopropanol was removed and replaced with 2ml of Oil Red O (ORO) solution (Abcam Cat.Ab150678) ready-to-use for 5 minutes. ORO solution was removed, and coverslips were washed with H₂O several times until clean. Nuclei were the stained with 2ml of ready-to-use haematoxylin solution (provided with the kit, Abcam Cat.Ab150678) for 1 min. Cells were then washed until clean and mounted into glass slide with water-based mounting medium.

2.9 MTS cell viability assay.

Colorimetric-based MTS cell viability assay was performed using MTS assay kit (Promega Cat.G3582) following manufacturer's instructions.

Cells were plated at a density of 1×10^4 in flat bottom 96-well plates. At the end of treatment, AQueus One Solution Reagent was equilibrated to room temperature before to be added into each well (20 μl) containing medium (100 μl) (the assay was performed in duplicate). Blank controls (20 μl of AQueus One Solution Reagent + 100 μl

of serum-free medium) were included in the assay. Plates were then incubated at 37°C for 3 hours in CO₂ incubator (5%). This reaction will allow the bio-reduction of MTS reagent into coloured formazan product from metabolically active cells. The formation of formazan can be detected by absorbance at 490nm which will be directly proportional to the number of living cells. Absorbances were recorded using multiplate reader and the average absorbances of the duplicates per each condition was corrected subtracting the blank background and plotted.

2.10 Oxidised-LDL (ox-LDL) treatment of hCASCs.

hCASCs were plated at a density of 2×10^5 cells/well into 6-well plates 2mL of complete medium. 24 hours after plating, cells were treated with Low-Density Lipoprotein from human plasma Dil conjugate (Dil-OxLDL) (Invitrogen, L34358) at a concentration of 30 or 60mg/ml in 0.2%FBS medium for 24 hours. After 24 hours from the treatment, cells were imaged through fluorescence microscopy to assess the uptake from hCASC in culture. Cells were then washed once in PBS, harvested in Qiazol and stored in -80°C for RNA extraction.

2.11 MTT cell viability assay.

MTT cell viability assay was performed using MTT assay kit (Abcam, ab211091) following manufacturer's instructions. Cells were plated at a density of 1×10^4 in flat bottom 96-well plates. At the end of the treatment, medium was removed, and cells were washed 2X with sterile PBS. Previously aliquoted ready-to-use MTT reagent and ready-to-use MTT solvent (both provided with the kit) were allowed to equilibrate at RT before to use them. 50µl of serum-free medium was replaced PBS into each well to and 50µl of MTT reagent were added to each sample (the assay was performed in duplicate). Blank controls (50µl of MTT + 50µl of serum-free medium) were included in the assay. Cells were incubated for 3 hours at 37°C protected from light. After incubation time, the MTT-serum medium was removed and replaced with 150µl of MTT solvent to each well. The plate was then placed in orbital shaker for 15 min

protected from light. Absorbances were read at 590nm using a plate reader. The average absorbances of the duplicates per each condition was corrected subtracting the blank background and plotted.

2.12 Cellular Fractionation.

HCASMCs were plated at a density of 1.8×10^5 cells/well into 6-well plates in 2mL of complete medium. 48 hours after plating, cells were harvested for RNA fractionation assay. RNA fractionation was performed using the PARIS™ Kit (Life Technologies AM1921) according to the manufacturer's instructions. Cells were trypsinised and centrifuged at $1.200 \times g$ for 5 min, supernatant discarded and washed once in PBS. Ice-cold cell fractionation buffer was then added to cells for 10 min incubation on ice. After centrifugation performed with same condition mentioned before ($1.200 \times g$, 5 min), supernatant containing the cytoplasmic fraction, was collected in another tube and placed on ice. The remaining pellet was washed in ice-cold cell fractionation buffer and re-pelleted at 4°C , $500 \times g$ for 1 min. The nuclear pellet was then resuspended in ice-cold cell disruption buffer. 500µl 2X of lysis/binding solution at RT was added to both, cytoplasmic and nuclear fractions followed by addition of 500µl of 100% ethanol. Samples were then added to filter cartilage columns and centrifuged at $12.000 \times g$, 1 min. 700µl of wash solution were then added for another wash cycle (centrifuged at $12.000 \times g$, 1 min) followed by addition of 50µl pre-heated 95°C elution solution. The eluted RNA was stored at -80°C for downstream analysis of gene expression.

2.13 RNA Fluorescent In-Situ Hybridisation.

Custom RNA-FISH tiled probe sets were generated to all exons of CARMN as well as UBC and SNORD3 as positive controls (Thermo Fisher Scientific). RNA-FISH was performed according to manufacturer's instructions (ViewRNA™ cell

FISH) with minor changes as previously published²⁴³. HCASMCs were grown on 16-mm coverslips to 80% confluency, washed in PBS and fixed in 4% paraformaldehyde with 1% glacial acetic acid for 1h. Following detergent QS permeabilization and 1:4000 protease digest, coverslips were incubated with CARMN, UBC, and SNORD3 probe sets. Following probe hybridisation, coverslips were incubated with branched tree technology pre-amplifier for 1h and then with the amplifier for 30 min. Coverslips were then mounted onto glass slides using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories) and imaged using Andor Revolution XDi spinning disk confocal microscope.

2.13 RNA extraction from cultured cells and tissue.

Total RNA was extracted using miRNeasy kit (Qiagen, Hilden, Germany Cat: 217004) following manufacturer's instructions. After treatment, cells were washed once in PBS and harvested in 700µl of Qiazol lysis reagent (Qiagen, Cat:79306). In the case of tissues, fresh tissues were first homogenised in Qiazol reagent and processed using a tissue homogeniser. 140µl of chloroform were added to each sample and after 3 min samples were centrifuged for 20 min at 12.000 x g at 4°C. The supernatant phase was then collected into a new 1.5µl tube and 550µl of 100% ethanol was then added to each sample. Samples were then placed into cartilage columns provided with the kit and centrifuged at 8.000 x g for 1 min. The eluted solution was discarded and 350µl of RWT buffer (previously combined with ethanol as manufacturer's instructions) was added. After centrifugation (at 8.000 x g for 1 min), samples were treated for 10 min with DNase enzyme (RNase-free DNase set, Qiagen Cat:79256) as indicated in the manufacturer's instructions at room temperature (80µl of a mix of DNase enzyme and RDD buffer was added to each sample). Samples were then washed again with 350µl of RWT. After centrifugation, the eluted buffer was discarded and 2X 500µl of RPE buffer (previously combined with ethanol) were added to each sample. Columns were then replaced with new 2ml tubes provided with the kit for a step of centrifugation at 8.000 x g for 1 min to allow any residual RPE buffer to be discarded.

RNA was then eluted in 30µl of RNase-free H₂O and concentration was quantified by using Nanodrop 1000 spectrometer (Thermo Scientific, Parsippany, NJ, USA) and stored into -80°C for following analysis.

2.14 Dissection of human carotid plaques.

Carotid atherosclerotic plaque tissue was isolated from symptomatic patient's through endarterectomy. Ethical approval (Edinburgh) was obtained via the NHS Lothian BioResource Tissue Governance committee (15/ES/0094). Each plaque sample was macroscopically dissected into "stable" and "unstable" sections based on the presence of typical features: unstable lesions were defined for the presence of intraplaque haemorrhage, hydroxyapatite crystals and large lipid-core, while stable regions were identified as adjacent less advanced regions. Samples were stored in RNA later (Sigma) and snap frozen to be stored at -80°C for downstream analysis of gene expression.

2.15 Evaluation of RNA quality.

Total RNA was subjected to quality control using the Bioanalyzer Agilent RNA 6000 Nano or Pico kit (in the case of LCM samples as described below in section 2.29) (Agilent technology Inc., California US) which calculates the RNA integrity number (RIN) considering the ratio between 28S and 18S RNAs (expected 2:1). RIN values above 8 were considered as high quality. This quality control was used before to send samples for RNA-Sequencing analysis and after Laser Capture Microdissection technique performed in animal plaques.

2.16 RNA-Sequencing (RNA-Seq).

RNA sequencing was performed in stimulated hCASCs (PDGF-BB treatment, scratch stimulus and cholesterol loading) or corresponding basal conditions (described 3 basal) following transfection with GapCARMN and GapCTR. PolyA-enriched strand-

specific RNA libraries were prepared by GENEWIZ, Inc. (South Plainfield, New Jersey, USA). PolyA-enriched strand-specific RNA libraries were prepared by GENEWIZ, Inc. (South Plainfield, New Jersey, USA) and libraries were sequenced with Illumina Hi-Seq at an average of 30 million reads per samples (paired end 2 x 150bp). Gene quantification (read count and normalised expression value as FPKM) was obtained using RSEM (options: -bowtie2 -forward-prob 0 -paired-end), based on GENCODE annotation version 26 (primary assembly). The differential expression was assessed using DESeq2 by comparing treated conditions with the untreated control cells. We considered a threshold of absolute Fold Change ≥ 2 and adjusted pvalue < 0.01 to identify significant changes between two conditions. We also applied an expression value threshold of 2 FPKM (average of the three replicates) in the considered groups. Sample clustering was evaluated using the Principal component analysis (PCA) tool available in DESeq2 on the regularized log transformed data. Heatmaps were generated using the CRAN package heatmap. The gene ontology analysis was done using topGO (Alexa A and Rahnenfuhrer J (2016)) on enriched genes over a background of expressed genes (FPKM >2 in at least one condition). Fisher's exact test was used to calculate the p-values.

2.17 RNA-Sequencing data analysis pipeline.

Once obtained sequencing data, the sequences were processed following the below pipeline:

- 1)** Sequences were checked for their quality using the fast.qc files generated during the sequencing. This step is important to assess the length if the sequences obtained in both directions, R1 and R2.
- 2)** Sequences were trimmed (using TrimGalore) to remove the adaptors added during the library preparation at both ends (20-30 nucleotides) and remove the nucleotides sequenced with low quality.
- 3)** Files output.log were assessed for their errors.
- 4)** Sequencing reads were mapped to the genome generating RSEM files. RSEM files contain information regarding

- the gene ID (based on Ensembl annotation)
 - gene isoforms
 - Fragments per million of Reads Mapped to the Genome (FPKM), which indicate the normalised expression values
 - percentage of reads mapping to the transcriptome. Expected percentage for good quality sequencing is > 50%.
- 5)** Gene quantification analysis was performed using tables generated using the information contained in RSEM files: Gene ID (GENECORE v 26), percentage of reads and FPKM.
- 6)** STAR files were analysed. These files contain the percentage of reads mapped to the genome (the percentage of accepted uniquely mapped reads is >80%) and contain other 2 sub-files:
- log.final.out: containing the percentage of reads mapped to multiple loci (if % > 40 the results are contaminated with ribosomal RNAs) and the percentage of unmapped reads (this percentage should be < 1).
 - BAM file contains information on the length of nucleotides in R1 and R2 sequencing (similar both directions)
- 7)** Differential expression was quantified comparing each treated condition to the corresponding untreated controls using DESeq2 method. The DESeq2 function transforms the read counts per transcripts to log scale which can be used for the Principal component analysis (PCA) plot.
- Fold Change ≥ 2 and p value < 0.01 were used to identify changes with significance between the conditions. Moreover, expression value threshold of ≥ 2 FPKM (as average of 3 replicates) in each condition was applied and genes with expression < 2 FPKM were not taken into account.
- 8)** Principal component analysis (PCA) was performed using 3 libraries: Bioconductor, Biolite, Pheatmap.
- 9)** Heatmaps were generated with CRAN package.
- 10)** Gene ontology analysis of significantly differential expressed genes was performed using topGO (Alexa A Rahnenfuhrer J 2016)

11) Fisher's exact test was used to calculate the p-values.

2.18 cDNA preparation for gene expression analysis by Reverse transcription chain reaction (RT-PCR).

To analyse gene expression in human and mouse samples, cDNA was produced from total RNA (100-300 ng per reaction) using the TaqMan Reverse Transcription kit (Applied Biosystems, Cat: N8080234). RNA samples were combined with 10x RT Buffer 5.5mM MgCl₂, 0.5 mM of each dNTP, 2.5µM random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl of Multiscribe Reverse Transcriptase. All reagents were provided with the kit. A final volume of 40 to 60 µl was achieved with the addition of nuclease-free H₂O. Samples were then subjected to the following steps performed in a thermal cycler: 10min at 25°C, 30 min at 48°C, 5 min at 95°C. At the end of the reaction, samples were stored at -20°C for downstream qRT-PCR analysis.

2.19 cDNA preparation for microRNA expression analysis by RT-PCR.

In the case of microRNA analysis, cDNA was generated using the Applied Biosystems kit (Cat:4366596). Samples were first diluted to a concentration of 1ng/µl and added 2.5 µl for cDNA preparation. To each sample a mix of 5µl of the following reagents was added: 1 x reverse transcription buffer, 0.25 U/µL RNase inhibitor, 3.33 U/µL Multiscribe Reverse Transcriptase, 1 mM of each dNTP (all provided with the kit), 1 x TaqMan miRNA reverse transcription primers. Synthesis of the final volume of 7.5 µl was performed using the thermal cycler with the following conditions: 30 min at 16°C, 42°C for 30 min and 85°C for 5 min. RNU48 and U6 housekeeping controls were added in the reaction for human and mouse samples, respectively. Samples were then stored at -20°C.

2.20 SYBR Green and TaqMan quantitative RT-PCR (qRT-PCR) analysis of (human and mouse) gene expression.

The abundance of lncRNA isoforms and genes mRNA was quantified using custom Sybr Green primers (Eurofins MWG, Ederseberg, Germany) (listed in Table 2.2 human and 2.3 mouse) and Taqman fluorescently labelled probes (listed in Table 2.4). Sybr green qRT-PCR was performed using Power SYBR Green dye (Applied Biosystem, Cat: 4367659) while for TaqMan qRT-PCR TaqMan Universal Master Mix was used (Applied Biosystem, Cat: 4427788). Forward and reverse Sybr Green primers were designed spanning intron-exon boundaries and were reconstituted in nuclease-free H₂O at 100µM, aliquoted and stored at -20°C. 2.5µl of diluted each sample cDNA (obtained as described in section 2.17) was added to a mix of: 6.25µl of Power Sybr Green, 2.75µl of H₂O and 1µl of diluted primers (to a concentration of 2.5µM) or 5µl of TaqMan Universal Master Mix, 3µl of H₂O and 0.5µl of fluorescent probes. For gene expression analysis, stable housekeeping genes Ubiquitin (UBC) for human and Peptidyl-prolyl cis-trans isomerase (Ppia) and 18s for mouse samples were used as reference throughout the study. In addition, blank controls where cDNA was substituted from water, were used as technical control. Reactions were performed in 384-well plates and run in QuantStudio-5 (Applied Biosystem) Real time PCR system. After centrifugation of the plate, in the case of Sybr Green samples were subjected to 2 minutes at 50 °C, 10 minutes at 95°C, 40 cycles of denaturation for 15 sec at 95°C, 1 min at 60°C. In the case of TaqMan reaction, centrifugated plate underwent to a first step of 2 min at 50°C followed by 10 min at 95°C and 40 cycles at 95°C for 15 sec to finish with 1 min at 60°C.

Human Sybr Green Primers	Sequence
<i>CARMN_001_Fw</i>	TGGGAATGGAAACCCTTGGT
<i>CARMN_001_Rev</i>	AAGCTGCATGTTCAAGGTCG
<i>CARMN_003_Fw</i>	CTGTAACAAGTGACAGGCAA
<i>CARMN_003_Rev</i>	TTCTGTTGGGAGGCTTGGAGA
<i>CARMN_005_FW</i>	CCAGGAAAGGCAAGGGCGCTA
<i>CARMN_005_Rev</i>	TGGGAGCGGATGTGGGTGCCG

<i>CARMN</i> _006_Fw	GCTGTAACAAGTGCACAGGCA
<i>CARMN</i> _006_Rev	GTGGGCTCACAGTTCTGTCT
<i>CARMN</i> _007_Fw	GATCCAGAGTAGGAGGGAGCC
<i>CARMN</i> _007_Rev	GGCCCTTGAATCTGCTTGCC
<i>CARMN</i> _008_Fw	AGCCTGGAAGTGGCTGGATGT
<i>CARMN</i> _008_Rev	AAACGCATGCCTGATGGTGT
<i>CARMN</i> _009_Fw	GCTCCCAAAGCAGGAAGACC
<i>CARMN</i> _009_Rev	GCCCAACCTCACAAATCCTCT
<i>CARMN</i> _010_Fw	AATGCAGGAGGCATGGGCCA
<i>CARMN</i> _010_Rev	GCCACTTGAGTCAGTGATGGTG
<i>CARMN</i> _011_Fw	AAAAGTCAGAGGCTGTGGGAC
<i>CARMN</i> _011_Rev	TTGCCACACAATGCCCTA
<i>CARMN</i> _012_Fw	TGCCTCTTCAGCTCATATAAG
<i>CARMN</i> _012_Rev	GCTAGCGCCCTTGCCTTTCCT
Common primer_Fw	CGCCATGCTGATGTCAGAGA
Common primer_Rev	GTTCTGTCTCCGGGCTGC
Ubc_Fw	TTGCCTTGACATTCTCGATG
Ubc_Rev	ATCGCTGTGATCGTCACTTG

Table 2.2 Name and sequences of human Sybr Green primers used to identify *CARMN* isoforms and sequence of Ubiquitin C (UBC) housekeeping reference gene.

Mouse Sybr Green Primers Name	Sequence
<i>CARMN</i> _01_Fw	CATTTGAGGGAGCCAGGGGT
<i>CARMN</i> _01_Rev	GTGGAAGGAACAGTAGGACA
<i>CARMN</i> _02_Fw	AGTGCCAGCCCTGAGGAAAG
<i>CARMN</i> _02_Rev	TCCCCAGATAACCTTTGCTTCGT
Common_Fw	GGTTCCAGTGCCAGTTGCTTA
Common_Rev	GTGGTTGTGGGTGTTATTGCT

Ppia_Fw	ATTTCTTTTGACTTGCGGGC
Ppia_Rev	AGACTTGAAGGGGAATG
18S_Fw	GTAACCCGTTGAACCCCAT
18S_Rev	CCATCCAATCGGTAGTAGCG

Table 2.3 Name and sequences of mouse Sybr Green primers used to identify *CARMN* isoforms and sequence of Peptidylprolyl Isomerase A (Ppia) and 18s housekeeping reference genes.

Human and mouse TaqMan probe name	Assay reference number
miR-143-3p	477912_mir
miR-145-5p	477916_mir
RNU48	Hs04931161_g1
U6-snRNA	Mm00505971_m1

Table 2.4 Name and assays reference number of Taqman probes used in human and mouse to identify microRNA miR-143-3p, miR-145-5p and housekeeping reference genes RNU48 for human and U6-snRNA used in mouse.

Human TaqMan probes	Assay reference number
CD68	Hs02836816_g1
LGALS3	Hs00173587_m1
ACTA2	Hs00426835_g1
MYH11	Hs00975796_m1
CCN1	Hs00975796_m1
TAGLN	Hs06633192_s1
KLF4	Hs00358836_s1
ABCA1	Hs01059137_m1
PDGFRa	Hs00998018_s1
EGFR	Hs01076090_s1
UBC	Hs01867132_s1

Table 2.5 Name and assays reference number of TaqMan probes used to assess human gene expression.

2.21 RT-PCR (qRT-PCR) analysis of (human and mouse) microRNAs expression.

TaqMan miRNA qRT-PCR probes (provided in the same assay with the primers used for reverse transcription, listed in Table 2.4) were used to detect microRNA expression using 384-well plates. To 1.5µl of cDNA, a mix of the following reagents was added to each sample: 5µl of TaqMan master mix (Applied Biosystem, Cat: 4427788), 0.5µl microRNA probe and 3µl of nuclease-free H₂O. Samples were analysed using QuantStudio-5 (Applied Biosystems) and subjected to 01 min at 95°C, 15 sec x 40 cycles at 95°C to end with 1 min at 60°C. Samples were then stored at -20°C.

2.22 Analysis of qRT-PCR.

The analysis of qRT-PCR data (Sybr Green, TaqMan) of gene and microRNA expression in human and mouse samples were performed using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method²⁸⁷). The analysis of results was performed considering the Ct value obtained during the exponential phase of qRT-PCR amplification for the gene of interest and the relative housekeeping gene (UBC, Ppia, 18s, RNU48 or U6). The housekeeping genes were chosen for the stability in their expression displayed across samples and different treatments. The difference between the Ct values for the reference gene minus the housekeeping gene is named ΔCt (dCT). The ΔCt average of the technical triplicates per each condition was calculated and used to calculate the $\Delta\Delta Ct$, which indicates the difference in gene expression among the conditions (with reference to the control group, for example GapCTR in the case of transfection with GapmeR reagents). This was calculated per each biological condition. $2^{-\Delta\Delta Ct}$ was then calculated to represent the fold increase or decrease in gene expression among

the conditions. The statistical analysis was performed considering the ΔC_t of each condition.

2.23 Agarose gel electrophoresis.

qRT-PCR amplification products obtained during the testing of Sybr Green primers were run in agarose gel (1.5% w/v) to assess their size in bp. Agarose was dissolved 1% Tris-Borate buffer (EDTA) (Gibco, Paisley) in microwave for 3-4 minutes (low power) to allow agarose to completely dissolve. The solution was then left to cool down for 2-3 minutes and pre-treated with Sybr Safe DNA Gel Staining (Thermo Fisher Cat.S33102) for visualization of DNA fragments in agarose gel following manufacturer's instructions. Each sample was combined with blue/orange 6X Loading dye (Promega Cat.G190A) dye before to be loaded into the gel. Moreover, 1KB or 500bp DNA ladder (provided in the same kit of DNA dye, Promega) marker was used to compare the sizes of each DNA fragment obtained. Gel was then run at constant 100 V using BIO-RAD Pac 300 electrophoresis machine and samples were checked for their size every 45 minutes. Bands were visualised using UV transilluminator.

2.24 Gel extraction.

Once visualised, DNA fragments of the right size were isolated and purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Cat. 28706). In particular, bands were excised by visualization using UV transilluminator using a scalpel blade and collected in clean 1.5ml tubes. Gel fragments were weighted and mixed with correspondent amount of buffer QG (in a proportion of 100 μ l per 100mg of agarose gel). Samples were placed in a thermo-block at 50°C until the gel was completely dissolved. Dissolved samples were then mixed with isopropanol (in a ration of 1 gel volume) to be then transferred into a mini column provided with the kit and centrifuged at 16.000 x g for 1 minute. Columns were then washed twice with 500 and 750 μ l QG buffer and buffer PE following centrifugation at 16.000 x g for 1 minute between the two cycles. Waste solution was discarded before the second wash cycle.

Columns were placed into a clean 1.5ml tube and DNA was eluted in 30-50 μ l of EB buffer following centrifugation at 16.000 x g for 1 minute. Samples were then stored at -20°C until needed.

2.25 Sequencing of qRT-PCR products.

Having assessed the right length of qRT-PCR products by gel run, samples were sent to be sequenced to validate their sequences. To each sample (100-200ng), 2uM of forward or reverse primers were added together with 1 μ l of Ready Reaction Mix (Thermo Fisher) and 4 μ l of 5X Big Dye Sequencing buffer (Thermo Fisher). H₂O was then added to each sample to obtain a volume of 20 μ l. Blank control reaction, containing all the reagents except the DNA template, was also included. Each reaction mix was performed in clean 0.5 μ l tubes and each sample was sequenced in duplicate. Samples underwent 25 PCR cycles at 96°C for 50 sec, 50°C for 20 sec, 60°C for 3 minutes. Following PCR reaction, samples were sent to The Gene Pool (Ashworth Laboratories, Edinburgh). Sequencing results were then blasted to the reference transcriptome (human or mouse) using Primer Blast and Multialign software.

2.26 In Situ Hybridization (ISH) in human plaques.

CARMN was detected in human carotid plaque samples (isolated by endarterectomy). The tissue used were part of 'Maastricht Pathology Tissue Collection' in agreement with the Dutch Code for Proper Secondary Use of Human Tissue, the Declaration of Helsinki, and was approved by the local Medical Ethical Committee (protocol number 16-4-181). Cross-sections of human plaques (5 μ m) were de-paraffinized with Xylene (5 minutes, RT) and graded concentrations of ethanol (100%-96%-70%). After rinsing in DEPC-treated H₂O and DEPC-treated PBS, sections were treated with proteinase K (Qiagen, miRCURY LNA buffer Cat.339450) (2 μ g/mL) for 10 minutes on a pre-heated hot plate at 37°C. After washing two times in DEPC-PBS, slides were hybridised with *CARMN* or Scramble control probe (100nM)

(probes listed in Table 2.6) in 1X miRCURY LNA buffer (Qiagen, miRCURY LNA buffer Cat.339450), overnight in hot humidified chamber at 55°C. The probe sequences are reported in Table 2.6). The following day, slides were washed three times in 5X SSC buffer (Invitrogen, AM9763) at 50°C and blocked in Roche DIG blocking buffer set (Roche, Cat. 11585762001) for 1 hour at room temperature. After blocking, sections were incubated with anti-DIG-AP 1:500 (Roche, Cat.11093274910) in blocking buffer for 1 ½ hour at room temperature. After 3 washes in 1X TBS-T, sections were incubated with NBT/BCIP AP (Roche, Cat.11697471001) (1 table in 10ml of H₂O) detection for 5 hours at room temperature and stained with nuclear fast red (Sigma, N3020) for 3/4 minutes. Slides were washed in H₂O, and de-hydrated in 96%, 100% ethanol, Xylene and mounted in coverslip with a drop of Xylene-based mounting media.

Name	Sequence			
Human Negative control probe	/5DigN/GTGTAACACGTCTATACGCCCA /3Dig_N/			
<i>CARMN</i> probe human	/5DigN/TCTGGTCCAGGTGTGGCTCCTT/3Dig_N/			
Mouse Negative Control probe	/5DigN/GTGTAACACGTCTATACGCCCA/3Dig_N/			
<i>CARMN</i> probe mouse	/5DigN/TGGGTGTTATTGCTTCTCAACTA/3Dig_N/			

Table 2.6 Human and mouse sequences of double-digoxigenin labelled ISH probes targeting a common region to the human or mouse *CARMN* transcripts and scramble negative controls sequences.

2.27 Co-localisation staining in human plaques.

CARMN was co-localised with makers for vSMC (ACTA2), activated macrophages (CD68) and Leucocyte (CD45) in human atherosclerotic plaque samples (isolated by endarterectomy) by combining ISH with IHC protocol as follows:

Human carotid sections (5µm) were de-paraffinized with Xylene (5 minutes, RT) and graded concentrations of ethanol (100%-96%-70%). After rinsing in DEPC-treated

H₂O and DEPC-treated PBS, sections were treated with proteinase K (Qiagen, miRCURY LNA buffer Cat.339450) (2µg/mL) for 10 minutes in pre-heated hot palate at 37°C. After washing two times in DEPC-PBS, slides were hybridised with *CARMN* or Scramble control probe (100nM) in 1X miRCURY LNA buffer (Qiagen, miRCURY LNA buffer Cat.339450), overnight in hot humidified chamber at 55°C. The following day, slides were washed three times in 5X SSC buffer (Invitrogen, AM9763) at 50°C and blocked in Roche DIG blocking buffer (Roche, Cat. 11585762001) for 1 hour at room temperature. After blocking, sections were incubated with anti-DIG-AP 1:500 (Roche, 11093274910) in blocking buffer for 1 ½ hour at room temperature. After 3 washes in 1X TBS-T, sections were incubated with NBT/BCIP AP (Roche, Cat.11697471001) (1 table in 10ml of H₂O) detection for 5 hours at room temperature. Following NTB/BCIP AP detection of *CARMN*, slides were processed to block endogenous peroxidase (0.3% in H₂O₂ in MetOH) for 15 min at RT. Slides were then sequentially washed in H₂O and TBST-0.1%BSA. Blocking of aspecific binding was performed subjecting slides to TBST-5% Goat serum blocking for 30 minutes at RT. Incubation with antibodies or IgG control was performed for 30 min at RT, ACTA2 (Dako, M051 clone 1A4, diluted 1:2500) or CD68 (Dako, M0814 clone KP1, diluted 1:250). After washing slides 2X in TBS, sections were incubated with Brightvision anti-mouse-HRP (Immunologic, VWR Cat. VWRKDPVM55HRP) for 30 min at RT and then washed 2X in TBS to perform develop staining with PolyDetector HRP (BioSB, Cat. BSB0130) diluted 1:2 as manufacturer's instructions for 2-3 minutes (depending on the antibody). The detection was then stopped in water and slides were counterstained with nuclear fast red (Sigma, N3020) for 3/4 minutes. before to be washed in H₂O, de-hydrated in 96%, 100% ethanol, Xylene and mounted in coverslip with a drop of Xylene-based mounting media.

Target antigen	Vendor or Source	Catalog #	Working concentration
PerCP anti-mouse CD45 Antibody, clone 30-F11	Biolegend	103129	1:100 (FACs)

V500 Rat anti-Mouse CD45R/B220 Clone RA3-6B2	BD Horizon	561227	1:50 (FACs)
CD3 Monoclonal Antibody (17A2), eFluor 450	eBioscience	48-0032-80	1:100 (FACs)
APC-H7 Rat anti-Mouse CD4 Clone GK1.5	BD Bioscience	560246	1:100 (FACs)
APC anti-mouse CD8a Antibody, Clone 53-6.7	eBioScience	100711	1:50 (FACs)
PE Mouse Anti-Mouse NK-1.1, Clone PK136	BD Pharmingen	561046	1:100 (FACs)
Ly-6C APC Antibody, anti-mouse, Clone 1G7.G10	Miltenyi Biotec	130-102- 899	1:10 (FACs)
PE-Cy™7 Rat Anti-CD11b Clone, M1/70	BD Pharmingen	561098	1:300 (FACs)
PE Rat Anti-Mouse Siglec- F Clone. E50-2440	BD Pharmingen	562068	1:100 (FACs)
CD16/CD32 Monoclonal Antibody (93)	eBioScience	14-0161-82	1:100 (FACs)
APC-H7 Rat Anti-Mouse Ly-6G, Clone 1A8	BD BioScience	565369	1:100 (FACs)
F4/80 Monoclonal Antibody (BM8), FITC,	eBioScience	11-4801-81	1:50 (FACs)
CD45R (B220) Monoclonal Antibody (RA3-6B2), PerCP-Cyanine5.5,	eBioscience	45-0452-80	1:100 (FACs)
CD3e Monoclonal Antibody (145- 2C11), PerCP-Cyanine5.5	eBioscience	45-0031-80	1:50 (FACs)
PerCP-Cy™5.5 Rat Anti-CD11b Clone M1/70	BD Pharmingen	561114	1:500 (FACs)
PerCP-Cy™5.5 Rat Anti-Mouse Ly-6G Clone 1A8	BD Pharmingen	560602	1:100 (FACs)
NK1.1 Monoclonal Antibody (PK136), PerCP- Cyanine5.5	eBioscience	45-5941-80	1:200 (FACs)
TER-119 Monoclonal Antibody (TER- 119), PerCP-Cyanine5.5	eBioscience	45-5921-80	1:400 (FACs)
CD117 (c-Kit) Monoclonal Antibody (2B8), APC-eFluor 780	eBioscience	47-1171-80	1:200 (FACs)

Ly-6A/E (Sca-1) Monoclonal Antibody (D7), APC	eBioscience	17-5981-81	1:500 (FACs)
CD16/CD32 Monoclonal Antibody (93), PE-Cyanine7	eBioscience	25-0161-81	1:1000 (FACs)
CD34 Monoclonal Antibody (RAM34), eFluor 450	eBioscience	48-0341-80	1:50 (FACs)
Monoclonal Anti-Actin, α -Smooth Muscle - FITC antibody produced in mouse	Sigma Aldrich	F3777	1:300 (IHC)
Anti-Mouse/Human Mac-2 (Galectin-3), Purified (Clone M3/38)	Cedarlane	CL8942AP	1:4000 (IHC)
Anti-smooth muscle Myosin heavy chain 11 antibody	Abcam	Ab53219	1:100 (IHC)
Anti-Digoxigenin-AP, Fab fragments	Roche	1.1093E+10	1:500 (in situ)
Monoclonal mouse anti-human Actin (Smooth Muscle) (Concentrate) Clone 1A4	Agilent Dako	M085129	1:2500 (in situ)
Monoclonal mouse anti-human CD68 (Concentrate) Clone KP1	Agilent Dako	M081401	1:250 (in situ)
Monoclonal mouse anti-human CD45, Leucocyte Common Antigen (Dako Omnis) Clone 2B11 + PD7/26	Agilent Dako	GA75161	1:400 (in situ)

Table 2.7 List of human and mouse antibodies used in the study.

2.28 Mice.

All *CARMN*^{+/+} and *CARMN*^{-/-} animal experiments were performed in accordance with the Animals (Scientific Procedures) Act (UK) 1986 and under the auspices of UK Home Office Project and Personal Licenses (number I34D4F056) held within The University of Edinburgh facilities. C57BL/6Ntac *CARMN* knock-out mice and C57BL/6Ntac littermate wild-type controls were created by TACONIC Bioscience, Inc. (NY, USA). To obtain constitutive knock-out for *CARMN*, the first exon of the *CARMN* transcripts and a portion of 4.8kb of the gene promoter were deleted, leaving exons 2/3 and the

miRNA stem loops intact (Figure 2.2). Meta-analysis studies registered a predominance in carotid atherosclerosis in men than women^{288,289}. In addition, studies in animals confirmed that sex is a biological variable, and generally male animals show more inflamed plaque phenotype²⁹⁰. Only one gender was used to minimize variation within groups and keep standard deviation low and power high. For the purpose of this study, only male animals were included to remain comparable with a dose-response study with AAV-PCSK9²⁹¹, and this therefore represents a limitation to the understanding of underlying mechanisms of sex-related differences. Male, 8 weeks-old *CARMN*^{-/-} (n=16) and *CARMN*^{+/+} (n=19) animals were injected intraperitoneally (IP) with 1X10¹² vg/mouse of Adeno-Associated Vector serotype 8 (AAV-8) expressing Protein Convertase Subtilisin/kexin type 9 (PCSK9). In this regard, power calculation was performed (σ = 47.5% and δ is 45 % (plaque area as parameter): $n = 15.7 * (\sigma/\delta)^2$ $n=15.7*(47.5/45)^2 \rightarrow n = 17.5+5\%$ drop out $n=18.4 \rightarrow 19$ mice per group. To prevent bias, all mice used in *in vivo* experiments underwent a process of randomisation prior to be subjected to experimental procedures and processing, where each animal was randomly assigned to random numbers in an excel sheet. Moreover, cages were placed in a random order and shelf on the rack, and number of mice per cage varied depending on nest size. AAV8-PCSK9 for the study was kindly provided from the AAV vector unit in ICGEB (Trieste). At 1-week post injection, all mice were fed with high cholesterol diet (DBM Food Hygiene Supply, Scotland) for 18 weeks. During the time of the study, plasma was collected from the tail for cholesterol measurements immediately previous injection and at 6-,10-weeks post-injection and at sacrifice. Twenty-four hours prior sacrifice, mice were injected IP with 200 mg/kg of sterile 5-ethynyl-2'-deoxyuridine (InvitrogenTM, A10044) in sterile PBS. All mice were then sacrificed at 27 weeks post injection for collection and processing of plasma and tissues. The processing of animal tissues used for downstream analysis was performed in a blinded way in which a random number was assigned to each sample. The key list of number and genotype was blinded during acquiring raw measurements and only revealed upon analysis. Not-responder mice (n=3), in which no change in plasma cholesterol and no formation of atherosclerotic plaque in their

aortic valves and brachiocephalic arteries were observed, were removed from the analysis of the plaque volume, size and composition. LDLR knock-out studies were carried out in accordance with institutional guidelines and regulations of the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences under project license number PV2018-011. Male low-density lipoprotein receptor deficient mice (LDLR^{-/-}), originated from Jaxx (B6.129S7-Ldlrtm1Her/J Stock No: 002207), of 8-12 weeks old were fed chow (controls) or high-cholesterol diet (HCD, 0.25%, 824171, Tecnilab-BMI) for 16 weeks. All mice were euthanized with an overdose pentobarbital (100mg/kg) injected intraperitoneally. Brachiocephalic arteries, aortic roots, spleen, lymph nodes and blood were collected for further analysis.

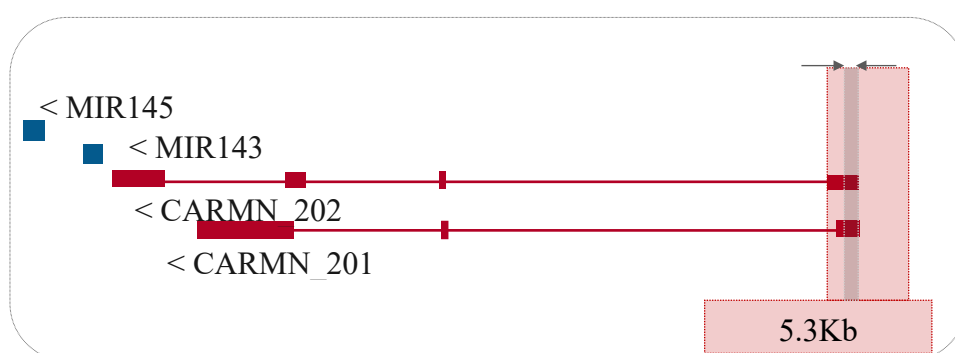


Figure 2.2 Schematic representation of *CARMN* locus located on mouse chromosome 18.

Schematic of the transcripts belonging to *CARMN* locus in mouse (exons and introns are represented in red) and the pre-microRNAs miR-143 and miR-145 (in blue). The arrows next to each transcript Ensembl ID indicate the sense of transcription. Red box represents the location of CRISPR/Cas9 deletion to genetically deplete *CARMN* transcripts. A portion of 5.3 kilobases, including part of the promoter and the first exon, was deleted. The grey arrows and box indicate where common primers to both isoforms were designed.

2.29 Isolation and maintenance of mouse aortic smooth muscle cells (ASMCs).

Aorta was isolated from 6-8 weeks mice. The dissection and isolation of aorta from *CARMN*^{+/+} and *CARMN*^{-/-} mice was performed taking into consideration the region

above the base of the heart (aortic trunk) until the thoracic region of the vessel. Aortas were then placed in sterile DMEM (no serum) medium provided with 1% Penicillin/Streptomycin. At the end of the isolation procedure, vessels were then moved into sterile environment and washed with PBS (excess of blood was removed with multiple washes). Using small scissors and blunt watchmakers, the vessel was cleaned from fat tissue (yellow-white appearance attached to the vessel). Using the same tools, the adventitial layer was peeled-off from each vessel. After this step, the vessel appeared translucent which indicated the successful removal of the adventitia and connective tissue. The vessels were then cut longitudinally, and the endothelium was gently scraped from the inside part of the vessel using flat edge of bent forceps. Each vessel was then cut into 1-2 mm pieces with scalpel. The cut vessels from 5 mice, were then placed into sterile 1.5ml tubes for the digestion step. Collagenase enzyme (Sigma C-0130), 3mg/ml in serum free DMEM + 1% PSG, sterile filtered and Elastase (Sigma E0127), 1mg/ml in serum-free DMEM + 1% PSG, sterile filtered were used as digestion enzymes. A mix of 2ml of collagenase (previously diluted) and 50ul of elastase (previously diluted) were added into each 1.5ml tube to cover the vessels and incubates into humidified atmosphere (5% CO₂, 37°C) for 4 hours. Every 30 minutes the tube was vortexed for few seconds. In the meantime, 6.25ul of Laminin (Sigma L2020, 1mg/ml) were added to 2ml of PBS and 1 T25 flask was coated per each 1.5ml tube (the culture aortic smooth muscle cells isolated from 5 mice was carried out) and incubated for at least 2 hours in humidified atmosphere (5% CO₂, 37°C). At the end of the 4 hours, digested vessels were resuspended with pipette and centrifuged at 1000rpm for 5 minutes at RT. The pellet was then resuspended into 1 ml of 10% FBS DMEM. The laminin was then aspirated from the T25 flask and washed once in warm medium. Culture medium (5ml) was then added to the flask and cell suspension was then added into the flask. Cells were then left undisturbed for 72 hours. When confluent cells were trypsinised and divided into 2 T25 flasks. Cells were then maintained in culture and used for experiments for maximum 6 passages.

2.30 Laser Capture Microdissection (LCM).

Aortic root plaque material from *LDLR*^{-/-} mice (fed with high cholesterol diet for 13-weeks or on chow diet) was captured using a PALM-Robo LCM microscope (Zeiss, Oberkochen, Germany) as previously described²⁹². 4-μm cryosections were dehydrated in ethanol solutions (2X 95% 5 min, 2X 100% 5min) and Xylene (2X 5min). Samples were then processed with laser capture microscope under direct visualisation of plaques and necrotic core. The plaque area was selected (without inclusion of necrotic core) and captured into RNase-free 0.5μl Eppendorf tube cap containing 75μl of Qiazol lysis reagent. Total RNA was then isolated using the microRNA RNeasy micro-Kit as described in section 2.13 (QIAGEN). The quality of RNA was measured by using Bioanalyzer Agilent RNA 6000 Pico kit (Agilent technology Inc., California US) as described in section 2.15.

2.31 Optical Projection Tomography (OPT).

OPT was performed in *CARMN*^{-/-} and *CARMN*^{+/+} aortic arches as previously described²⁹³. Formalin (Sigma)-fixed aortic arches and major branches (left carotid artery, left subclavian artery and brachiocephalic trunk) from *CARMN*^{-/-} and *CARMN*^{+/+} were embedded in 1.5% low melting point agarose (Invitrogen,16520-050) and dehydrated with 24 hours serial incubation washes with 100% MetOH, BABB solution made up with Benzyl alcohol (Sigma,402834) and benzyl benzoate 1:2 (Sigma, B6630) and 100% MetOH for 24 hours. Cleared samples were then placed in a calibrated tomography machine (Edinburgh, UK). A resolution of 1,024 x 1,024 and a magnification of 4X was set up to see the entire area of interest. Tomographic reconstruction of the 400 pictures was obtained using CTAn software (Bruker, Billerica, US Version 1.17.7.2). For each scan, the region of interest was defined (which encompassed the section of vessel where the lesion was present) and adjusted with a vertical orientation. The binary image produced will have white pixels representing the plaque and black pixels represent the lumen. The parameters considered include the object volume (lesion volume), percentage of object volume, mean cross-sectional area and object cross-sectional area (lesion area). The

percentage of maximum lesion area was calculated by the software per each sample and plotted for statistical analysis.

2.32 Blood count and flow cytometry.

Immune cell subsets were quantified in blood, spleen and lymph nodes using flow cytometry and absolute cell counts were obtained using TruCount (BD, Cat No 340334). Blood was subjected to erythrocyte lysis prior to antibody labeling (8.4g NH₄CL + 0.84g NaHCO₃ in 1 litre H₂O, pH 7.2-7.4). Combinations of different antibodies were used to identify leukocytes (CD45 Biolegend clone 30-F11), B-cells (B220 BD clone RA3-6B2), T-helper cells (CD3 eBioscience clone 17A2/CD4 BD clone GK1.5), cytotoxic T-cells (CD3/CD8 eBioscience clone 53-6.7), NK cells (CD8/NK1.1 BD clone PK136) NKT cells (CD3/NK1.1), monocyte subsets (Ly6C Milteni clone 1G7.G10/CD11b BD clone M1/70) and eosinophils (SiglecF BD clone E50-2440/CD11b clone M1/70). Spleens and lymph nodes were dissociated into single-cell suspensions and enzymatically digested for dendritic cell separation using liberase and DNase (both 0.2 mg/ml, Roche) for 30 min in RPMI medium. Spleen and lymph nodes were then subjected to erythrocyte lysis as stated above. Antibody staining was performed using the following antibodies to detect granulocytes/neutrophils (CD11b^{high}, Ly6G^{high}), T cells (CD3⁺; Miltenyi), T helper cells (CD4⁺; BD), cytotoxic T cells (CD8a⁺; BD), effector/memory T cells (CD44^{high}, CD62^{low}; BD, eBioscience respectively), naïve T cells (CD44^{low}, CD62^{high}), regulatory T cells (CD4⁺, CD25⁺, FoxP3⁺; eBioscience), B cells (B220⁺; BD), NK cells (NK1.1⁺; eBioscience) and monocytes (CD11b^{high}, Ly6G⁻, Ly6C^{high/int/low}; BD, eBioscience, Miltenyi respectively). The gating strategy is depicted in Figure 5.13 and 5.14. Tibia and fibula were flushed with PBS using a 23G needle and gently pressed through a 70 µm strainer. Bone marrow cells were subjected to erythrocyte lysis as stated before. All lineage negative cells (CD3⁻, B220⁻, CD11b⁻, Ly6G⁻, NK1.1⁻ and Ter-119⁻) were analyzed further for bone marrow stem cells (lin/Sca-1/c-kit; eBioscience, Bd), common myeloid progenitors (CD16/32^{int}, CD34^{int}; eBioscience, BD respectively), granulocyte-macrophage progenitors (CD16/32^{high}, CD34^{high}), erythrocyte-megakaryocyte progenitors (CD16/32⁻, CD34⁻). Gating strategy

for bone marrow progenitors is shown in Figure 5.15. All data was acquired and analyzed using a FACSCanto II and FACSdiva software (BD Bioscience).

2.33 Immunohistochemistry (IHC).

Cross-sections from brachiocephalic arteries and aortic roots from *CARMN*^{-/-} and *CARMN*^{+/+} and *LDLR*^{-/-} animals were first deparaffinized in xylene (2X) and then re-hydrated in a graded series of ethanol concentrations (2X 100% 5 min, 2X 96% 5min, 2X 70% 5min). Sections were then boiled for 10 minutes (90W) in antigen retrieval buffer HIER (made up with 1X citrate antigen retrieval buffer, pH 6 diluted from stock (S231, Dako). Slides were then left to cool down and after 3X washes in TBS buffer, sections were then incubated with goat serum (MP-7404 Impress kit) for 30 min at RT. Primary antibodies were then added to the slides: anti- α SMA-FITC (1:300 Sigma F3777), anti-Mac2 (1:4000 Cedarlane CL8942AP), anti-Myh11 (Abcam Ab53219 1:100) in 1% goat serum in PBS at room temperature for 30 min. After 3X washes in TBS, slides were incubated for 30 min at RT with secondary antibodies: Poly anti-FITC for α SMA 1:6000 for SMA, Goat anti-rat IgG ready-to-use for Mac2 (provided with the Impress kit MP-7404) or swine anti-rabbit biotin (1:250) for MYH11 staining. Parallel slides were used for isotype controls (incubated with IgG control during primary antibody incubation step). In the case of MYH11, incubation with a tertiary antibody ABC-AP (1:200 in TBT, vector labs) was performed incubating sections for 30 min at RT. Tissues were then developed with DAB chromogen (Impact DAB HRP substrate, SK-4105) (for approximately 2 minutes) and counterstained in haematoxylin and washed in H₂O until clean from blue colour reaction. Slides underwent serial washed with 2X 96% ethanol, 2X 100% ethanol, 2X Xylene to be then mounted in coverslip with Xylene-based mounting media. Sections were then imaged using brightfield microscope and the signal quantified using QuPath software.

2.34 Picrosirius Red staining (collagen).

Cross sections of brachiocephalic artery and aortic roots from *CARMN*^{-/-} and *CARMN*^{+/+} animals were de-waxed and hydrated with 2X Xylene washes and ethanol graded washes: 2X 100% 5 min, 2X 96% 5min, 2X 70% 5min. After washes in H₂O, sections were incubated in 0.2% Molybdotophosphoric solution for 5 minutes. Slides were then washed in H₂O and incubated in 0.1% Sirius Red solution (Sirius red in saturated picric acid solution to a concentration of 0.1%) for 90 min. Section were then rinsed in 0.01M HCl solution and after washes in H₂O, sections were then de-hydrated in graded ethanol concentrations (1X 70% ethanol 45 sec, 2X 96%- 2X 100%, 2X Xylene for 5 min each. Slides were then mounted in coverslips with Xylene-based mounting media. Sections were then imaged using the polarised microscopy and the signal quantified using Fiji software.

2.35 EdU detection in tissue.

EdU was detected in cross sections of brachiocephalic artery using the Click-IT EdU Proliferation kit for Imaging kit (Life Technologies, C10337) accordingly with manufacturer's instructions. Slides were deparaffinised in xylene (2X 5min) and ethanol (100%, 96%, 70%, 50%) solutions at room temperature and washed in H₂O and PBS. Tissues were then incubated (microwave, 90W) with pre-boiled antigen retrieval buffer (made up with 1X citrate antigen retrieval buffer, pH 6.0 diluted from 10X stock (S2031, Dako) in H₂O) for 10 minutes at ~90°C. After rinsing in cold water, slides were then incubated for 3 min in 3%BSA in PBS followed by treatment with PBS-TritonX-0.5% for 20 minutes. EdU cocktail was prepared mixing the following reagents: 1X reaction buffer (previously diluted in H₂O as per instructions), CuSO₄, Alexa Fluor azide, reaction buffer additive. The EdU mix was prepared not more than 15 min before to apply it on tissue. Tissues were incubated with 100µl of EdU cocktail (per each section in each slide) for 30 min at room temperature and protected from light. After washes in 3%BSA in PBS and PBS/Twin-20 (3 minutes), slides were stained with DAPI at a dilution of 1:1000 in 2X PBS for 3 min (protected from light) and mounted with water-based mounting medium. Sections were then imaged using the confocal microscope and the signal quantified using Fiji software.

2.36 Blood collection from mice and measurement of total cholesterol in plasma.

Blood samples were collected from mouse veins at 0- 6- , and 10-weeks post infection with AAV-PCSK9 vector and at sacrifice in anti-coagulant coated EDTA 1.5ml tubes. Cells were removed from plasma by centrifugation at 4°C for 10 min at 2.000 x g. The supernatant containing the plasma fraction, was then collected and aliquoted into clean 0.5µl tubes (steps performed on ice). Samples were stored into -80°C until usage and freeze-thaw cycles were avoided.

Quantitative cholesterol determination was performed in mouse plasma using the colorimetric Cholesterol FS assay (DiaSys, Holzheim, Germany cat.1-1300-99-10-021) following manufacturer's instructions. Samples defrosted in ice. 8 serial dilutions of the standard provided with the kit in NaCl 0.9% solution. Blank samples, where H₂O was added instead of the sample, were also included in the experiment using a 96-well flat bottom plate (transparent plastic). 2µl of plasma or standard dilutions was added in each well in duplicate, to which 180µl of reagent provided with the kit were added to each sample. Samples were incubated for 10 minutes protected from light at 37°C. Absorbances were then measured within 60 min using a plate reader at 490nm and analysed using the standard curve method and results converted into mmol/L using the conversion factor provided with the kit's instructions.

2.37 Processing of aortic roots and brachiocephalic arteries.

Aortic roots of LDLR^{-/-} mice were embedded in OCT and frozen at 80°C till further use for laser capture microdissection. Aortic roots of *CARMN*^{+/+} and *CARMN*^{-/-} were fixed in paraformaldehyde (1%, 24h), paraffin embedded, and serially sectioned (4µm per section). Five equally dispersed sections were stained with haematoxylin and eosin (H&E) and imaged. Sections were used for immunohistochemical staining within a 100 µm interval where a fully developed media within the aortic valves was present

on H&E. The sum of plaque area in all three valves was analysed in five consecutive H&E sections at 20 μm intervals using computerized morphometry (QuPath v0.1.2, Open-source software for digital pathology image analysis) and averaged per mouse. Two sections per mouse were stained with alpha-SMA, Lgals3, EdU and Sirius Red. The signal was quantified and averaged per mouse.

Following OPT analysis, brachiocephalic arteries were serially sectioned (5 μm per section). Five equally dispersed sections were stained with haematoxylin and eosin (H&E) and imaged. Sections within the plaque interval were used for immunohistochemical staining. Plaque areas were analysed five consecutive H&E sections using ImageJ software and averaged per mouse. Two sections per mouse were stained with alpha-SMA, Lgals3, MYH11, EdU and Sirius Red and the signal was averaged per mouse. The signal was quantified only in the plaque area and the medial layer was not included in the quantification of the immunostainings.

2.38 Statistical analysis.

Data in the graphs are shown as datapoints including the mean $\pm$ standard error of the mean. Test used to assess statistical significance is indicated in each figure legend. For *in vitro* experiments, all biological replicates using primary cells correspond to independent experiments from distinct expansions and passages numbers, performed in technical triplicates (except were clearly indicated in the figure legends). As each experimental data set is an average of a large number of cultured cells, it is assumed the data was normally distributed based on the central limit theorem. Statistical analysis of biological replicates was performed using unpaired t-test (2 groups comparison) or one-way Anova with Bonferroni correction for multiple comparisons (> group comparison). QRT-PCR data in graphs is shown as relative expression to housekeeping control as described by Livak and Schmittgen. Statistical analysis was performed using GraphPad Prism 9.0.0. All the data obtained from *in vivo* experiments were tested normal distribution using the Kolmogorov-Smirnov test. Data following a normal distribution was analysed using unpaired t-test. In the

case of data not-normally distributed or N too small ($n < 6$) to test for normality, Mann-Witney was used to assess significance. The data obtained *in vivo*, have been tested for outliers using GraphPad Prism 9.0.0 and samples have been excluded in the case positive for outlier test.

Chapter3 Characterisation of the lnc *CARMN*'s expression and effects of its modulation in basal and stimulated human Coronary Artery Smooth Muscle Cells (hCASMCs).

3.1. Introduction

In the past decade, long non-coding RNAs (lncRNAs) transcripts have been characterised as important molecules widely involved in the regulation of different cellular processes in physiological and pathological conditions. Although in the past years their importance has been increasingly recognised¹⁸⁰ and efforts have been made to understand their molecular role and association with human diseases, lncRNAs remain very complex molecules still not entirely characterised. Considering the high level of complexity of lncRNA transcripts, their annotation is in most of the cases incomplete and challenging. Factors that generally contribute to this are their low expression levels, weak conservation across species and lack of complete knowledge of key domains that would make easier their identification on the genome. Moreover, their cell-specific expression profile, complicates the characterisation of their transcripts. Therefore, many lncRNA molecules remain still uncharacterised or misannotated on the main publicly annotated databases (GENECODE, Ensembl).

Different strategies have been developed to define the sequences and expression levels of specific lncRNAs in different cell types. Rapid amplification of cDNA ends (RACE) combined with sequencing allows for information on transcript ends (5' and 3' sequence extremities)²⁹⁴. However, this strategy does not provide complete sequence and it requires the knowledge of specific sequences of interest to design oligonucleotides required for the experimental reaction. Cap analysis gene

expression (CAGE) is a method that can be used in the case of polyadenylated lncRNAs, however less efficient in the case of not polyadenylated transcripts which would require previous polyadenylation treatment²⁹⁵. Recently, a new technology, Nanopore long-reads sequencing, has been optimised to obtain information on complex lncRNA transcripts, allowing characterisation of a large number of splicing isoforms²⁹⁶. This novel powerful approach yields high quality full-length sequence information that can be more easily mapped to the reference transcriptome, for example, exon-exon boundaries, transcriptional start sites and this can be used as a novel transcript discovery tool. Moreover, in the case of low-abundance transcripts, this technology can be coupled with a previous capture step using capture probes. In this regard, *CARMN* belongs to the lncRNAs which were first studied in 2017 as an example to validate this innovative strategy. Importantly, Lagarde J, Uszczynska-Ratajczak B. *et al* in 2018 confirmed the previously identified isoforms and extended the reference annotation to include 7 more unannotated novel transcripts expressed from the same locus. This approach allowed the development of strong tools for the study of this complex locus.

lncRNAs have been found differentially expressed in a wide spectrum of diverse cellular contexts. Therefore, profiling the expression changes of these molecules is an essential 1st step for the characterisation of their molecular role. Importantly, the modulation of their expression has proven to be strategic to dissect the molecular role of lncRNAs from their interactors, although it might be very difficult in the case of complex lncRNAs with multi-transcripts profile. Loss and gain of function strategies have been developed and new methodologies have been perfected to specifically manipulate lncRNAs based on their structural characteristics.

RNA interference, which is based on the use of small antisense RNA molecules (Dicer-substrate Short Interfering RNAs, Short Harpin RNAs), has been extensively used to induce post-transcriptional gene silencing of lncRNAs for *in vitro* applications. These strategies can be used to transiently (in the case of DsiRNAs) or stably (for ShRNAs)

reduce gene expression for downstream functional studies by inducing RNA cleavage or transcription repression. However, both methods are not highly effective in the targeting of nuclear lncRNAs²⁹⁷. Therefore, considering that some lncRNAs display entirely nuclear localisation and some others can shuttle between nucleus and cytoplasm¹⁹⁴, the design of new technologies has been developed to overcome this disadvantage. Antisense Oligonucleotides (ASOs), modified to enhance their stability against the action of nucleases, overcome this difficulty by efficiently targeting nucleic RNAs and inducing RNase-H mediated transcript degradation²⁹⁸. Moreover, recent advances in genome editing strategies have allowed the development of CRISPR/Cas9 technology to perform highly efficient and specific gene knockout studies. This method is based on the capability of Cas9 enzyme to bind and perform double-stranded DNA cut with high specificity to a determined target region. Cas9 endonucleases uses single guide RNA sequences (sgRNAs) which bind the target DNA, therefore guiding the Cas9 to the region of interest. However, one limitation of this method is the efficiency of the endogenous cut and repair mechanisms which might induce indel mutations. New advanced variations of this strategy have been generated to perform gene silencing (CRISPRi) and gene activation (CRISPRa), which exploits the use of deactivated Cas9 (dCas9) reducing double strand breaks caused DNA instability and toxicity¹⁹⁴. This highly specialised technology is widely used for *in vivo* and *in vitro* applications however, it requires time and resources to be optimised for the desired purpose. In addition, the challenges associated with effective delivery of the CRISPR machinery into terminally differentiated cells reveal RNA interference (by GapmeRs, DsiRNAs) as a more suitable option for *in vitro* screenings of lncRNAs.

Following the discovery of lncRNAs of interest, high-throughput strategies have been developed to study *in vitro* and *ex vivo* their role and assess their function in the regulation of cellular processes. One of the first methods used to explore differential gene expression in the context of loss or gain of function studies was based on the hybridization of custom designed probes of known sequences, which complementarily bind to fluorescently labelled cDNA in one specific condition,

microarrays. Using this strategy, the quantitation of differences in gene expression can be performed detecting the fluorescent signal after probe-cDNA hybridization. However, this technology has the limitation of being highly influenced by the density of probes hybridizing to reach good coverage and good knowledge of the genomic sequence. Tag-based technologies (serial analysis of gene expression, SAGE, or cap analysis of gene expression, CAGE) overcome these limitations by using a collection of short random sequences derived from the cDNA sample which are then sequenced to investigate gene expression. Nevertheless, these strategies do not always uniquely map to the reference genome and do not allow the study the dynamic expression of complex multi-transcript loci.

RNA Sequencing (RNA-Seq) is a widely used technology for the study of differentially expressed genes, quantification of transcriptional isoforms belonging to the same locus and powerful novel transcript discovery tool. Many RNA-Sequencing platforms have been developed (such as Illumina, Life technologies, Oxford Nanopore²⁹⁹), and are generally based on the generation of cDNAs (using oligo dT or random primers) cloned into a library to be sequenced to generate a pool of short sequences (50-400 bp) which are then mapped to the genome and transcriptome. Bulk RNA-Seq for the characterisation of gene expression and differential gene expression at large scale level in cells or tissues, and single-cell resolution RNA sequencing (scRNA-seq) for to profile cell expression patterns related to cell heterogeneity in development and disease, have been widely used from the scientific community³⁰⁰. Importantly, these methodologies have strategically allowed the investigation of the functional consequence of the modulation of lncRNA molecules in cellular processes *in vitro* with the goal of translating the acquired knowledge for *in vivo* applications.

In the context of understanding the functional readout of the modulation of lncRNA expression, one of the challenges is to effectively dissect their molecular role from the one of their molecular interactors. In regard to this, the presence of microRNAs co-located within lncRNAs loci constitutes another level of complexity. Recently,

studies have begun to unravel the functional interactions between lncRNAs and their hosted microRNAs characterising lncRNA inter and independent roles. However, although efforts for the identification of lncRNA-microRNAs networks have been made, dissecting their molecular function through the modulation of their expression can be challenging. This is mostly because current strategies might contemporarily modulate the expression of noncoding molecules, especially in the case lncRNA and mature microRNA share common exon sequences.

3.2 Aims

The expression and function of *CARMN* were initially studied *in vitro* in vSMC and the main hypothesis of this chapter is:

CARMN is involved in the regulation of vSMC phenotypical switch through a mechanism of action which is in part independent from miR-143/145.

- 1) To characterise the expression of human *CARMN* transcript variants in hCASC.
- 2) Develop a strategy to modulate the expression of *CARMN* transcripts to explore the functional consequence of this modulation on hCASC phenotypes.
- 3) To functionally assess whether the mechanism of action of *CARMN* is in the regulation of miR-143/145 exclusively or whether miR-independent effects are evident.

3.3 Results

3.3.1 Expression of *CARMN* in unstimulated human Coronary Arterial Smooth Muscle Cells (hCASMCs)

The characterisation of lncRNA expression profile is extremely important to give strong basics to a research study. Ensembl database 98 was taken as public database reference for the characterisation of *CARMN* isoforms in human coronary artery smooth muscle cells (hCASMCs) in unstimulated basal conditions. At the beginning of this study, the annotation counted 12 transcriptional isoforms belonging to *CARMN* locus (Figure 3.1)

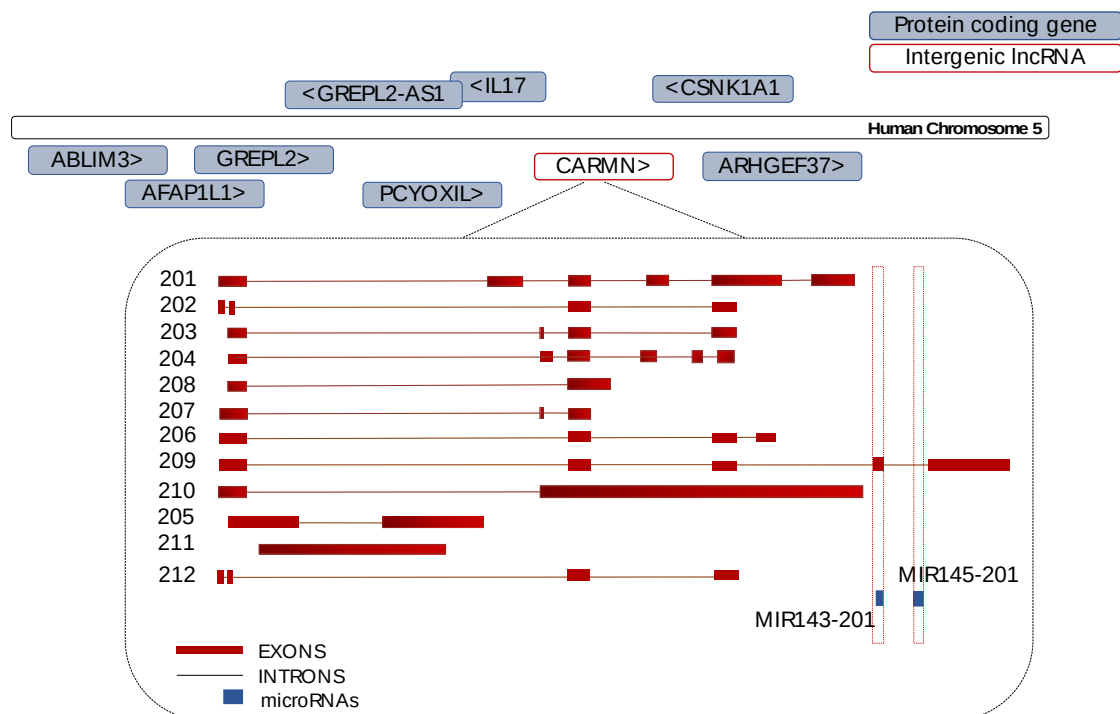


Figure 3.1 Schematic representation of human *CARMN* locus.

CARMN is a multi-transcript long intergenic RNA located in the human chromosome 5 (grey bar). This picture displays neighbour genes to *CARMN* locus (light blue boxes) and their transcription direction (indicated by the arrows next to each gene name). *CARMN* transcripts are represented with a multi-exonic structure where exons are

indicated by red boxes and introns by red lines. *CARMN* locus is host genes for miR-143/145 and the pre-microRNA is represented by dark blue boxes in this picture. The annotation used throughout the study is Ensembl database (GRCh38/hg38 version). Independent transcript-specific primer sets were designed spanning exon-exon junctions to selectively amplify each isoform (Figure 3.2). It was not possible to design specific primer sets for the amplification of isoform 204 as the sequence of this transcript entirely overlaps with isoform 210. Therefore, in this case, primers amplifying both isoforms were designed. The primer sequences were blasted against the human *CARMN* sequences using online Primer Blast software to ascertain their target specificity.

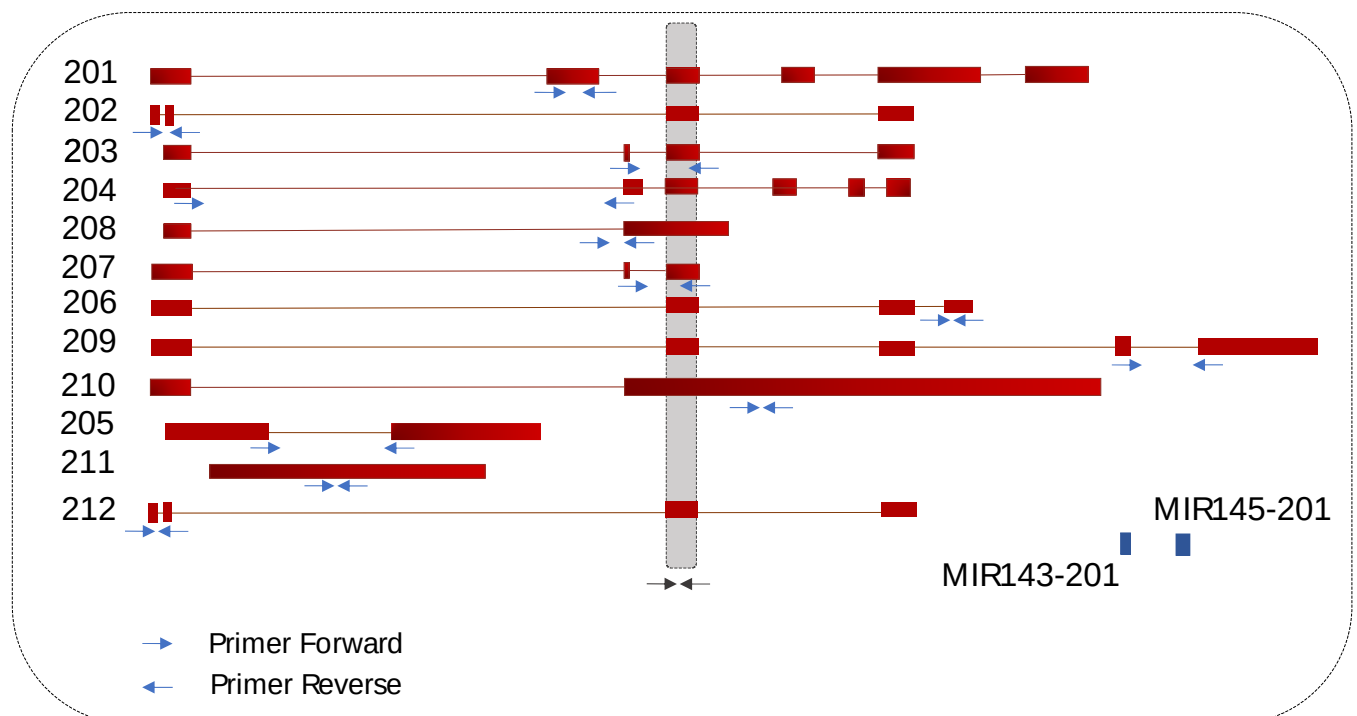


Figure 3.2 Localisation of transcript-specific primer sets.

Isoform-specific pairs of forward and reverse primers (blue arrows) were designed to uniquely identify each transcript belonging to the human *CARMN*. The design was performed into exon-exon junctions where possible, otherwise amplifying exons. The expression of isoform 204 was not possible to be identified separately from isoform

210 as the two isoforms overlap in their sequence. The grey box and arrows indicate common primer set used in the study, amplifying a common region to most of the transcripts (except 205 and 211).

Primer qRT-PCR reactions were checked for the presence of a single amplification point through analysis of their melting curves. Each set of primers producing multiple points were excluded and substituted with a new set. Moreover, qRT-PCR reaction products were run in agarose gel to assess the length of amplification products and sequenced using both, forward and reverse primers in separate reactions for sequence confirmation in both directions (Figure 3.3). It is important to emphasise that this strategy was used to assess the presence of the annotated isoforms in our hCASMCS samples to fully characterise the locus. However, it is not possible to have specific information about transcripts ends or abundance of each transcript using Sybr Green primers-based strategy. Nevertheless, a set of common primers amplifying most of the isoforms (except transcript 4, 10 and 11) were used throughout this study and referred to as *CARMN* primers.

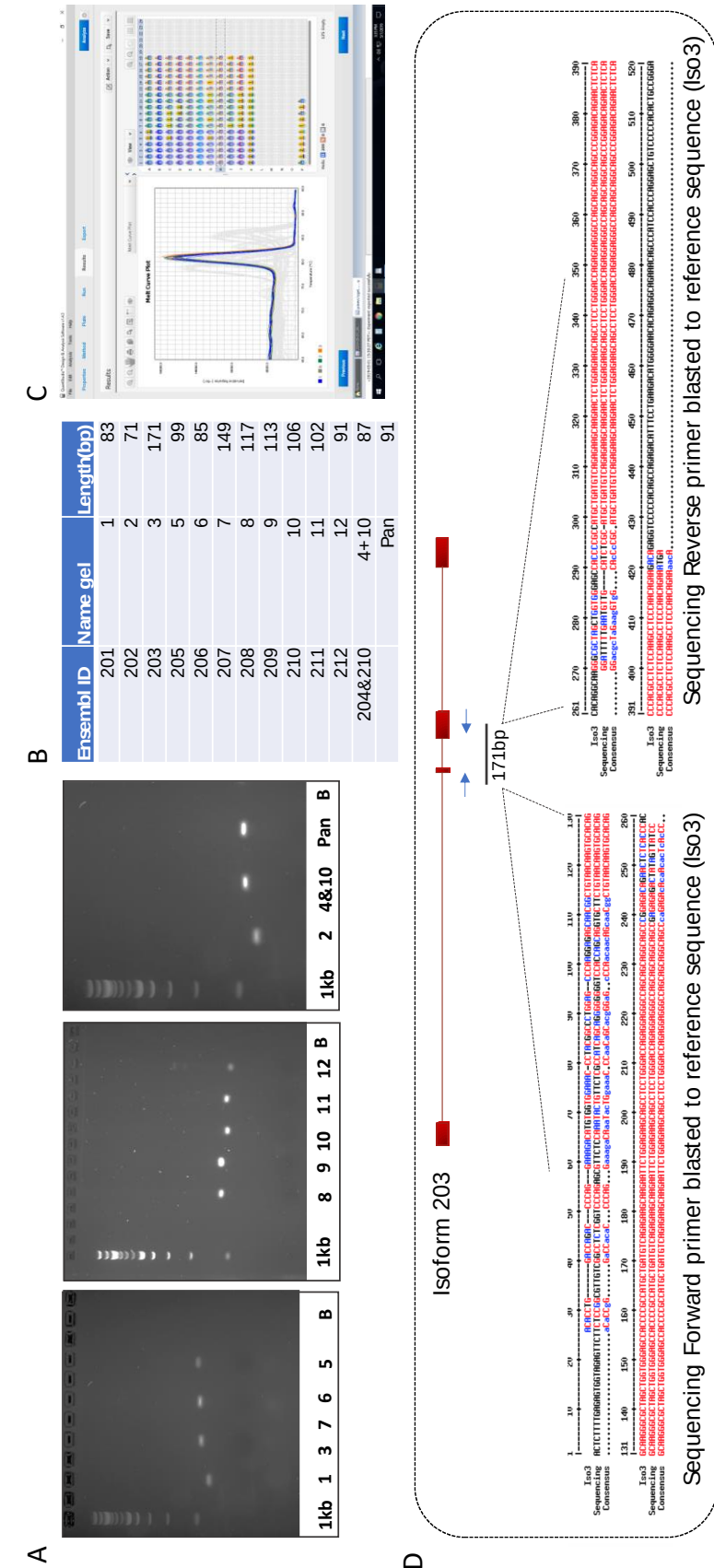


Figure 3.3 Assessment of primer sets efficiency and specificity.
A) 2% Agarose gel pictures showing qRT-PCR amplification products and 1Kb DNA ladder marker. Blank sample (B) with no cDNA insert was also considered in each gel. B) Table showing the Ensembl ID for each isoform with correspondent names given to each isoform in the agarose gel and the length of each amplification product. C) Example of efficient melting curve with only one amplification product. D) Example of sequencing results of amplification product (for CARMN transcript 203, 171bp length) obtained sequencing the amplicon in both directions using forward and reverse primer sets.

Figure 3.3 Assessment of primer sets efficiency and specificity.

Following primers optimisation, the presence of *CARMN* transcripts was assessed in unstimulated hCAsMCs (Figure 3.4).

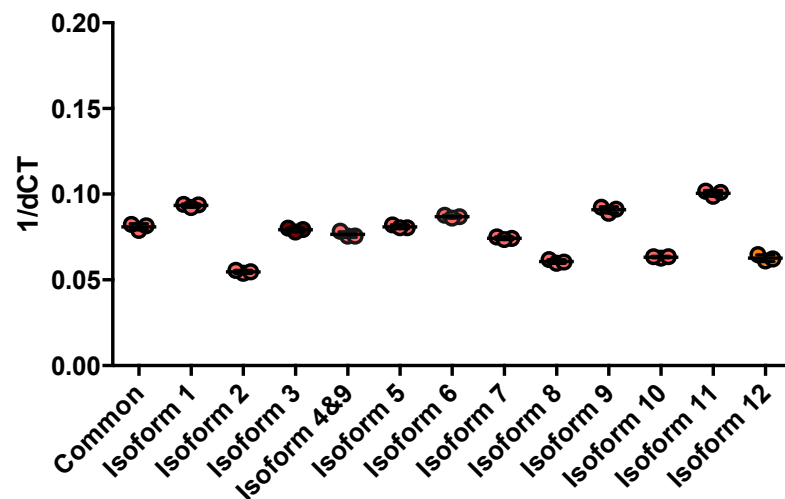


Figure 3.4 Expression levels of *CARMN* transcripts in hCAsMCs at basal conditions.

To profile *CARMN* annotated transcripts in hCAsMCs, cells at basal conditions (0.2% FBS) were harvested and RNA was extracted for downstream gene expression analysis. The graph represents qRT-PCR data in hCAsMCs (n=3 independent experiments each with technical triplicates. Passage number 4-5) using isoform-specific primers (Isoform 1,2,3,4+9,5,6,7,8,9,10,11, and 12) and common primers (amplifying all isoforms except 4, 10 and 11). Data are represented as the reverse of delta CT values (higher values in the plot correspond to highly expressed transcripts, and vice versa). Graphs are shown as datapoints including the mean $\pm$ standard error of the mean. UBC was used as housekeeper gene because of the stability in its expression across the samples.

3.3.2 Expression of *CARMN* and microRNAs in hCASMCM phenotypes.

Human SMCs can switch from their contractile phenotype to a synthetic state during vascular remodelling in atherosclerosis. The synthetic stage is characterised by increase in proliferation rate, migration, differential expression of SMC identity genes and de-differentiation to pro-inflammatory foam-cell like phenotype. The capability of SMC to switch to a pro-pathological state in response to pathogenic environmental factors has previously been studied *in vitro* and studies had contributed to enhancing our knowledge of the pathophysiology of this process *in vivo*. In this section, the expression of *CARMN* was interrogated following the induction of SMC proliferation, migration and de-differentiation phenotype mediated by cholesterol loading of cells in culture.

3.3.2.1 *CARMN* and miR-143 and miR-145 expression in proliferating hCASMCM

The exposure to increased cytokine presence such as Platelet-derived Growth Factor BB (PDGF-BB) is one of the factors driving the activation of hCASMCMs proliferation in atherosclerotic plaques. Cells were first induced to quiescence to synchronise to their G0 cell cycle phase by treatment with 0.2%FBS (basal medium), prior to stimulation with 20ng/ml human PDGF-BB in basal medium (0.2% FBS) for 48 hours (Figure 3.5). This dosage was chosen as previously used in literature to induce SMC proliferation *in vitro*.

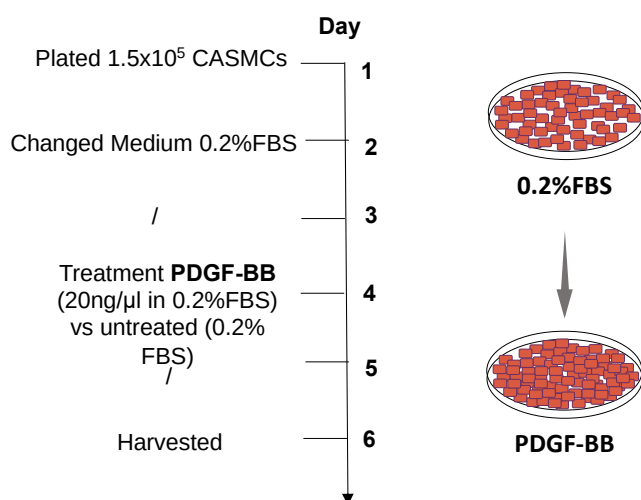


Figure 3.5 Workflow of hCASCs proliferation experiment.

hCASCs were plated in a density of 1.5×10^5 in complete medium (15% FBS), induced to quiescence in basal medium (0.2% FBS) and stimulated with PDGF-BB cytokine at a concentration of 20ng/ μ l in basal medium (0.2% FBS) for 48 hours. Cells were then harvested for downstream analysis of gene expression through RNA extraction or flow cytometry analysis to assess the percentage of EdU incorporating cells. In this experiment 3 independent experiments each with technical triplicates were performed as described below.

To check the effective induction of hCASC proliferation by PDGF-BB treatment, flow cytometry examining EDU incorporation was performed in stimulated and unstimulated (0.2%FBS) hCASCs. The gating strategy was designed using untreated and unstained cells (Figure 3.6). Percentages of EdU positive cells were analysed and plotted using FlowJo software.

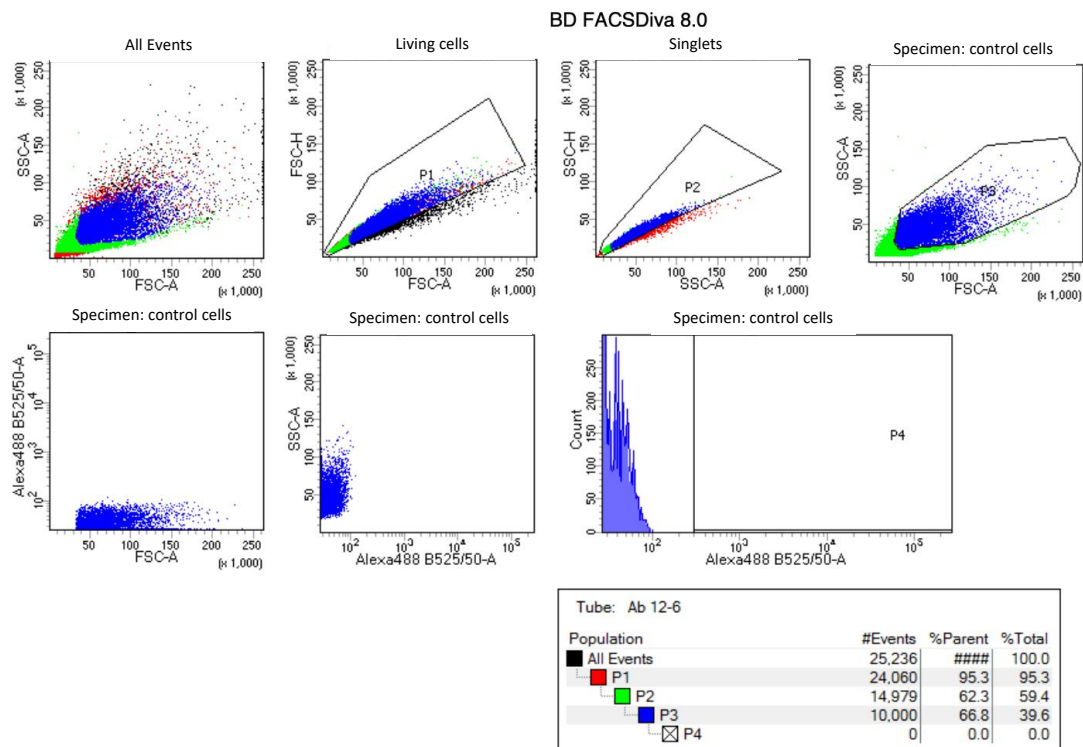


Figure 3.6 Gating strategy for Flow Cytometry analysis for the detection of EdU positive cells.

The gating strategy for all the experiments throughout the study was designed with the supervision of the Flow Cytometry core facility (QMRI) and applied to all the experiments to consistently analyse and compare samples across different replicates and times. This is one example of the strategy used to assess the percentage of positive cells to incorporation of EdU. Briefly, not-EdU stained cells (technical control) were used to assess autofluorescence and cell availability. Moreover, only single cells were considered for analysis (and aggregates of cells not very well separate were excluded). The percentage of events indicate the % of cells positive to staining (P4 gate in the picture). These experiments were performed in 3 independent experiments each with technical triplicates using the same gating strategy.

PDGF-BB treatment induced 6.3 ± 2 fold increase of cells positive to EdU incorporation compared to control (Figure 3.7).

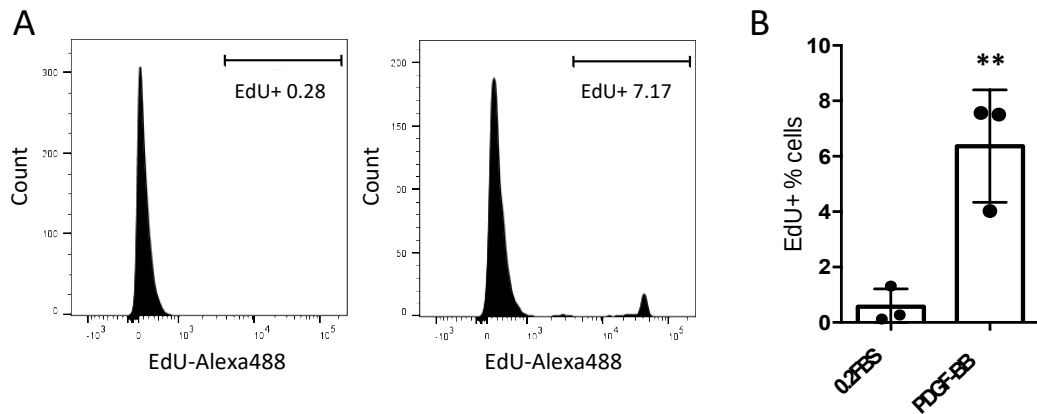


Figure 3.7 hCASCs proliferate following PDGF-BB treatment.

hCASCs were plated and treated for 48 hours with PDGF-BB (20ng/ml) or 0.2% FBS following starvation in 0.2%FBS medium. A), B) FACS plot and graph showing the percentage of hCASCs (n=3 independent experiments each with technical triplicates. Passage number used 4 and 5) incorporating EdU following PDGF-BB treatment and in basal (0.2% FBS) conditions obtained with FlowJo data analysis software. Student's *t*-test was used to assess **P<0.01. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Having assessed the induction of PDGF-BB pathway by increased cell proliferation, the expression of *CARMN* and the microRNAs miR-143 and miR-145 were assessed in the same conditions (Figure 3.8). Interestingly, *CARMN* expression was found decreased by 2.9 ± 0.44 fold versus control together with decreased levels of miR-143 (2.21 ± 0.67) and miR-145 (2.23 ± 0.31) being observed, indicating that the all axis is affected by PDGF-BB stimuli.

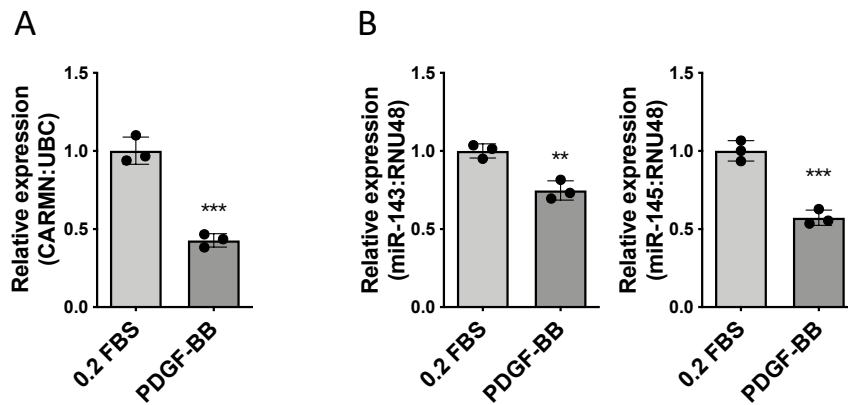


Figure 3.8 Expression of *CARMN* axis following PDGF-BB treatment of hCASMCs.

A) B) qRT-PCR (n=3 independent experiments each with technical triplicates. Passage number used 4 and 5.) expression of *CARMN* and microRNAs in hCASMCs at basal (0.2% FBS) and PDGF-BB stimulated conditions. Results are represented as relative expression to UBC (in the case of *CARMN*) and RNU48 (in the case of microRNAs) housekeeping genes. Student's *t*-test was used to assess **P<0.01 and ***P<0.001. Graphs are shown as datapoints including the mean ± standard error of the mean.

3.3.2.2 *CARMN* and microRNAs expression in migrating hCASMCs

In response to mechanical or chemical cues in vascular injury, pro-pathological SMCs migrate from the medial to the neointimal compartment of the arterial wall thus contributing to the cluster of dysregulated pro-inflammatory cells forming the early plaque. hCASMCs were induced to migrate using a wound healing assay, by which a physical scratch is performed in a two-dimensional confluent monolayer of cells and migration distances are measured following cell migration forward into the wound. It is important to underline that cells were induced to quiescence in starvation media (0.2%FBS) prior to the scratch and the wound healing assay was then performed in starvation media. These conditions suppress cell proliferation during the migratory

process to wound closure, therefore allowing for the measurement of the ability of cells to migrate (Figure 3.9).

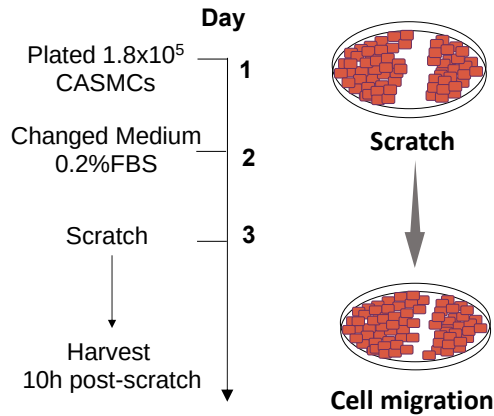


Figure 3.9 Workflow of migration assay performed in hCASCs.

hCASCs were plated, induced to quiescence in 0.2%FBS and stimulated to migrate by scratch assay in starvation medium (0.2% FBS). Scratch was performed using p200 sterile tip and wash with warm medium. Cells were then harvested following 5, 10 or 24 hours post scratch assay for downstream analysis of gene expression through RNA extraction. In this experiment, 3 independent experiments each with technical triplicates were performed as described below.

A scratch was induced in a monolayer of hCASCs. Following 5, 10 or 24 hours from the induction of the scratch, cells were harvested to assess *CARMN* and microRNA-143/145 expression. Contrarily to SMCs proliferation, the induction of cell migration did not affect the expression levels of *CARMN* or microRNAs indicating that *CARMN* axis might not be affected by signalling pathways activated during the matrix remodelling of migratory SMCs (Figure 3.10).

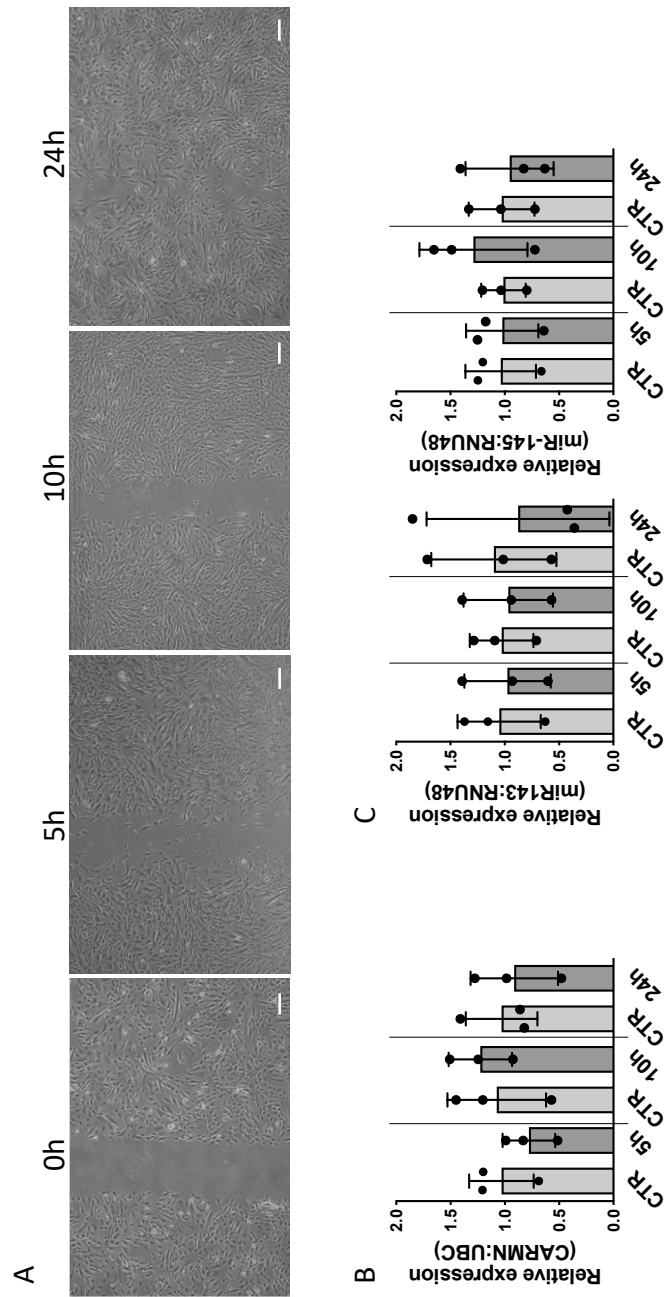


Figure 3.10 CARMN expression in migrating hCASCs.

Figure 3.10 CARMN expression in migrating hCASCs.
A) Micrographs representing hCASCs at 0-5-10-24 hours following induction of migration by scratch assay in basal conditions (0.2% FBS medium). Pictures were acquired at 10X magnification, scale bar 100µm B), C) qRT-PCR data (n=3 biological replicate each with technical replicates from same donor and independently cultured. Passage number 5 and 6) relative to CARMN and microRNA expression levels relative to housekeeping genes, UBC and RNU48 respectively. Student's t-test was used to assess significance.

3.3.2.3 *CARMN* and microRNA-143/145 expression in foam-cell like hCASCs induced by cholesterol loading.

Previous studies have used the loading with cholesterol of vSMCs to study the effect of the accumulation of cholesterol excess and uptake by vSMCs *in vitro*⁹⁸. The formation of lipid loaded vSMCs has been observed *in vivo* in plaque and described to be one of the main contributors to the progression of the plaque. Interestingly, miR-143/145 expression was previously found downregulated during this process¹¹³. Using a methodology similarly to what was published before, we loaded hCASCs in culture with water soluble cholesterol-methyl- β -cyclodextrin (CMBCD) for 72 hours (Figure 3.11), assessed the cholesterol uptake by Red Oil O staining and checked cell viability at different concentrations (5, 10, 20, 40 μ g/ml) (Figure 3.12).

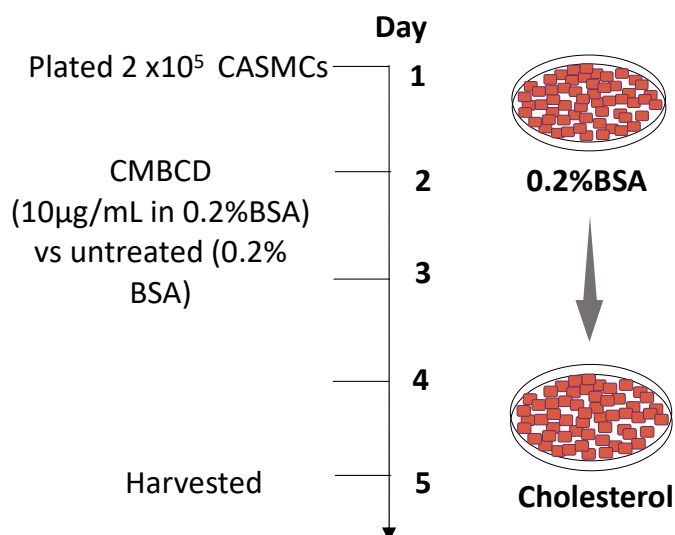


Figure 3.11 Workflow of loading hCASCs with water-soluble cholesterol (CMBCD).

hCASCs were plated at a confluence of 2×10^5 , induced to quiescence in 0.2%BSA and stimulated to de-differentiate into foam-cell like phenotype through treatment with CMBCD (10 μ g/ml) for 72 hours. Cells were then harvested for downstream analysis of gene expression through RNA extraction. In this experiment, 3

independent experiments each with technical triplicates were performed as described below.

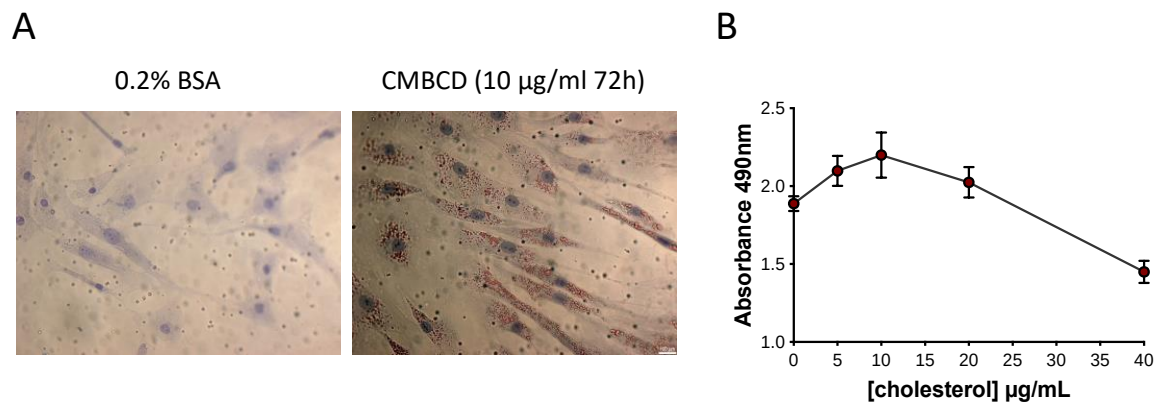


Figure 3.12 CMBCD loading of hCASMCs *in vitro*.

hCASMCs were plated and stimulated with water soluble cholesterol-methyl- β -cyclodextrin cholesterol (CMBCD) for 72 hours (10 µg/ml) or in 0.2%BSA as control.

A) Oil Red O (ORO) staining of hCASMC following loading with water soluble cholesterol or 0.2%BSA control. Nuclei were stained with Eosin (blue) and intracellular lipids were stained with Oil Red O solution (red). Pictures were acquired at 20X magnification, scale bar 100 µm. B) Cell viability assay to measure hCASMC viability following 5-10-20-40 µg/ml treatment with CMBCD for 72 h. n=1 experiment with technical triplicates, cell passage used 6. Data expressed as absorbance at 490nm. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Considering the efficient uptake and the low cytotoxicity effect observed following loading with 10 µg/ml methyl- β -cyclodextrin, this concentration was chosen throughout the study. To assess the efficiency of the model to induce a de-differentiation process towards a foam-cell like phenotype, the expression of typical vSMC identity genes and activated macrophage markers were assessed in the same

samples (Figure 3.13). We observed an increase of 4.4 ± 0.22 and 1.8 ± 0.03 fold increase in CD68 and LGALS3 macrophage genes respectively. A concomitant decrease of 23 ± 0.14 and 5 ± 0.19 fold in the expression of ACTA2 and MYH11, SMC marker genes respectively, was also observed suggesting the effective induction of foam-cell like cell in hCASCs.

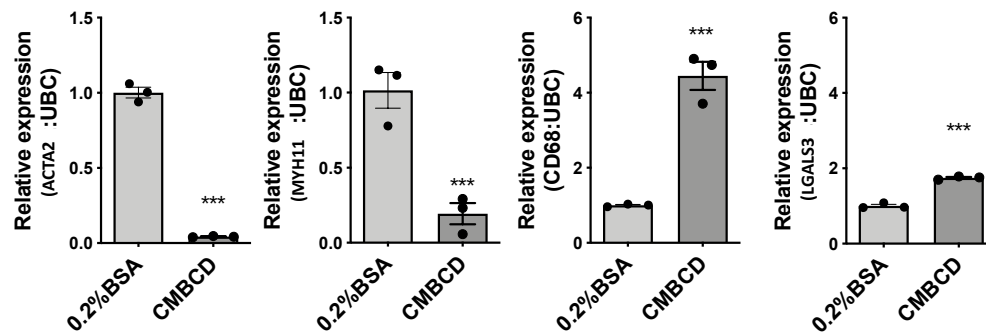


Figure 3.13 hCASCs de-differentiate following CMBCD stimulation.

Expression of ACTA2, MYH11, CD68 and LGALS3 in hCASCs (n=3 independent experiments each with technical triplicates. Cell passage used 5 and 6) at basal (0.2% BSA) and following 72 hours of stimulation with 10 μ g/ml of CMBCD (relative to UBC). Student's *t*-test was used to assess ***P<0.001. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Having assessed the efficiency of the model in hCASCs, the expression of *CARMN* and microRNA-143/145 was checked in the same samples. Interestingly, the expression of *CARMN* was found significantly downregulated by 6.3 ± 0.12 fold following CMBCD versus control (Figure 3.14). Moreover, miR-143 and miR-145 expression was decreased of 2.33 ± 0.07 and 2.9 ± 0.06 fold respectively, confirming previous findings¹¹³.

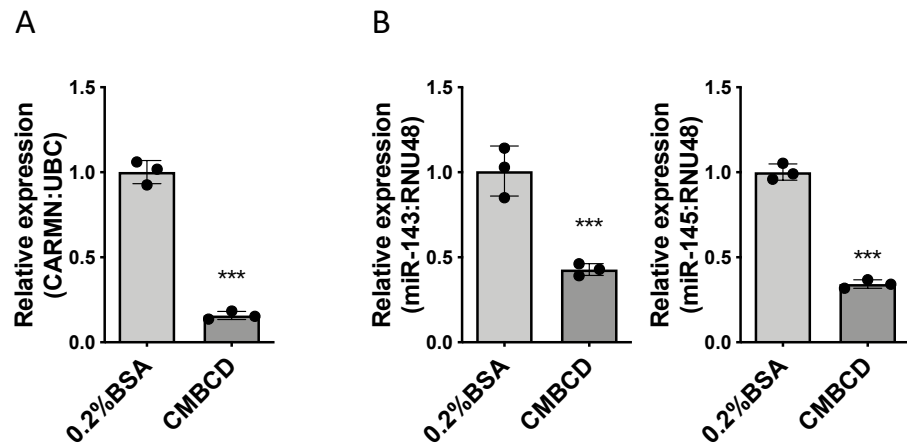


Figure 3.14 Expression of *CARMN* axis in cholesterol-loaded hCASCs.

A), B) qRT-PCR data (n=3 independent experiments each with technical triplicates, cell passage used 5 and 6) of *CARMN* and microRNAs miR-143/145 in hCASCs at basal (0.2% BSA) and following 72 hours of stimulation with 10 $\mu\text{g/ml}$ of CMBCD (relative to UBC or RNU48 housekeeper genes). Student's *t*-test was used to assess *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.2.4 The expression of *CARMN* axis following ox-LDL treatment of hCASCs.

The impact of lipid accumulation on the vSMC phenotype was studied *in vitro* through the stimulation of cells with oxidised Low-Density Lipoproteins (ox-LDL) isolated from hypercholesterolemic plasma³⁰¹. The efficiency of the model was firstly assessed by loading hCASCs with Dil-conjugated human ox-LDL particles for 24 hours (Figure 3.15).

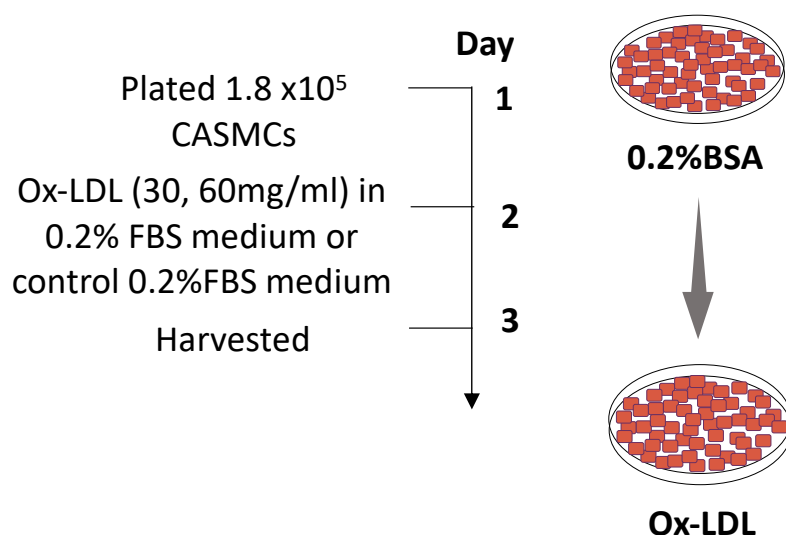


Figure 3.15 Workflow of hCASCs laden with ox-LDL.

hCASCs were plated at a confluence of 1.8×10^5 , induced to quiescence in 0.2%FBS and stimulated to transdifferentiate into foam-cell like through treatment with oxidised Low-Density Lipoprotein (Ox-LDL) in a concentration of 30 or 60mg/ml for 24 hours. Cells were then harvested for downstream analysis of gene expression through RNA extraction. In this experiment, 3 independent experiments each with technical triplicates were performed for downstream gene expression analysis performed by qRT-PCR.

In addition, cells were assessed for the uptake of Dil-conjugated ox-LDL particles through microscopy and cell viability were evaluated by MTT assay (Figure 3.16).

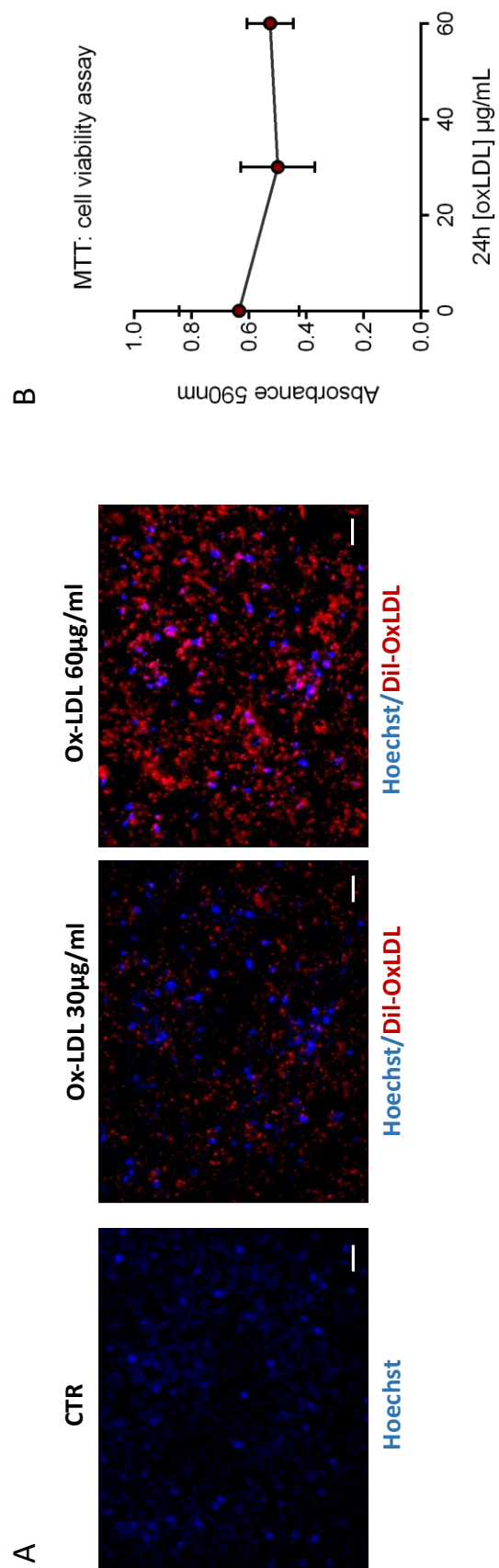


Figure 3.16 hCASMC uptake labelled ox-LDL in culture.

A) Representative micrographs of hCASMC loaded with ox-LDL (30 or 60 µg/ml) for 24 hours. Hoechst was used as nuclear staining. Pictures were acquired at 100X magnification, scale bar 100µm. B) MTT viability assay performed in hCASMC (n=1 biological performed in technical triplicates, cell passage 6) loaded with 30 or 60µg/ml for 25 hours. Data show the absorbance detected at 590nm.

Figure 3.16 hCASMC uptake labelled ox-LDL in culture.

Having established the uptake of ox-LDL into hCASCs, the expression of *CARMN* and microRNAs miR145/143 were then assessed in ox-LDL-induced foam cells. Moreover, the expression of the membrane protein ATP-binding cassette transporter 1 (*ABCA1*), whose upregulation was previously described before in response to ox-LDL, was assessed in the same samples as a marker of this model (Figure 3.17). *ABCA1* expression was found 2.3 ± 0.16 and 2.5 ± 0.09 fold increased following treatment with 30 or 60 $\mu\text{g/ml}$ ox-LDL respectively, which confirmed previous findings and functionality of the model in hCASCs. Interestingly, *CARMN* levels were downregulated in a dosage-dependent mode with a 2.2 ± 0.073 and 3.76 ± 0.07 fold decrease following treatment with 30 or 60 $\mu\text{g/ml}$ ox-LDL versus control, suggesting the potential involvement of the lncRNA as downstream modulator affected by modified lipids-mediated activated pathways. In contrast, the expression of microRNAs was not affected by ox-LDL loading of hCASCs, which indicates that miR-143/145 cluster might not be involved in the early response to oxidised lipids molecules activated in pro-pathogenic hCASCs.

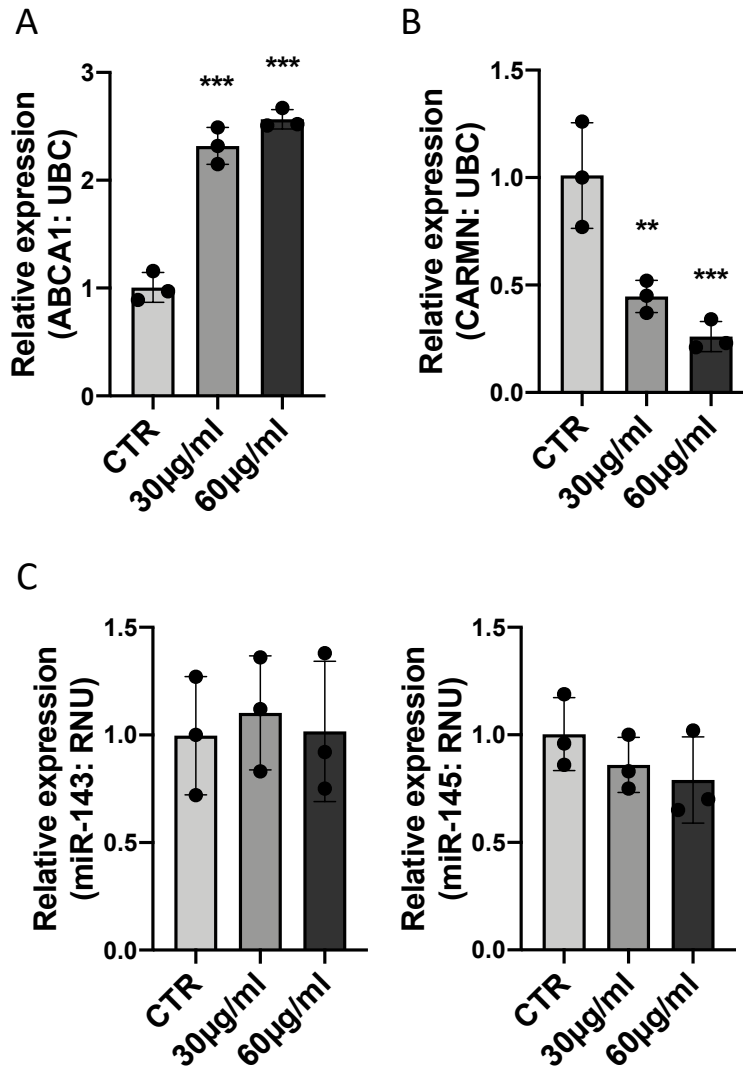


Figure 3.17 The effect of ox-LDL on the expression of *CARMN* axis in hCASCs.

hCASCs were treated in a dosage dependent way with 30 or 60 µg/ml of human ox-LDL for 24 hours or 0.2%FBS in the control condition. Cells were then harvested for downstream analysis of gene expression. A) qRT-PCR data relative to the expression of ABCA1 in n=3 biological replicates (n=3 independent experiments each with technical triplicates, cell passage used 5 and 6) following treatment with ox-LDL or control. B) Expression of *CARMN* using pan primer in hCASC treated or not with dosages of ox-LDL. C) Expression of miR-143 and miR-145 in cells treated with ox-LDL or 0.2% FBS control. Housekeeping genes (UBC for gene expression or RNU48 for

microRNAs) were taken as reference genes. Student's *t*-test was used to assess ** $P < 0.01$ and *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.2.5 Investigating *CARMN* downregulation in hCASM C de-differentiation process.

Considering the phenotypical effect that the activation of cholesterol pathways, through CMBCD and ox-LDL treatment, had in the modulation of the expression of *CARMN*/miR-143/145 locus, the current annotated promoter region was interrogated to assess the presence of specific transcriptional binding sites that may play a role in the transcriptional downregulation of *CARMN*.

In 2015, the promoter of *CARMN* and miRNA-143/145 locus was mapped and the presence of several putative binding sites for nuclear Liver X Receptor (LXR) and Retinoid X Receptor (RXR) were identified at -600 and -1000bp from the Transcriptional Start Site (TSS) (Figure 3.18)²⁷¹. These two factors dimerise together for DNA binding and regulation of gene expression. In particular, LXR controls the reverse cholesterol transport by positively regulating the expression of ATP Binding Cassette 1 (ABCA1) and inhibit pro-inflammatory pathways. Mice knocked-out for this factor developed severe atherosclerosis caused by the intracellular accumulation of toxic cholesterol which was reversed by the administration of LXR agonists³⁰². Moreover, the expression of this factor is attenuated during atherosclerosis, leading to inefficient cholesterol removal and accumulation of foamy-cells laden with toxic modified cholesterol³⁰².

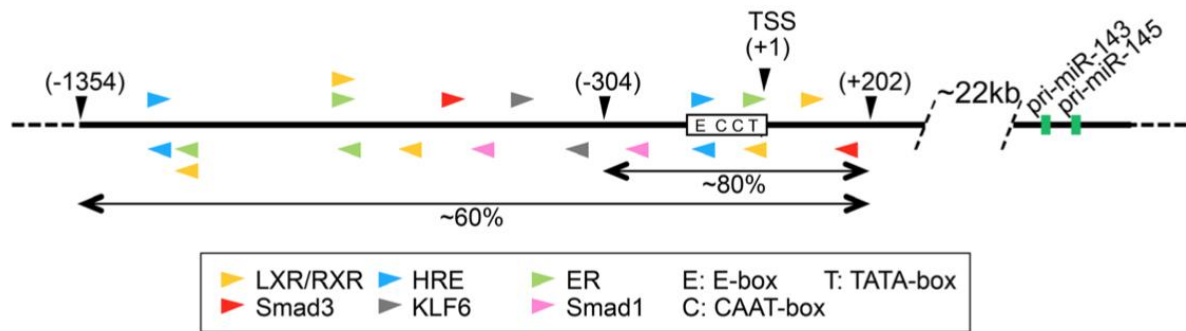


Figure 3.18 Schematic representation of *CARMN* promoter with details of putative LXR/RXR binding sites in yellow. Homology percentage among species is indicated.

Image taken from Deng L, Blanco F *et al* 2015, Circulation Research.

Therefore, to test whether *CARMN* downregulation in hCASCs was in part mediated by the inactivation of LXR pathway recapitulating the previous *in vivo* experiment, the effect of LXR antagonist was assessed in hCASCs. GSK2033 was selected as efficient antagonist previously studied in literature to inhibit LXR pathway *in vitro*³⁰³. Different dosages of GSK2033 were tested (0.5, 1, 3 μ M) in hCASCs for 24 hours and the expression of LXR and ABCA1 was assessed to verify the efficacy of LXR pathway inhibition (Figure 3.19). Cells treated with DMSO (the vehicle control) were used as control since the chemical compound was resuspended in DMSO-conditioned medium. The results show the expression of LXR gene was inhibited by 7, 11 and 10-fold following 0.5, 1, 3 μ M treatment with GSK2033 respectively. This confirms previous findings³⁰⁴ demonstrating the positive feedback loop of LXR on the regulation of LXR promoter itself. Moreover, ABCA1 expression was reduced 4.8, 6.4 and 5.7-fold in the same conditions therefore suggesting a functional readout of the inhibition of the pathway (Figure 3.19).

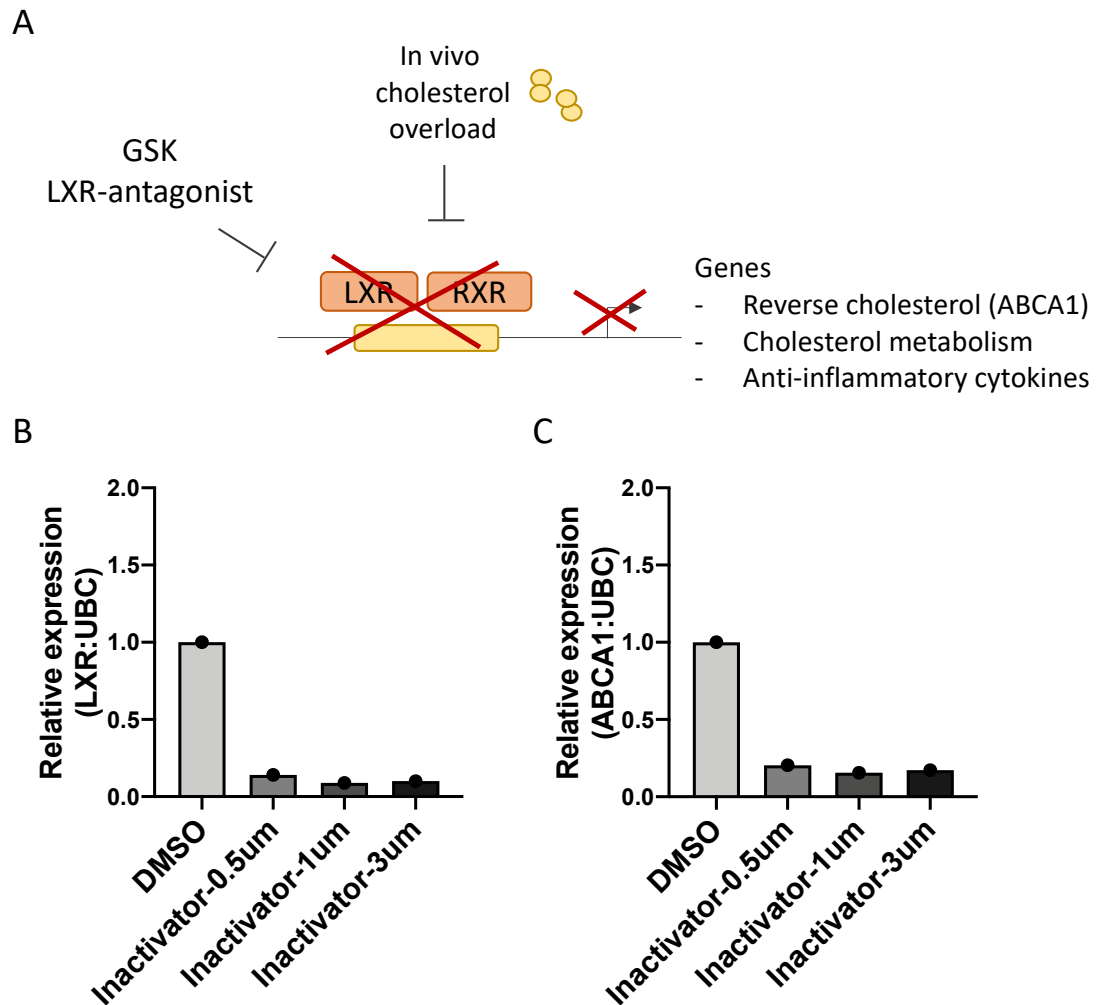


Figure 3.19 Inhibition of LXR pathway in hCASCs.

A) Diagram of LXR/RXR pathway inhibited by accumulation of toxic modified cholesterol *in vivo*. GSK2033 antagonist has similar effect on LXR pathway blocking the transcription of genes activated by LXR, such as ABCA1. B), C) Expression data relative to LXR and ABCA1 genes in hCASCs (n=1 experiment performed in technical triplicates, passage used 6) following 24 hours of treatment with 0.5, 1, 3 μ M of GSK2033 inhibitor in 0.2%FBS medium or DMSO in 0.2% FBS medium as control. UBC was used as housekeeping gene. Graphs are shown as datapoints.

Having verified the effective repression of LXR gene expression and inhibition of genes activated by the pathway, the expression of *CARMN* was assessed following

treatment with 0.5 μ M of GSK2033 for 24 hours. *CARMN* levels were not significantly affected by the treatment with the synthetic inhibitor when compared to the DMSO control (Figure 3.20), indicating that the *CARMN* promoter might not be directly regulated by the functional dimer LXR/RXR and more studies would be required to experimentally validate the presence of binding sites. Nonetheless, even in the case of a direct interaction, *CARMN* might not be among the early response genes in the LXR/RXR cascade. Moreover, belonging to a class of nuclear receptor mostly expressed in the liver and adipose tissue, more exploratory experiments should be conducted in these tissues to understand the effect that LXR knock-out might have in *CARMN* expression *in vivo*.

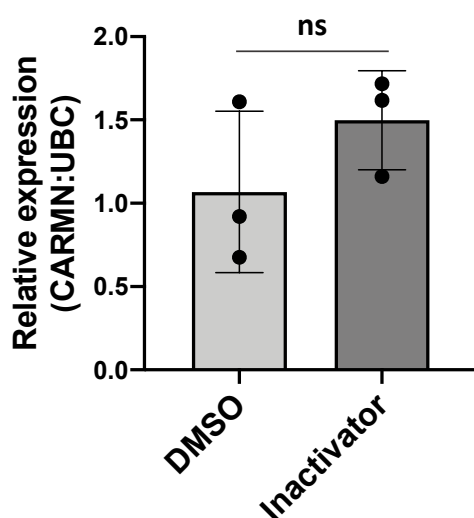


Figure 3.20 *CARMN* expression in LXR repressed pathway in hCASMCs.

hCASMCs were plated and treated with 0.5 μ M of GSK2033 to inactivate LXR/RXR pathway for 24 hours or with DMSO in 0.2%FBS as control. Cells were then harvested to perform qRT-PCR analysis. The graph represents the expression of *CARMN* in hCASMC (n=3 independent experiments each with technical triplicates, passage used 5 or 6) treated with the inactivator (GSK2033) or in control cells. UBC was used as

housekeeping gene. Statistics was performed with Student's *t*-test, ns= not significant. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.3 GapmeR-mediated *CARMN* depletion affected miR-143/145 expression.

CARMN expression was found dysregulated in disease in a tissue-specific and disease stage-specific way. The next scientific question of this study was to interrogate whether *CARMN* modulation affected hCASM function. LncRNA widely vary in their subcellular localisation which is important information to be able to apply efficient knock-down strategies.

To choose the most efficient approach to downregulate *CARMN* expression, the lncRNA localisation was assessed in hCASM in basal conditions (0.2% FBS). Subcellular fractionation results indicated that *CARMN* localised mostly in the nucleic compartment of cells in basal conditions (Figure 3.21 A, B). Considering that the purification of the nucleic fraction is a critical step in this procedure, it was important to include a positive control localising mostly in the nucleic fraction of the cells. Therefore, the lncRNA NEAT1 was taken as positive control for the isolation of the nucleus. Cytoplasmic lncRNAs can localise also in the nucleus, and many lncRNAs can shuffle between compartments, therefore it is difficult to identify an entirely cytoplasmic lncRNA¹⁹⁴. A second technique, Fluorescent In-Situ Hybridisation, was used to validate the mostly nuclear localisation of *CARMN* in hCASM cells at basal conditions (Figure 3.21 C). Ubiquitin C (UBC) and Small Nucleolar RNA 3 (SNORD3) were used as confirmation of cellular localisation.

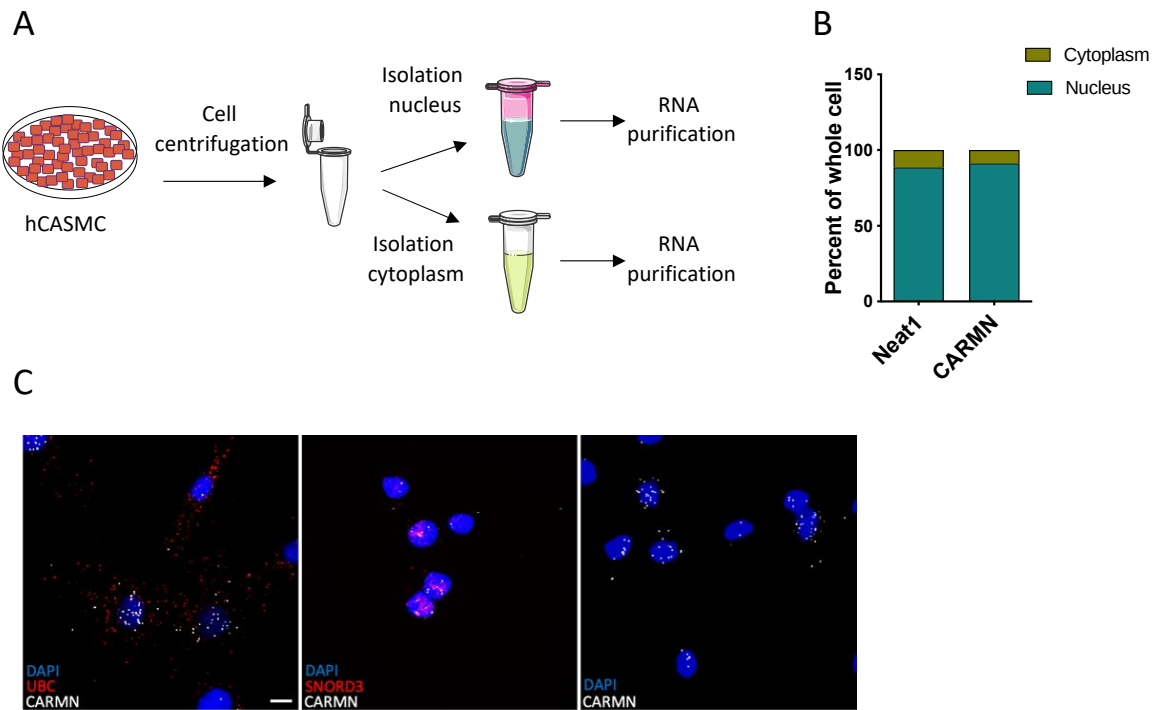


Figure 3.21 Subcellular fractionation and Fluorescent In-Situ Hybridisation in hCASCs.

hCASCs were seeded at basal conditions and used to perform subcellular fractionation experiment. A) Diagram representing the main steps of subcellular fractionation of hCASCs where the nuclear component was separated from the cytoplasm. Both fractions were then used for RNA extraction for gene expression analysis. B) qRT-PCR data (n=1 independent replicate) expressed as percentage of whole cell, of *CARMN* and the long noncoding RNA *NEAT1* in the nuclear or cytoplasmic fraction isolated from hCASCs. *Neat1* was taken as positive control of the nucleic fraction. C) Fluorescent In-Situ Hybridisation performed in hCASCs at basal conditions (n=1 independent experiment, cell passage 6). Probes targeting *CARMN* (white), *SNORD3* (red) and *UBC* (red) were used to identify each transcript within the nucleic and cytoplasmic compartment. Scale bar 100um.

Considering that *CARMN* is a nuclear lncRNA, the GapmeR approach was selected as potential strategy to achieve effective knock-down of all the transcripts. GapmeR sequences were primarily designed to widely target and affect the expression of all

the isoforms belonging to the locus. GapmeR have generally been designed to target exonic sequences of RNA target molecules to ensure knock-down effectiveness. However, recently intron-targeting GapmeR have been described as more efficient targeting the intron sequences of their target as these are more susceptible to RNase-H mediated cleavage³⁰⁵.

Considering that the complex structure of the *CARMN* transcripts and the lack of a shared exonic region, an intronic sequence common to all the isoforms was selected for the design of two GapmeRs (named throughout the study, Gap*CARMN* and Gap*CARMN2*) (Figure 3.22)

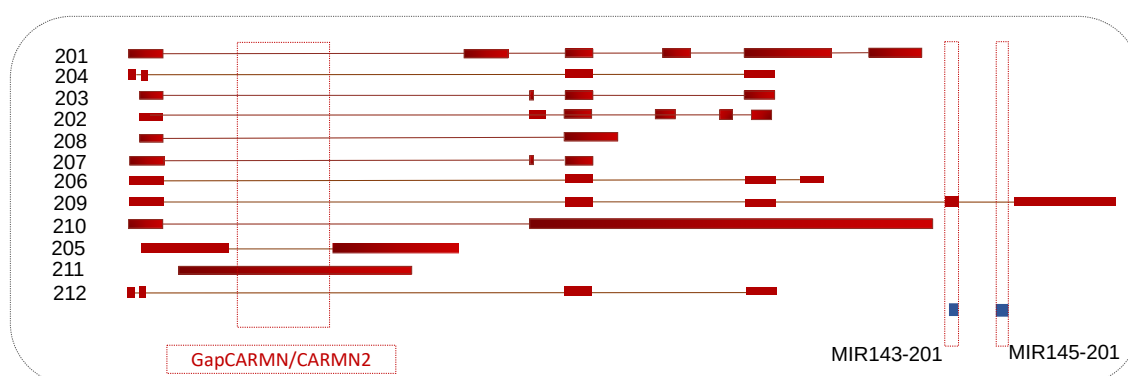


Figure 3.22 GapmeR design strategy to target *CARMN* isoforms.

Schematic representation of target region selected to design GapmeRs to downregulate *CARMN* isoforms, Gap*CARMN1* and Gap*CARMN2*. The intronic sequence or most isoforms, represented by red line, was used as target sequence. The red box indicates the region targeted by both GapmeRs.

Having chosen the GapmeR target region, GapmeR sequences were designed using the Exiqon (Qiagen) designing tool which considers different parameters to reduce target-off effect and ensure RNA strand specific knock-down. Two GapmeRs with the highest score in the ranking were selected and their sequences were then blasted

against the human sequence to assess target specificity of the design, followed by the optimisation the transfection conditions to have efficient knock-down of *CARMN* transcripts. To this end, fluorescently labelled GapmeR was transfected to hCASCs and transfection efficiency was assessed at 24-48-72 hours post-transfection, this revealed increased signal intensity and transfection effectiveness compared to untransfected control (Figure 3.23).

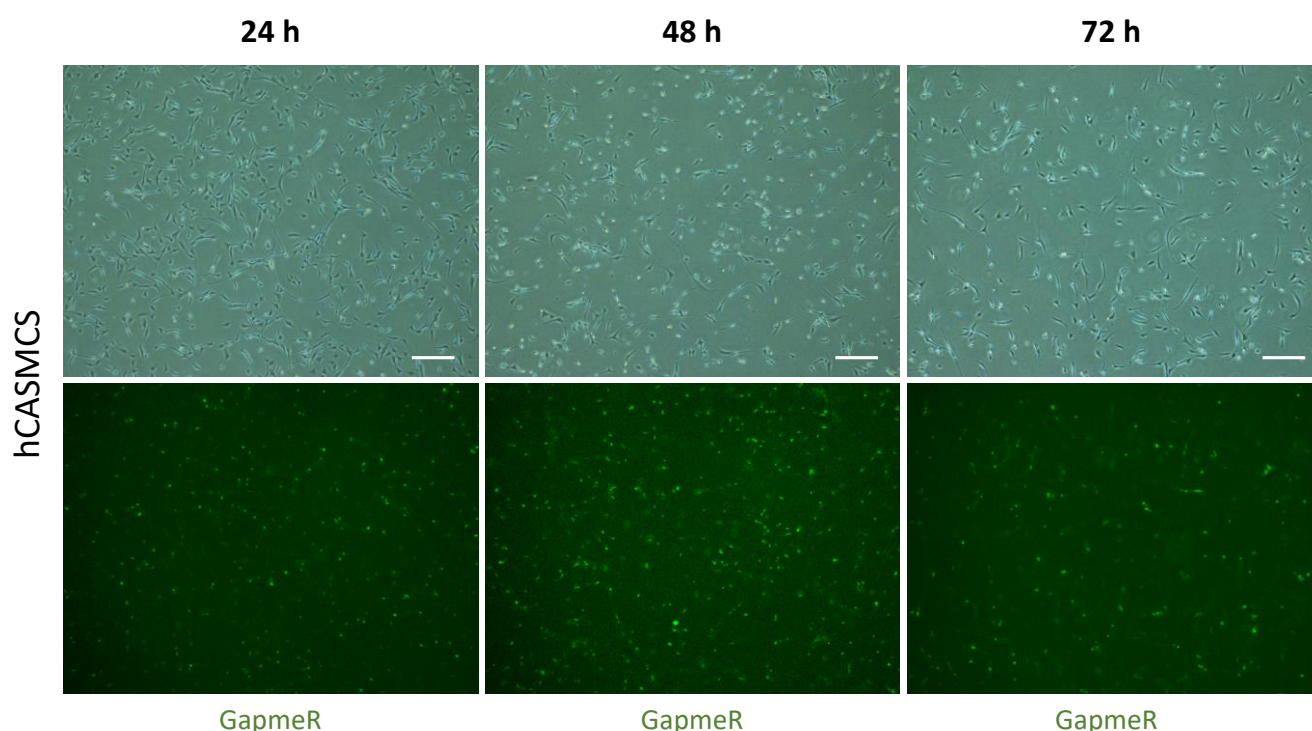


Figure 3.23 Assessing transfection efficiency of GapmeR reagents in hCASCs.

hCASCs (n=1 independent experiment, passage used 6) were plated and transfected with labelled GapmeR using RNAiMAX lipofectamine reagent. The pictures display representative micrographs of hCASCs at basal conditions transfected for 24, 48 and 72 hours with fluorescent GapmeR. Pictures acquired with 400X magnification, scale bar 200µm.

To assess the knock-down efficiency of *CARMN*, hCASCs transfected with Gap*CARMN* and Gap*CARMN2* were harvested and the expression of *CARMN* was

assessed at 48 hours following transfection (Figure 3.24). GapmeR control (GapCTR), with a scrambled sequence not targeting genomic sequences, was used as negative control. The sequence of this GapmeR was also blasted to the human genome to validate the non-specific targeting of human sequences.

Following GapmeR knockdown, *CARMN* expression was reduced 6.73 ± 0.01 and 3.67 ± 0.09 fold with Gap*CARMN* and Gap*CARMN2*, respectively (Figure 3.24). Importantly, GapmeR control did not affect the expression of *CARMN* indicating the absence of significant transfection efficiency effect compared to Mock control, which included cells treated with transfection medium without any GapmeR reagents.

Considering the more pronounced levels of *CARMN* knock-down obtained with Gap*CARMN* versus Gap*CARMN2* and considering it was the highest in the ranking of designed GapmeRs, Gap*CARMN* was used as primary GapmeR reagent throughout the study and Gap*CARMN2* as secondary GapmeR to confirm the observed phenotypes.

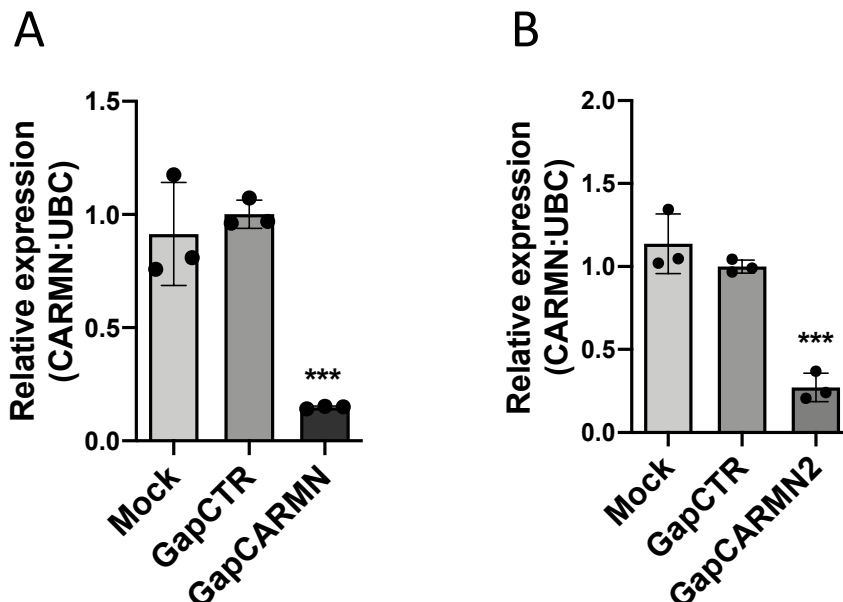


Figure 3.24 *CARMN* expression following transfection with Gap*CARMN* and Gap*CARMN2*.

hCASCs were plated and transfected with 40nM of Gap*CARMN1* or Gap*CARMN2* in 0.2% BSA. Cells were then harvested for gene expression analysis. A), B) Expression of *CARMN* obtained by qRT-PCR in hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) following 48 hours of transfection with Gap*CARMN* and Gap*CARMN2* versus GapCTR. Mock indicates un-transfected control cells. UBC was used as housekeeping gene. One-way-Anova was used to assess ***P< 0.001. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Furthermore, the expression of individual *CARMN* transcripts was checked using the isoform-specific primer sets (Figure 3.25) and a significant downregulation of all of them following Gap*CARMN* treatment versus GapCTR was observed. Importantly, these data confirmed the effectiveness of the intron-targeting strategy in efficiently deplete *CARMN* transcripts.

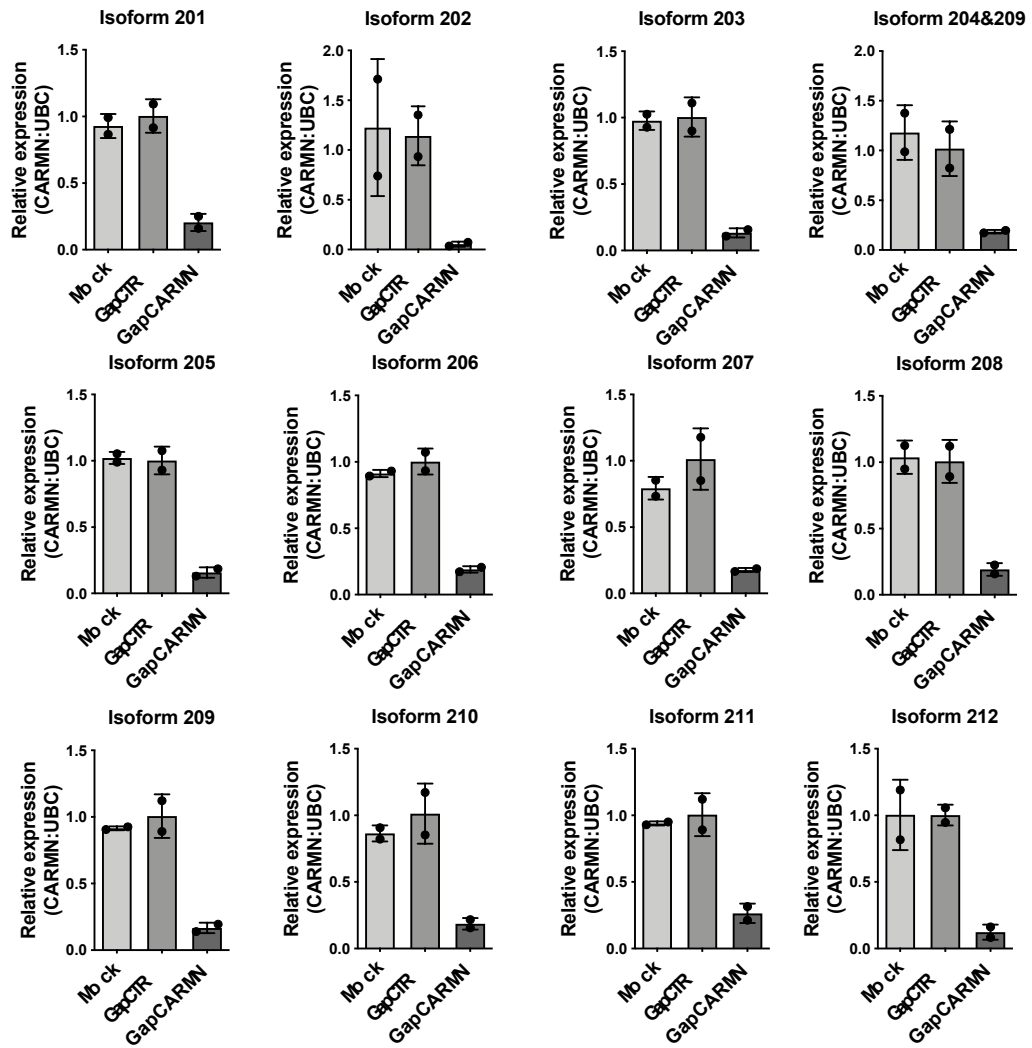


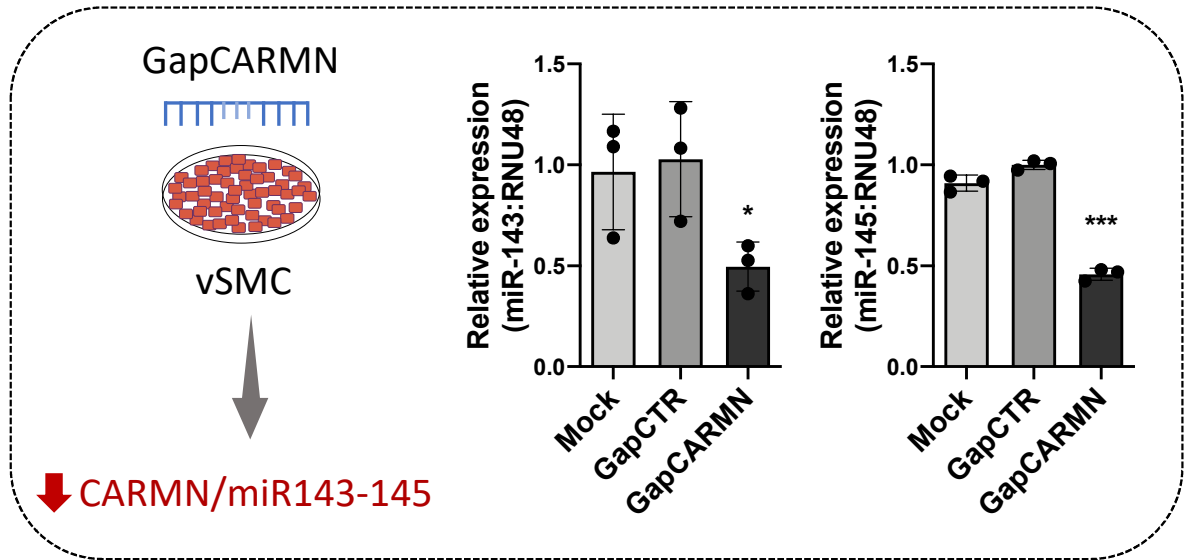
Figure 3.25 Isoform-specific expression of *CARMN* transcripts following Gap*CARMN*-mediated depletion.

Expression of isoform 20-1,2,3,4&9,5,6,7,8,9,210,211,212 obtained with transcript-specific primer sets in hCAsMCs (n=2 independent experiments each with technical triplicates, passage used 5 and 6) following Gap*CARMN* transfection versus GapCTR. UBC was used as housekeeping gene. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Given the genomic location of the hosted microRNA cluster in the *CARMN* locus and considering that there is no clear evidence of the molecular interaction between the

two noncoding molecules, it was fundamental to assess the expression of miR-143 and miR-145 in the same samples (Figure 3.26). Interestingly, the GapmeR-mediated *CARMN* knock-down reduced miR-143 and miR-145 expression by 2 ± 0.12 and 2.18 ± 0.03 following Gap*CARMN* transfection and 1.73 ± 0.09 and 4 ± 0.09 after transfection with Gap*CARMN2*, respectively (Figure 3.26). These results suggest that miR-143/145 cluster might be transcribed from the same promoter as *CARMN* with the primary microRNA transcript being affected by GapmeR-mediated *CARMN* knock-down. At the same time, lncRNAs host genes have been described to regulate microRNAs in proximity through cis or trans effects. To determine whether *CARMN* knock-down is the first event causing the depletion of microRNA expression or if the miR-143/145 downregulation is a consequence of the depletion of their primary transcript by GapmeRs would require more study.

A



B

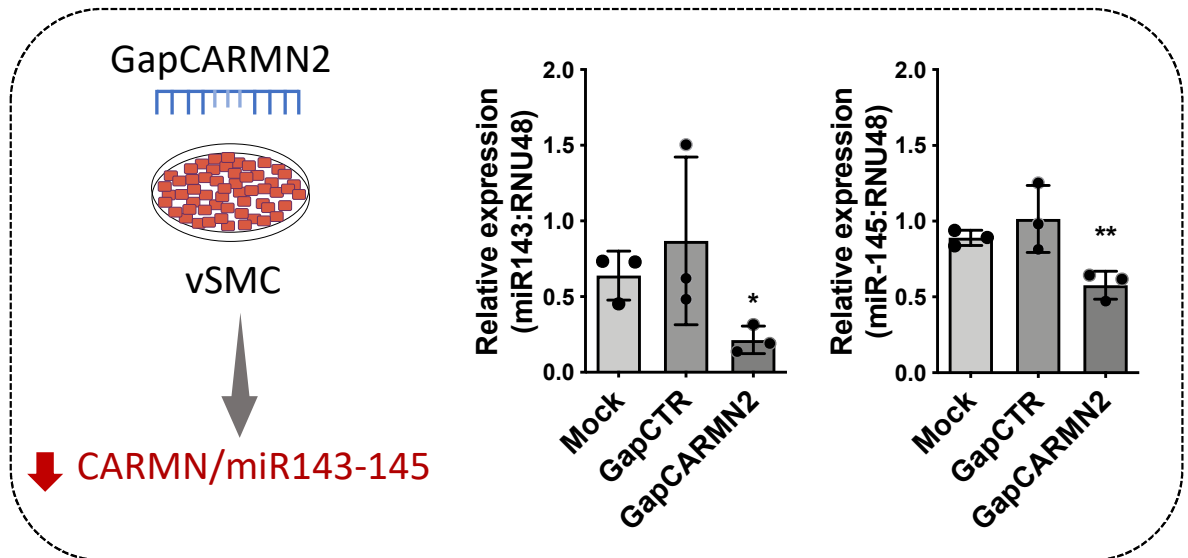


Figure 3.26 Expression of microRNA cluster following GapmeR-mediated *CARMN* knock-down.

A), B) Expression levels of miR-143 and miR-145 (n=3 independent experiments each with technical triplicates, passage used 5 and 6) following 48 hours treatment of hCASCs with GapCARMN and GapCARMN2, respectively, versus GapCTR. RNU48 was used as housekeeper control gene. One-way Anova was used to assess *P< 0.05, **P< 0.01, ***P< 0.001. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

To assess if *CARMN* axis, composed by the microRNAs and lncRNA transcripts, was downregulated in a similar way by exon-targeting strategy, the expression of the axis was evaluated following transfection with a third Gapmer, Gap*CARMN3*, previously published by Pedrazzini's laboratory²⁸⁶ (Figure 3.27). Gap*CARMN3* was designed in one of the common exons to most of *CARMN* transcripts (except isoforms 205 and 211). *CARMN*, miR-143 and miR145 expression was downregulated by 4.9 ± 0.16 , 5 ± 0.03 and 3.9 ± 0.13 respectively in Gap*CARMN3* versus GapCTR condition, therefore confirming the downregulation observed in miR-143/145 by Pedrazzini's laboratory following transfection with this reagent.

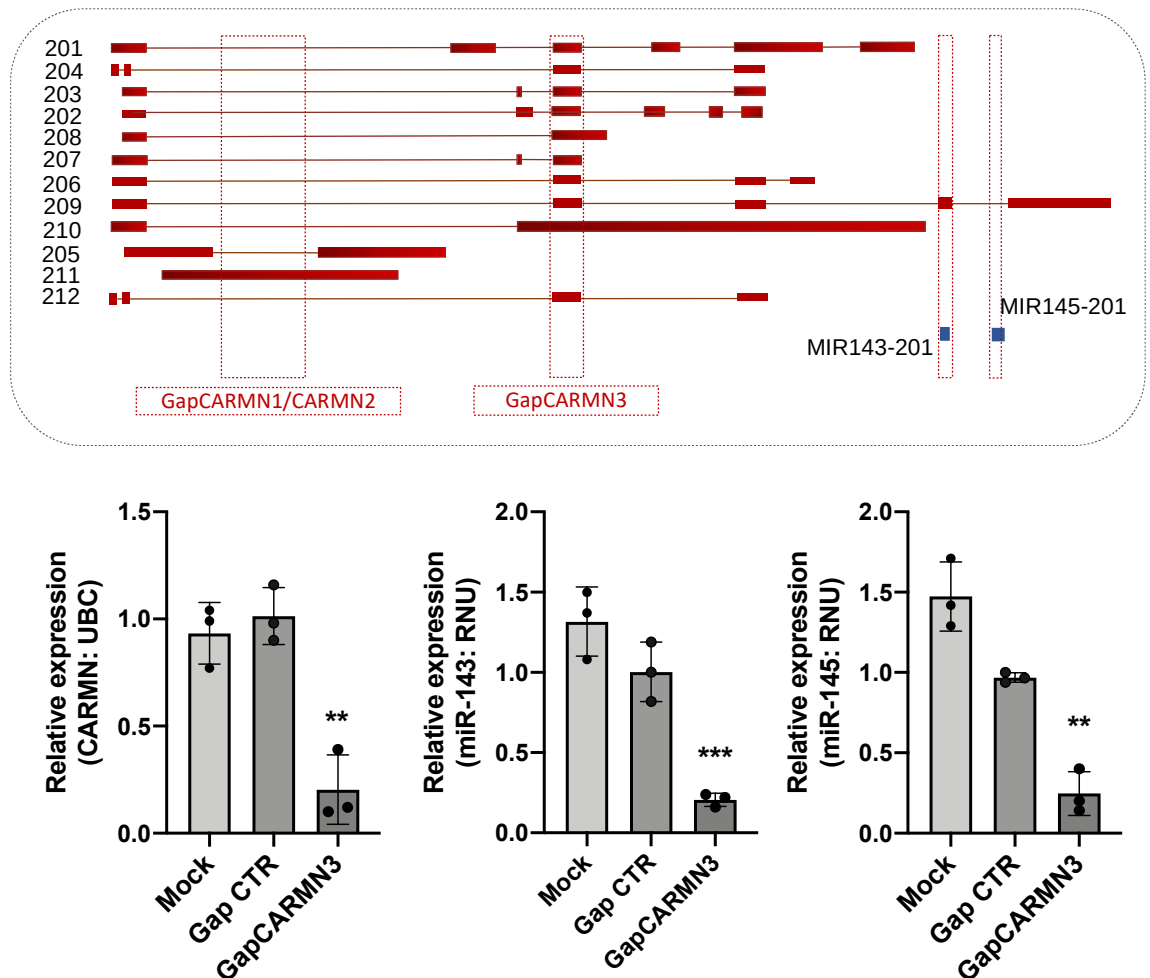


Figure 3.27 Expression of *CARMN* axis in hCASMCs transfected with exon-targeting Gap*CARMN3*.

Schematic representation of Gap*CARMN3* sequence target site in *CARMN* locus (in grey) and Gap*CARMN*/Gap*CARMN2* binding region (in red). Expression of *CARMN* and miR-143/145 following Gap*CARMN3* depletion in hCASMCS (n=3 independent experiments each with technical triplicates, passage used 5 and 6) versus GapCTR. UBC was used as housekeeper control. One-way Anova was used to assess **P< 0.01, ***P< 0.001. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.4 Changes in the Ensembl annotation revealed the presence of other transcriptional variants belonging to *CARMN*.

LncRNA annotations are frequently updated as the strategies to identify their sequences rapidly advance. Of note, during the course of the experiments in 2018, Lagarde J. Uszczyńska-Ratajczak B. et colleagues performing long read RNA sequencing were able to confirm and expand the previous Ensembl annotation for *CARMN*. In particular, they were able to identify 7 new transcriptional isoforms belonging to *CARMN* loci (Figure 3.28). However, since this study aims at the wide characterisation of *CARMN* isoforms, the common primer set previously designed were used in the following experiments as still an efficient strategy to assess the expression levels of *CARMN* considering that the new isoforms share the sequence selected for primer design (Figure 3.28).

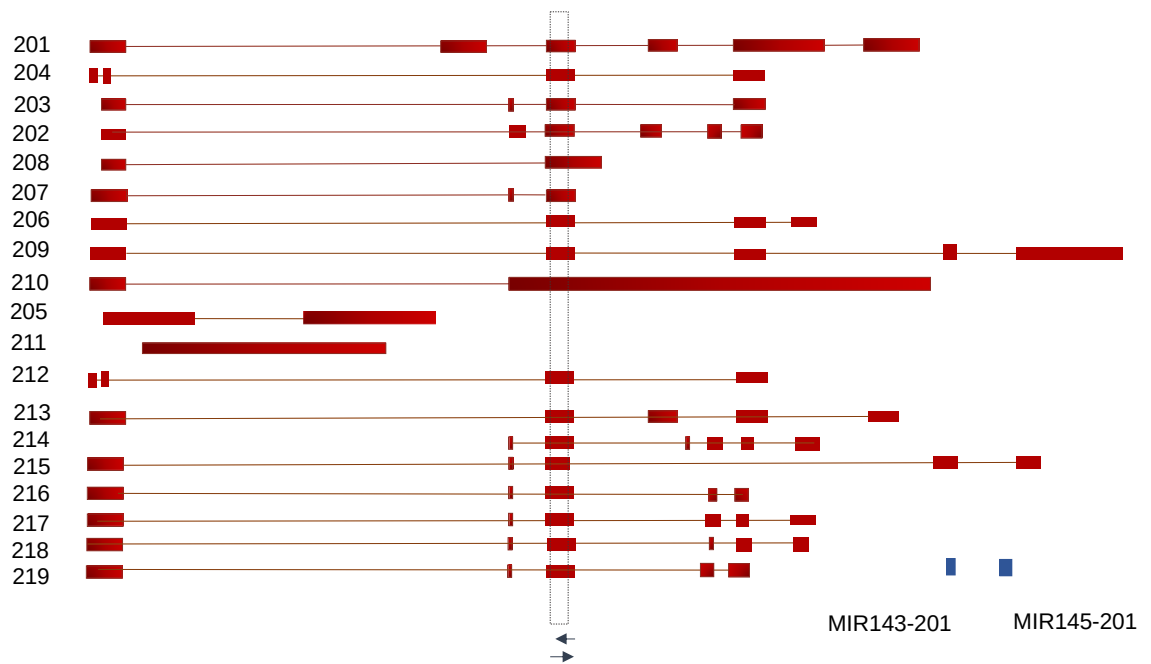


Figure 3.28 Schematic representation of human *CARMN* locus based on the latest release of Ensembl 98.

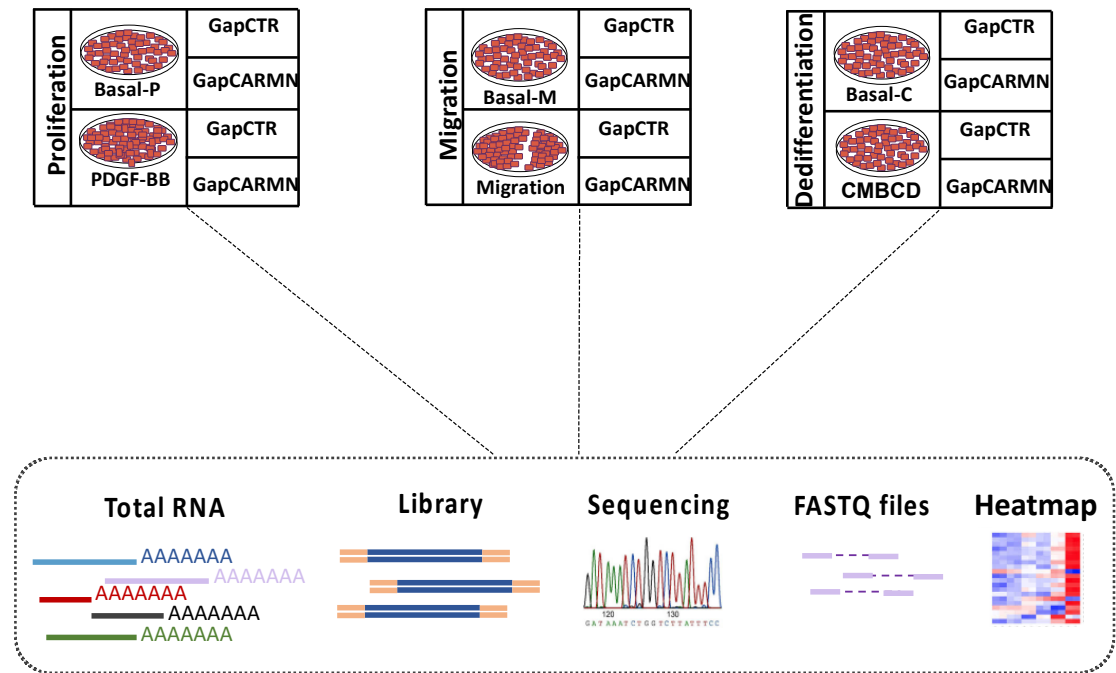
This scheme represents the current annotation which counts a total of 19 transcriptional isoforms for *CARMN* lncRNA (red) and the pri-miR143/145 (dark blue). The grey box and arrows indicate the region amplified by common primer set used in this study to evaluate *CARMN* expression.

3.3.5 The knock-down of *CARMN* axis affects vSMCs function.

Following the quality control steps performed to assess GapmeR-mediated knock-down strategy, the effect of *CARMN* axis downregulation was interrogated in hCASCs. In the past decade, RNA Sequencing (RNASeq) technology has improved and has been perfected to be considered one of the best tools to investigate differences in gene expression patterns in different contexts. The RNA-Sequencing analysis was performed under the direct supervision and guidance of a senior scientist, in the group. Therefore, RNASeq was used to explore the molecular consequences of *CARMN* depletion in hCASCs in basal conditions. Moreover, considering that the pro-pathogenic phenotypes previously tested, including cell

proliferation and foam-cell formation, affected the expression of *CARMN* axis, hCASMCs induced to proliferate, migrate and de-differentiate to foam-cell like phenotype were included in the experimental design. The experimental conditions are illustrated in detail in Figure 3.29. Importantly, considering that each protocol (proliferation, migration, de-differentiation) varies for number of plated cells, days of treatment following transfection and condition medium, treatment-specific basal conditions were included in the experimental design as specified in Figure 3.29.

A



B

Condition	GapmeR targeting CARMN	GapmeR control	Days treatments following transfection	Density of cells plated
Basal P	0.2% FBS Untreated	0.2% FBS Untreated	Proliferation- 5	Proliferation- 1x10 ⁵
Basal M	0.2% FBS	0.2% FBS	Migration- 3	Migration- 1.8x10 ⁵
Basal C	Unscratched	Unscratched	CMBCD - 4	CMBCD- 2x10 ⁵
PDGF-BB	0.2% BSA Untreated	0.2% BSA		
PDGF-BB	PDGF-BB 20ng/ml in 0.2%FBS	PDGF-BB 20ng/ml in 0.2%FBS		
Migration	Scratch in 0.2% FBS	Scratch in 0.2% FBS		
Migration	CMBCD 10µgml in 0.2% FBS	CMBCD 10µgml in 0.2% FBS		
CMBCD	0.2% BSA	0.2% BSA		

Figure 3.29 RNA-Sequencing experimental design on *CARMN*-depleted hCASCs.

A) Schematic representation of the experimental conditions included in the RNASeq performed in hCASCs following transfection with GapCARMN and GapCTR in basal (0.2% FBS or 0.2% BSA) and stimulated conditions with PDGF-BB, scratch or CMBCD. n=1 independent experiment performed with technical triplicates; cell passage used

4. B) Details of the experimental conditions included with specified differences in condition medium, number of cells plated and days following *CARMN* axis depletion.

RNA-Seq quality control was performed on the samples before to be sequenced and included the assessment of the expression of *CARMN* across the different conditions to ensure the effective knock-down (Figure 3.30). *CARMN* expression was found decreased > 7 fold in each condition.

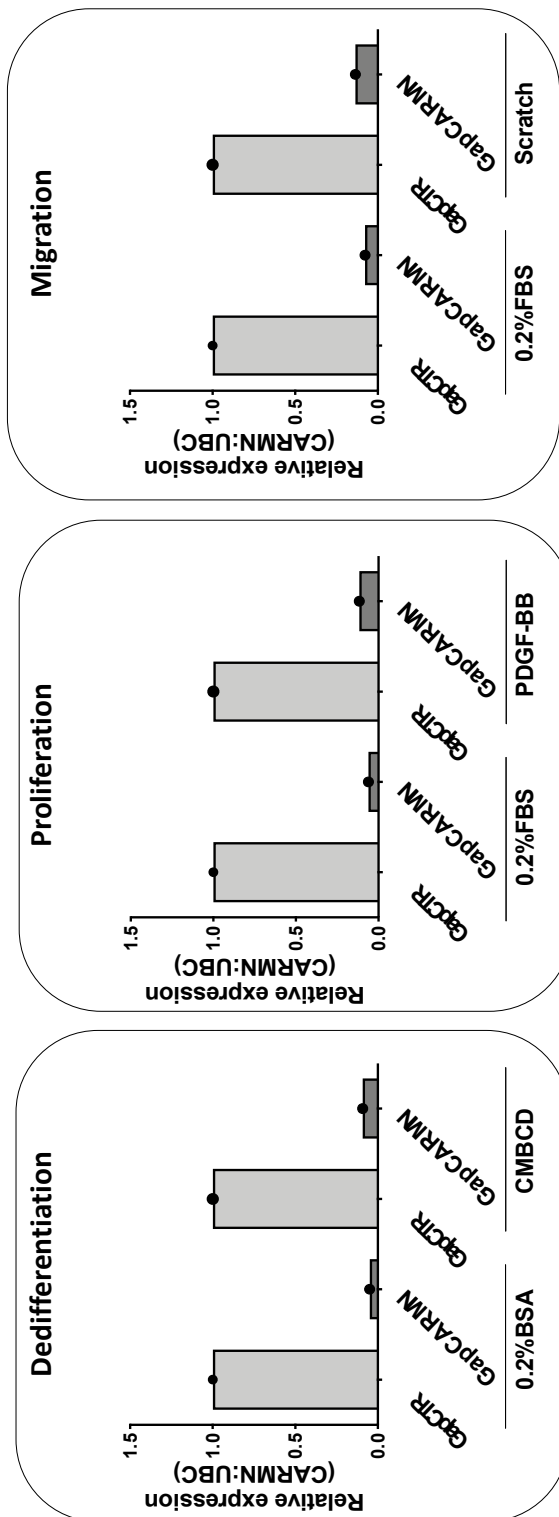


Figure 3.30 CARMN expression in samples subjected to RNA-Sequencing experiment.

qRT-PCR data relative to the expression of CARMN relative to UBC gene. CARMN expression was assessed in proliferating (PDGF-BB treated), migrating (scratched) and de-differentiating (CMBCD treated) hCASCs and all the relative basal conditions 0.2% FBS (control for proliferation), 0.2% FBS (control for migration) and 0.2% BSA (control for CMBCD treatment). n=1 independent experiment performed with technical triplicates; cell passage used 4.

Figure 3.30 CARMN expression in samples subjected to RNA-Sequencing experiment.

Prior to sequencing all samples were subjected to RNA quality control, using Agilent Bioanalyzer (Agilent RNA 6000 Nano kit) which provides information on RNA purity, quantity, size and integrity. The principle of this assay is based on the use of a gel electrophoresis technique performed on an automated chip for the analysis of DNA, RNA and proteins. The experiment includes the use of a chip with microchannels filled with the gel polymer and fluorescent dye. Each microchannel is provided with electrodes to allow the migration of molecules towards the opposite charge, similarly to gel electrophoresis. The gel loading includes the use of marker containing fragments of a predefined size range by which it is possible to compare the size of each sample which is then automatically calculated by their migration time. The readout obtained from the machine is a migration scheme where it is possible to check for the quality of the samples: intact total eucaryotic RNA is characterised by defined bands for 18S and 28S rRNAs while the presence of smears in the gel indicates RNA degradation (Figure 3.31). The ratio between 18S and 28S should approximately be 1.8 to 2. Moreover, the machine provides the electropherogram indicating RNA Integrity Number (RIN) and concentration in ng/ μ l. RIN above 8 are generally considered acceptable for RNA-Sequencing analysis. Samples with RIN > 9 to 10 were used for RNA-Seq analysis.

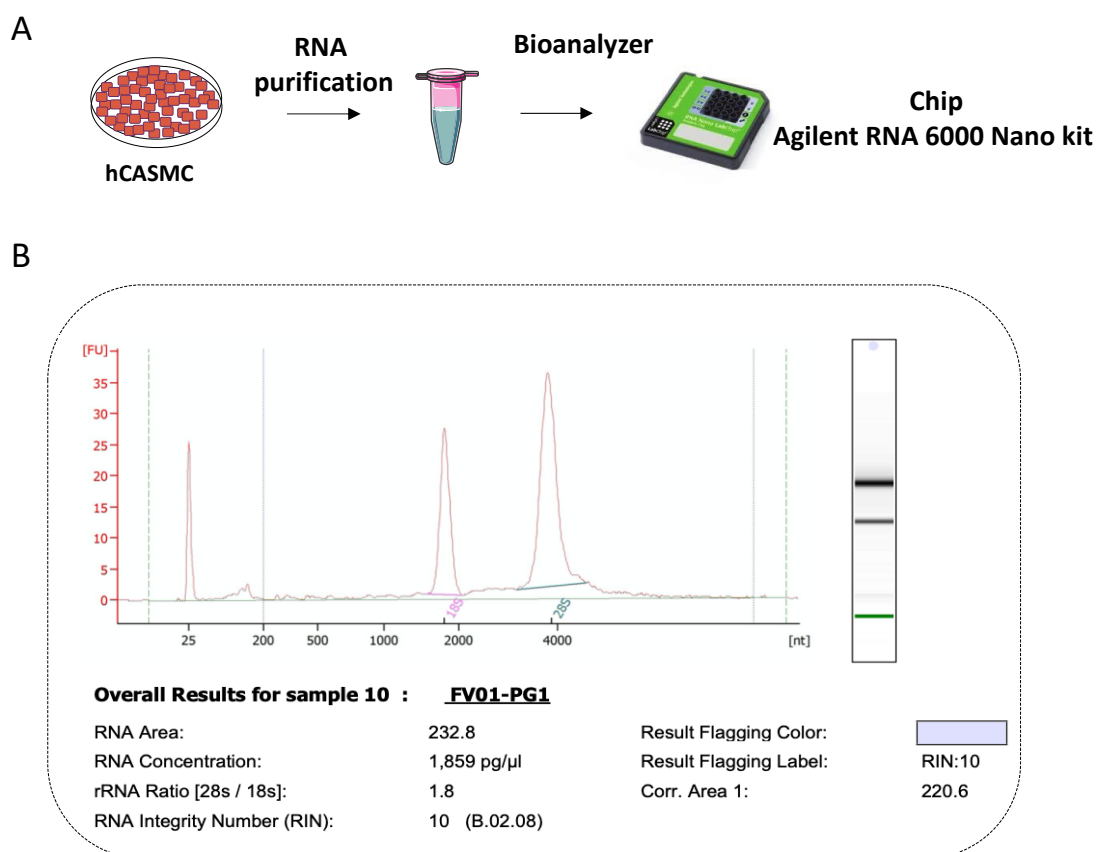


Figure 3.31 Bioanalyzer of RNA-Sequencing samples.

A) Diagram summarizing the main steps of bioanalyzer assay performed in samples prior to RNA-Seq analysis. B) Example of electropherogram for one example sample indicating RNA Integrity Number (RIN), concentration in ng/μl and 18S/28S ratio.

CARMN expression was evaluated in the different conditions included in the RNA Seq and FPKM are represented in Figure 3.32. *CARMN* resulted to be downregulated from an average FPKM 2.01 (hCASCs treated with GapmeR control) to average FPKM 0.11 (hCASCs treated with Gap*CARMN*), thus confirming the efficiency of knock-down.

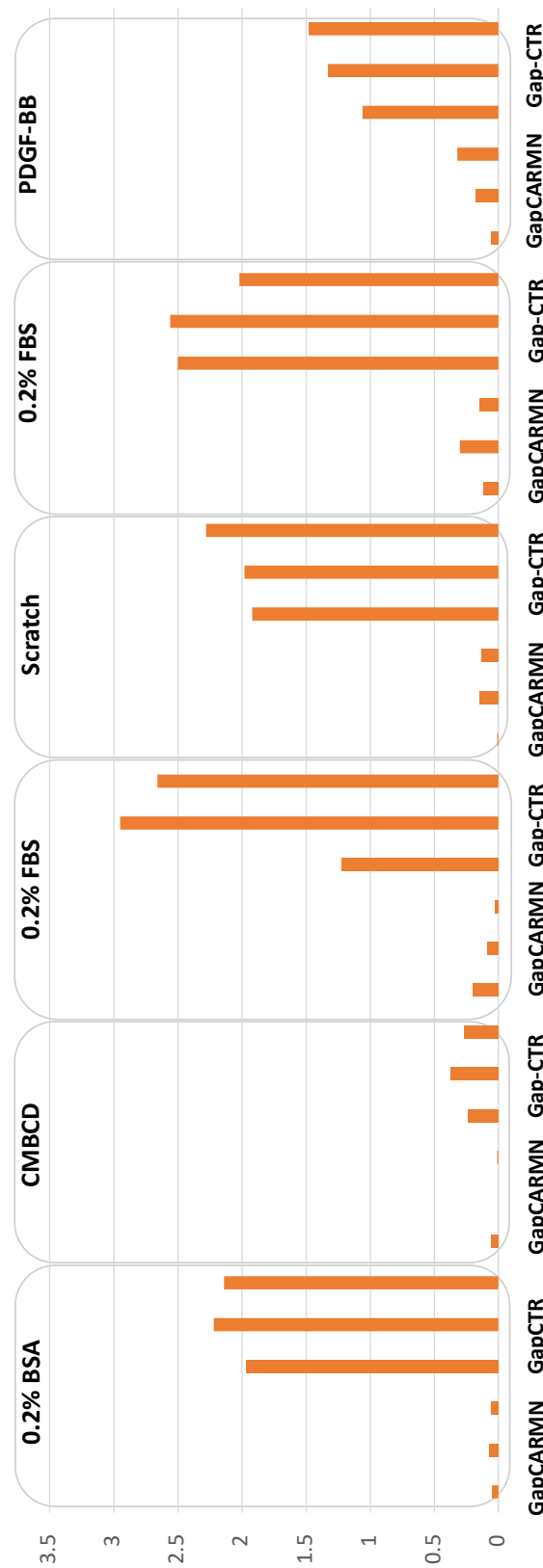


Figure 3.32 CARMN expression as FPKM in RNA-Sequencing samples.
 FPKM relative to CARMN expression assessed in each treatment (CMBCD loading, migration and proliferation) and in the relative basal conditions (0.2%BSA, 0.2%FBS, 0.2%FBS). Triplicates were considered per each condition.

Figure 3. 32 CARMN expression as FPKM in RNA-Sequencing samples.

Principal Component Analysis (PCA) was performed to determine the variance among the replicates of the same condition and among different conditions. PCA confirmed the close clustering of the replicates per condition (which indicates the consistency in the results of each replicate) and showed a clear separation of the GapCARMN-treated samples from the GapCTR-treated hCASCs in all analysed conditions, thus confirming the efficiency of knockdown treatment versus controls (Figure 3.33).

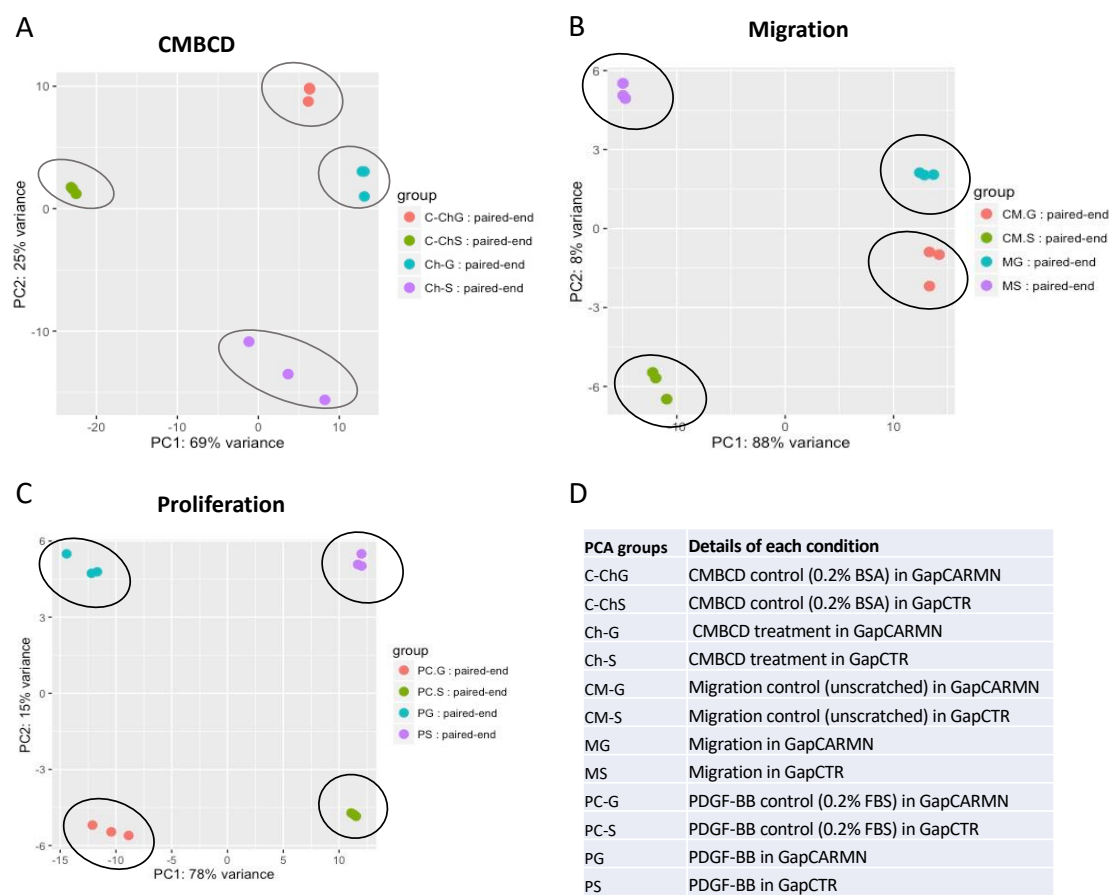


Figure 3.33 Principal Component Analysis for CMBCD, scratch and PDGF-BB treated and non-treated (0.2%BSA, un-scratched in 0.2%FBS, 0.2% FBS) samples.

A) PCA plots (obtained DESeq2 function) expressing the variance among replicates per each condition, highlighted with black circles. B) Table indicating the complete

name of each group indicated in the PCA plots. n=1 independent experiment performed with technical triplicates; cell passage used 4.

Differential expression in gene expression was analysed in samples Gap*CARMN*-treated versus GapCTR in each condition. Analysis showed that *CARMN* depletion affected a large number of genes, in average 910 genes per condition with 411 up-regulated and 499 down-regulated genes. In total, 2315 genes were significantly affected in their expression by *CARMN* depletion versus GapCTR (Figure 3.34). These genes were then analysed for their expression profile and a heatmap was generated. Interestingly, the heatmap profile revealed a clear clustering of all Gap*CARMN*-treated versus GapCTR samples, showing a consistent effect following *CARMN* depletion independently of hCASC treatment. Intriguingly, the genes affected by CMBCD treatment in GapCTR condition (Figure 3.4, red box) clustered among the Gap*CARMN*-dependent dysregulated genes (Figure 3.34, purple box). These results indicate that some of the transcriptional changes observed in CMBCD-treated cells could be induced by *CARMN* knock-down in absence of treatment, which might suggest that the manipulation of *CARMN* expression might itself be enough to prime SMC towards the transdifferentiation towards pro-pathological phenotypes.

A

Significant changes
GapCARMN vs GapCTR
 (abs(LogFC) $\geq$ 1 & padj $\leq$ 0.01 & FPKM $\geq$ 2)

Condition	Total	up	down
Basal-P	469	216	253
PDGF-BB	649	338	311
Basal-M	1103	480	623
Migration	1180	481	699
Basal-C	1322	615	707
Cholesterol	734	336	398

B

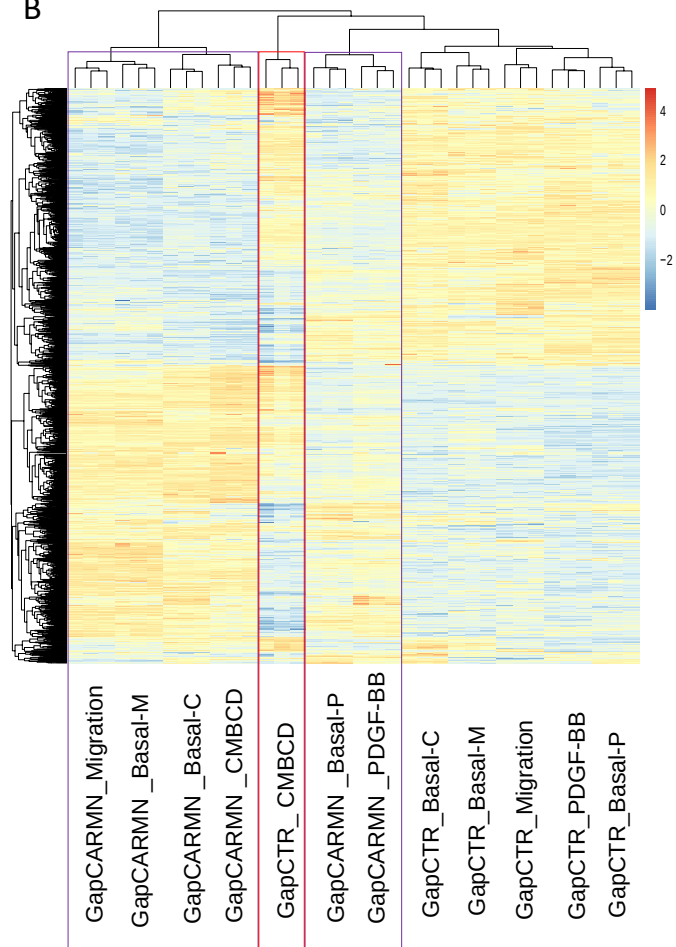


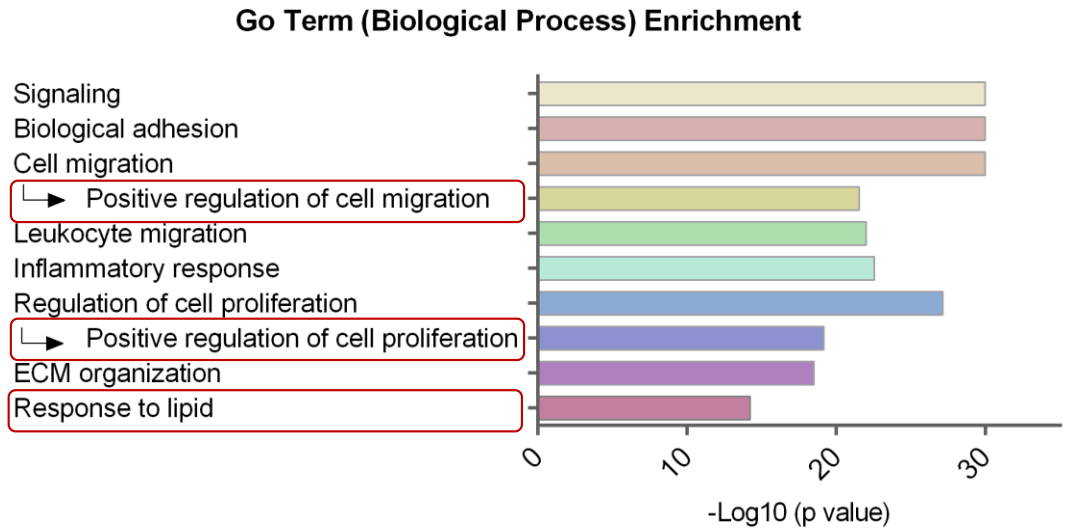
Figure 3.34 Analysis of *CARMN*-depletion dysregulated genes versus GapCTR.

A) Table showing the number of significant changes (abs LogFC) $\geq$ 1 & padj $\leq$ 0.01 & FPKM $\geq$ 2) upon *CARMN* knockdown samples in the 6 independent conditions versus control. B) Heatmap (as z-score of log₂(FPKM+1)) of differentially regulated genes by *CARMN* depletion in all conditions. In total, 2315 genes were included in the analysis. The purple box highlights the clustering of all *CARMN*_KD samples while the red box shows the CMBCD treated replicates (Cont_KD). n=1 independent experiment performed with technical triplicates; cell passage used 4.

To understand the pathways related to the dysregulated genes in Gap*CARMN* versus GapCTR, Gene Ontology (GoTerm) analysis was performed considering the upregulated and downregulated genes. Interestingly, GoTerm analysis of the *CARMN*-dependent dysregulated genes in basal conditions already revealed the

enrichment of several processes related to the SMC phenotypic switch (Figure 3.35). In particular, the analysis highlighted an enrichment of terms linked to a positive effect on cell migration and proliferation (Figure 3.35, red boxes). Genes affected by *CARMN* depletion were also involved in lipid metabolism and cholesterol homeostasis. Moreover, *CARMN* knock-down upregulated the expression of pro-inflammatory cytokines, such as interleukins²⁵ (IL1-beta, IL-6), associated with atherosclerosis progression and CXCL and CCL chemokines (Figure 3.35), responsible for leucocyte migration and recruitment to the developing lesions⁶³. Furthermore, we noted a significant upregulation of metalloproteases (MMP1) and a significant downregulation of collagen and elastase genes (COL3A1 and ELN) (Figure 3.35) whose expression decreases in the context of extracellular matrix remodelling and plaque vulnerability³⁰⁶.

A



B

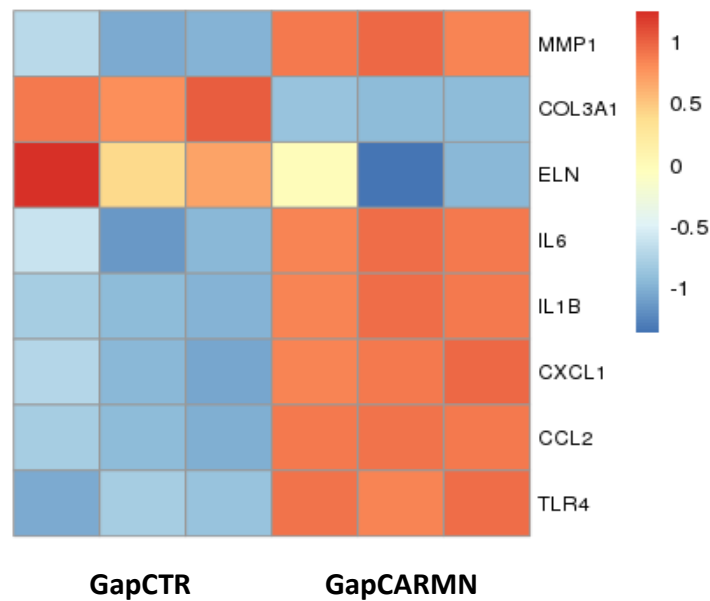


Figure 3.35 Analysis of dysregulated cellular processes and genes following GapCARMN transfection in hCASCs versus GapCTR in basal conditions.

A) Histogram of selected Go Term (Gene Ontology Biological Process). The analysis was performed on the 2315 genes regulated by *CARMN* depletion versus GapCTR in all the conditions. E) Heatmap (as z-score of $\log_2(\text{FPKM}+1)$) of selected differentially regulated genes by GapCARMN and GapCTR in the basal condition with the shortest

time of GapmeR transfection (migration). n=1 independent experiment performed with technical triplicates; cell passage used 4.

3.3.6 *CARMN* depletion affects hCASC proliferation, migration and identity genes in hCASCs *in vitro*.

Considering that *CARMN* depletion affected different genes involved in the regulation of vSMC function, such as positive regulation of cell proliferation, positive regulation of cell migration and cholesterol metabolism, the consequence of *CARMN* knock-down on these pro-atherogenic critical processes were validated in hCASCs.

In particular, hCASCs were induced to de-differentiate to foam-cell through treatment with CMBCD, migrate by performing scratch assays and proliferate with PDGF-BB stimulation. These treatments were performed in both *CARMN*-depleted cells transfected with Gap*CARMN* and cells transfected with GapCTR. Moreover, the same basal conditions used in the RNA-Sequencing experimental design were included in each phenotypical assay: 0.2% BSA in the case of CMBCD, unscratched in 0.2% FBS for migration and 0.2% FBS as control for proliferation.

The proliferation rate of *CARMN*-depleted hCASCs was assessed using EdU incorporation revealed by FACS assay (as previously optimised). Following transfection with Gap*CARMN* or GapCTR, cells were starved for 48 hours in 0.2%FBS medium followed by treatment with 20ng/ml of PDGF-BB. FACS analysis revealed that Gap*CARMN* treated cells shown 2.15 ± 0.8 -fold increase in cell proliferation rate versus control (Figure 3.36). Moreover, under PDGF-BB stimuli, *CARMN* knock-down increased 9.7 ± 3.4 fold proliferation versus GapCTR (Figure 3.36).

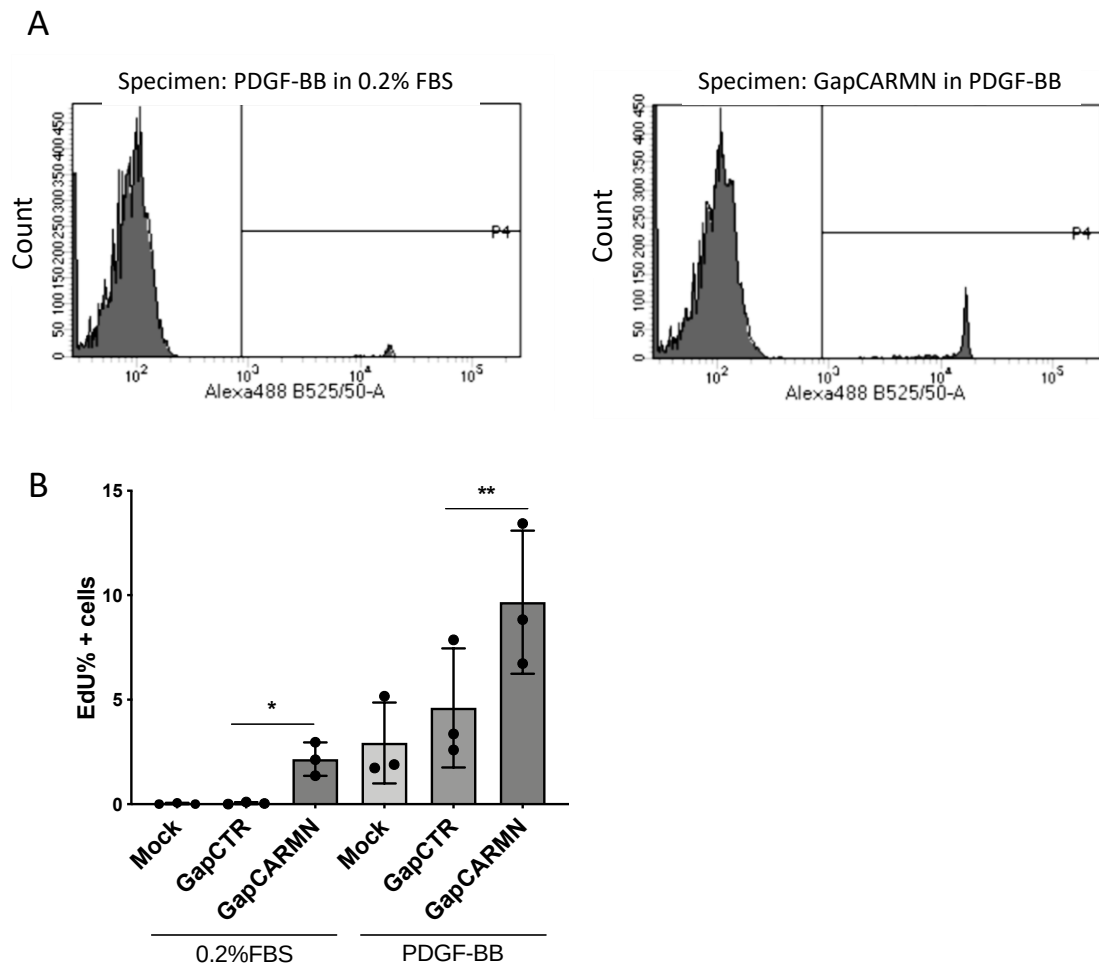


Figure 3.36 Assessment of hCASCs proliferation rate following GapCARMN treatment versus GapCTR in PDGF-BB treated and untreated (0.2% FBS) cells.

A) FACS plots showing the number of positive cells to Alexa488 staining detecting EdU incorporation in PDGF-BB treated control cells and cells depleted for *CARMN* in the presence of PDGF-BB stimuli. B) Graph representing the percentage of EdU positive hCASCs (n=3 independent experiments each with technical triplicates, passage used 4 and 5) in different conditions obtained using FlowJo data analysis software. One-way Anova was used to assess *P< 0.05 and **P< 0.01. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

The modulation of genes linked to the positive regulation of cell migration was validated by phenotypic assay. In this case, hCASCs transfected with GapCARMN

and GapCTR were quiesced and then induced to migrate by wound healing assay. Cells were harvested at 10 hours post induction of the scratch, as the most usable time-point to quantitate cell migration distances (using migration tool in ImageJ software) before the closure of the gap. In particular, pictures were acquired at 0- and 10- hours post scratch and relative migration distances were quantified using ImageJ (Figure 3.37). Interestingly, analysis of the results revealed an increase of 1.87 ± 0.20 fold in *CARMN*-depleted hCASCs versus cells transfected with GapCTR (Figure 3.37).

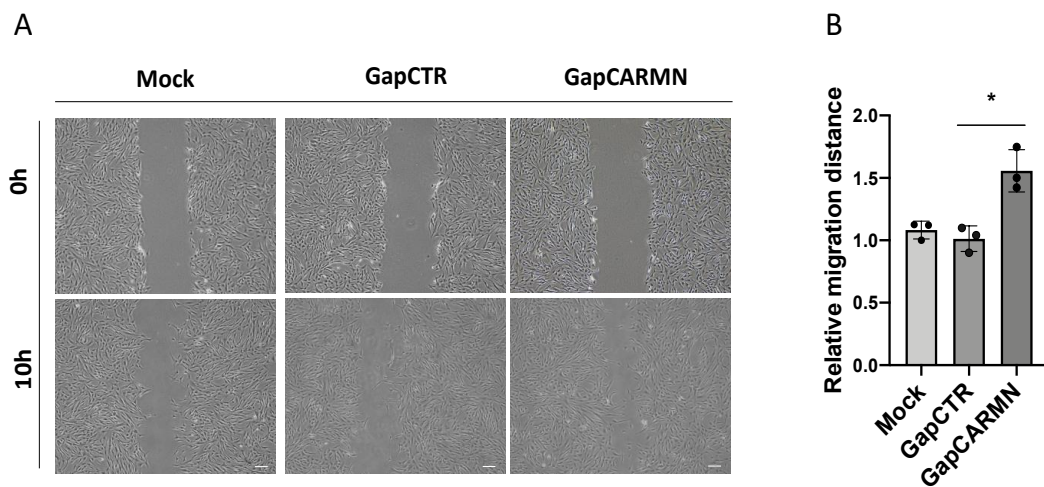


Figure 3.37 Assessment of hCASC migration following *CARMN* depletion versus GapCTR in quiescent hCASCs (0.2% FBS).

hCASCs were plated, starved and transfected with GapCARMN or GapCTR before to perform cell scratch assay. A) Representative micrographs of hCASCs following 0 or 10 hours from the induction of migration by scratch assay. Pictures were acquired at 100X magnification, scale bar 100µm B) Quantification of hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) migration distances following 10 hours from the scratch relative to 0 hours post scratch. Data were obtained with ImageJ wound healing tool. One-way Anova was used to assess $*P < 0.05$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Finally, the effect of *CARMN* downregulation on SMC identity genes and cholesterol metabolism pathways was investigated in hCASMSC treated with CMBCD. More specifically, cells transfected with Gap*CARMN* and GapCTR were treated for 72 hours with CMBCD in serum free medium (0.2% BSA) to de-differentiate to foamy cell like cells or in control condition (only 0.2% BSA) (Figure 3.38). The expression of typical SMC markers, such as ACTA2 or MYH11, was interrogated together with the levels of macrophage marker genes, CD68 and LGALS3. Interestingly, the expression of ACTA2 and MYH11 was found downregulated of 10 ± 0.07 fold and 13.5 ± 0.06 fold following Gap*CARMN* versus GapCTR in quiescent conditions. In contrast Acta2 was found significantly decreased by 77 ± 0.001 following CMBCD treatment in Gap*CARMN* versus GapCTR (Figure 3.38). A trend of decrease was found in MYH11 expression in Gap*CARMN* versus control post CMBCD stimuli, but the observed change was not significant (Figure 3.38). Furthermore, *CARMN* depletion upregulated CD68 expression, a macrophage marker¹⁰¹, 1.69 ± 0.45 fold versus GapCTR in basal condition and 6.5 ± 1.6 fold following CMBCD stimuli, even though not significantly in the latter condition. Lgals3 expression displayed a trend to be increased but this did not reach statistical significance (1.73 ± 0.65 fold increase in Gap*CARMN* versus GapCTR following CMBCD treatment), while its expression remained unchanged under basal conditions (Figure 3.38).

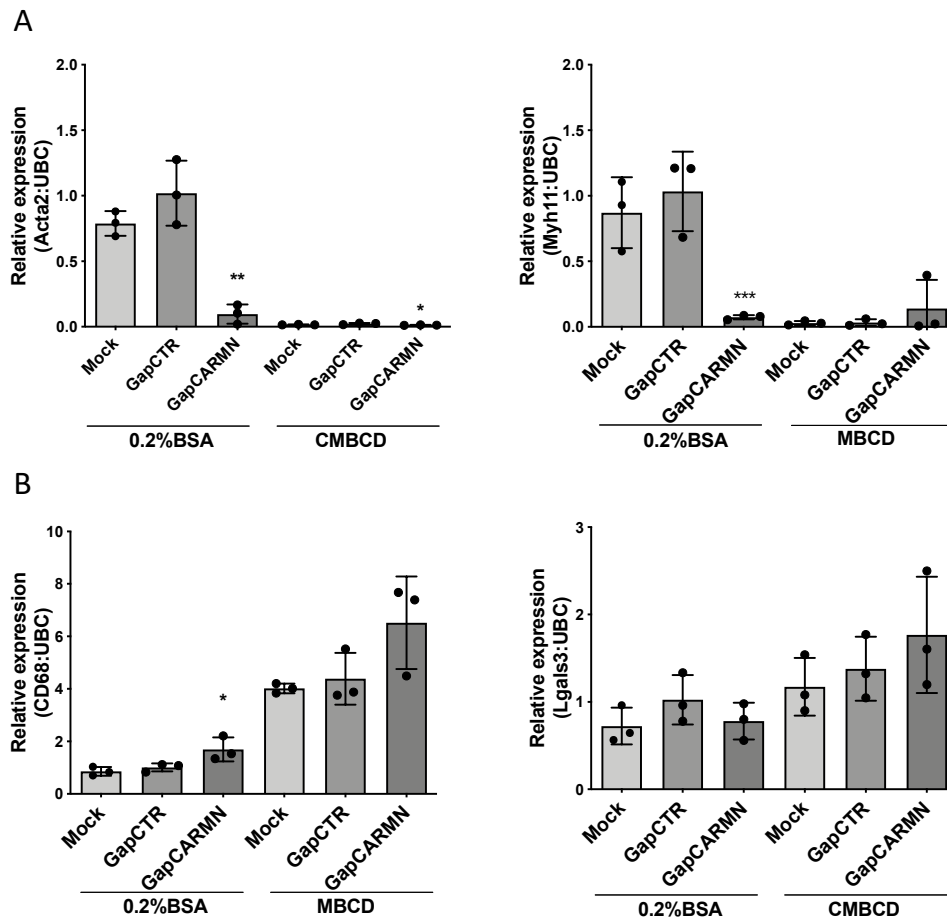


Figure 3.38 Expression of de-differentiation genes in hCASCs depleted for *CARMN* versus GapCTR in treated and untreated hCASCs.

A), B) qRT-PCR results (n=3 independent experiments each with technical triplicates, cell passage used 5 and 6) relative to vSMC gene expression, ACTA2 and MYH11, and levels of macrophage-like genes, CD68 and LGALS3, respectively. CMBCD indicates the treatment with water-soluble cholesterol methyl-beta-cyclodextrin and 0.2% BSA are control samples. UBC was used as housekeeping gene. One-way Anova was used to assess * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.7 Gap*CARMN2* confirms *CARMN*-depletion induced hCASC phenotype.

To determine whether the observed phenotypes are caused by the knock-down of *CARMN* and not result of the targeting of a specific sequence, a second GapmerR (Gap*CARMN2*) was added in the study to validate previous findings by functional assays.

The hCASCs proliferation, migration and change in vSMC identity genes was interrogated following transfection with Gap*CARMN2*.

The proliferation rate of *CARMN*-depleted hCASCs was examined after transfection with Gap*CARMN2*. Considering the wide effect of *CARMN* knock-down observed in basal quiescent conditions, this context was further explored in the presence of Gap*CARMN* versus GapCTR. Therefore, hCASCs transfected with Gap*CARMN2* and GapCTR were induced to quiescence, as previously optimised, and treated with EdU (10 μ M) in 0.2% FBS starvation medium. Importantly, Gap*CARMN2* induced 2.86 $\pm$ 1.31 fold increase in hCASCs EdU incorporation versus GapCTR, thus confirming the alteration of cell proliferation following depletion of the *CARMN* axis (Figure 3.39).

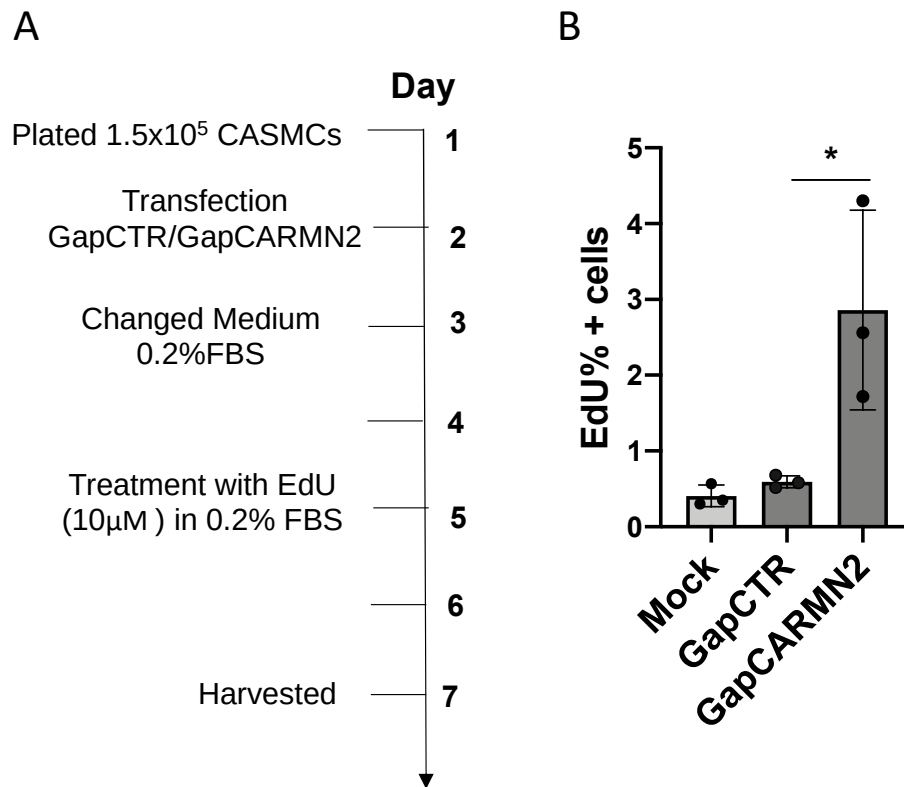


Figure 3.39 *CARMN* depletion GapCARMN2 mediated affects hCASC proliferation in quiescent conditions.

A) Workflow for the assessment of hCASCs proliferation in basal conditions by EdU incorporation revealed through FACS analysis. B) Plot expressing the percentage of EdU positive hCASCs following transfection with GapCARMN2 versus GapCTR. Data obtained by FACS and analysed through FlowJo software. n=3 independent experiments each with technical triplicates, passage used 5 and 6. One-way Anova was used to assess * $P < 0.05$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Furthermore, hCASCs migration capability was interrogated following *CARMN* depletion mediated by GapCARMN2 versus control. In particular, following GapCARMN2 transfection cells were induced to quiescence to be consecutively subjected to scratch assay. Cells were then harvested at 10 hours, same time point used to analyse migration rate following GapCARMN transfection. Also, in this case,

hCASCs relative migration to control was increased of 1.52 ± 0.07 fold compared to GapCTR (Figure 3.40).

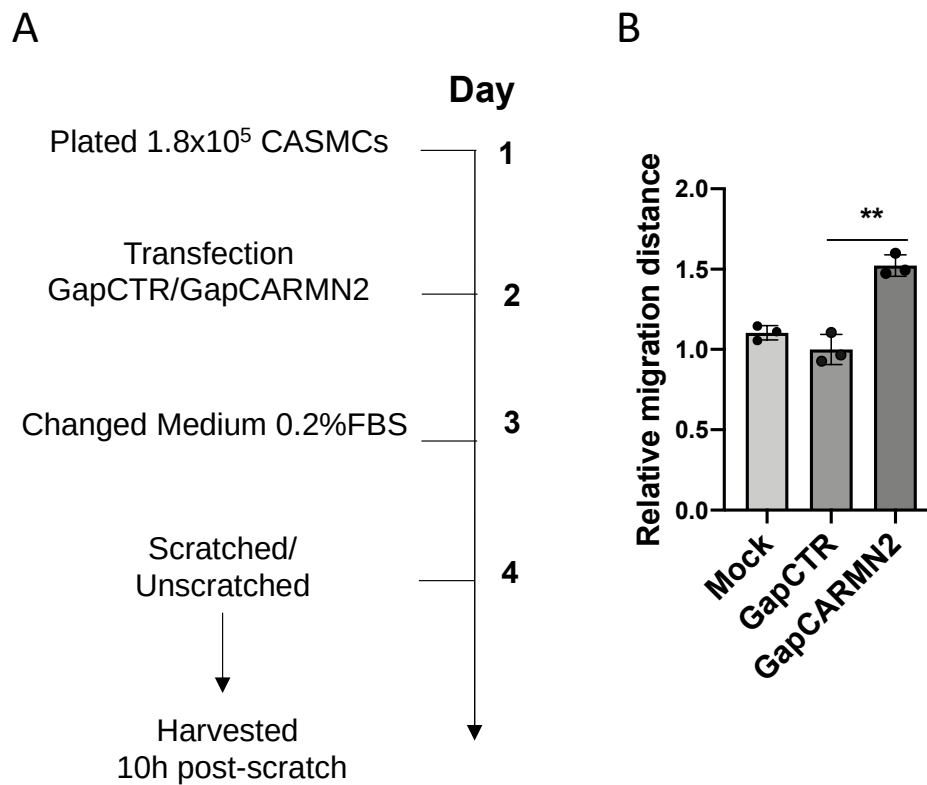


Figure 3.40 hCASC migration is increased following GapCARMN2 CARMN depletion versus control.

A) Workflow of wound healing assay following GapCTR or GapCARMN2 transfection in hCASCs. B) Quantification of hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) migration distances at 10 hours post scratch relative to 0 hours control. Quantifications obtained using ImageJ wound healing tool. One-way Anova was used to assess ** $P < 0.01$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean

Since *CARMN* depletion dysregulates the expression of vSMC identity genes, inducing the expression of macrophage markers, their expression was interrogated in Gap*CARMN2* transfected hCASCs. Cells were transfected with Gap*CARMN2* or GapCTR and induced to de-differentiate by treatment with CMBCD or untreated control (0.2% FBS). Expression of vSMC gene *ACTA2* and macrophage gene *CD68* were analysed. Interestingly, *ACTA2* expression was reduced 3.2 ±0.02 fold in untreated cells (0.2% BSA) compared to control, which confirms previous trend observed after the treatment with Gap*CARMN*. However, *ACTA2* was not significantly reduced following Gap*CARMN2* versus GapCTR in CMBCD treatment. *CD68* expression were moreover interrogated, however no significant change was observed in the levels of this macrophage typical gene following Gap*CARMN2* versus control in both, stimulated and unstimulated hCASCs (Figure 3.41).

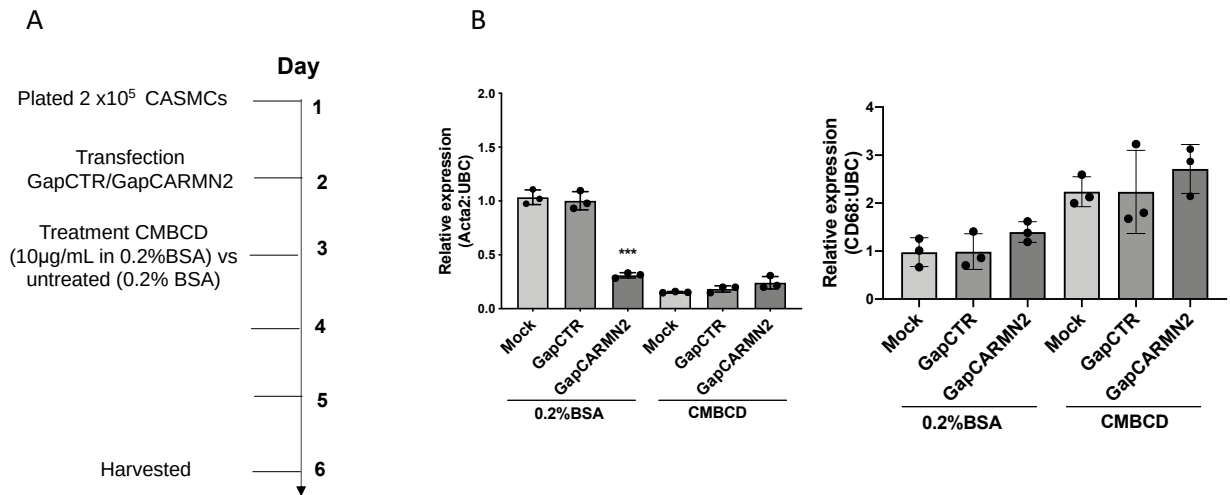


Figure 3.41 Expression of vSMC and macrophage genes in hCASCs following treatment with Gap*CARMN2* versus GapCTR.

A) Workflow for CMBCD-mediated de-differentiation process following transfection with Gap*CARMN2* or GapCTR. B) Expression of *ACTA2* and *CD68* in hCASCs treated with CMBCD or under basal conditions post transfection with Gap*CARMN2* or control. n=3 independent experiments each with technical triplicates, passage used 5 and 6.

UBC was used as reference housekeeping gene. One-way Anova was used to assess *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.8 *CARMN*-depletion regulates hCASCs proliferation independently from microRNAs.

The GapmeR-mediated *CARMN* knock-down strategy gives the advantage of efficiently depleting all the transcriptional variants belonging to *CARMN* locus, at the same time it affects the expression of the hosted microRNAs, miR-143 and miR-145. Hence, the effect of *CARMN* modulation on microRNAs expression does not allow to functionally dissect the lncRNA role in determining hCASC phenotypes from the function played by miR-143/145.

To functionally detangle the role of *CARMN* and gain more insight to the function of the lncRNA per se, a co-transfection strategy was optimised to contemporaneously restore the microRNAs expression in the presence of GapmeR-mediated *CARMN* depletion (Figure 3.42). Double-stranded Mimics reagents were used to exogenously upregulate the expression of both microRNAs.

The first step was to validate the efficiency of this strategy. Effective overexpression of miR-143 and miR-145 in the presence of GapmeR reagents was confirmed by checking the microRNAs levels following co-transfection in hCASCs. Cells were transfected with 5nM of miR-143/145 mimics (mimic143/145) or mimic control (mimicCTR) and co-transfected with 40nM (as previously optimised) of Gap*CARMN* in hCASCs at basal conditions. MiR-143 and miR-145 expression was confirmed downregulated following co-transfection of Gap*CARMN* together with mimic control (Gap + mimicCTR) of 1.8 ± 0.16 and 2.63 ± 0.09 fold respectively (Figure 3.42). This result was similar to the decrease obtained following Gap*CARMN* transfection versus GapCTR, of 2.4 ± 0.2 fold for miR-143 and 3.3 ± 0.06 fold for miR-145, indicating that

mimic control sequence had no effect on the regulation of microRNAs expression. The results show that miR-143/145 expression was increased 134 ± 15.2 and $1,240.3 \pm 34$ fold following co-transfection with GapmeR (Gap + mimic143/145) versus Gap + mimicCTR condition (Figure 3.42).

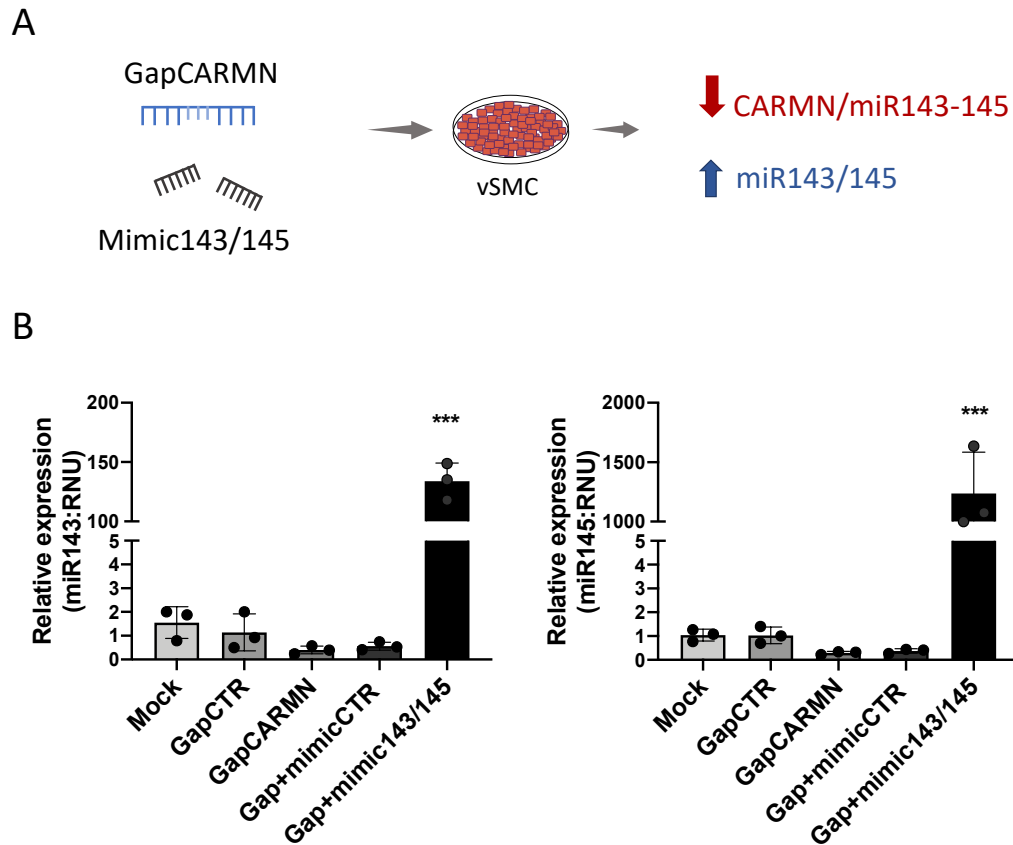


Figure 3.42 Assessment of GapmeR and Mimics co-transfection efficiency in hCASCs.

A) Schematic of co-transfection strategy. 40nM of GapmeR GapCARMN were co-transfected together with 5nM of miR-143/145 (mimic143/145) or mimic control (mimicCTR) in hCASCs in basal conditions. B) qRT-PCR results relative to miR-143 and miR-145 in hCASCs (n=3 independent experiments each with technical triplicates, cell passage used 5 and 6) transfected with GapCARMN and GapCTR and

co-transfected with Gap*CARMN* in combination with mimic control or mimics overexpressing miR-143 and miR-145. RNU48 was used as housekeeping gene. One-way Anova was used to assess *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

To assess whether miR-143/145 overexpression had a functional effect on hCASCs, the expression of two targets for microRNAs was interrogated on the same samples. Platelet-Derived Growth Factor Receptor alpha (PDGFRa) and Epidermal Growth Factor Receptor (EGFR) are two experimentally validated direct target for miR-143/145 and their expression was found significantly downregulated following overexpression of microRNA cluster^{307,279}. Confirming previous findings, the expression of PDGFRa and EGFR was found decreased of 2.2 ± 0.03 and 2.6 ± 0.01 fold following co-transfection of Gap*CARMN* with mimic143/145 versus mimic control (Figure 3.43).

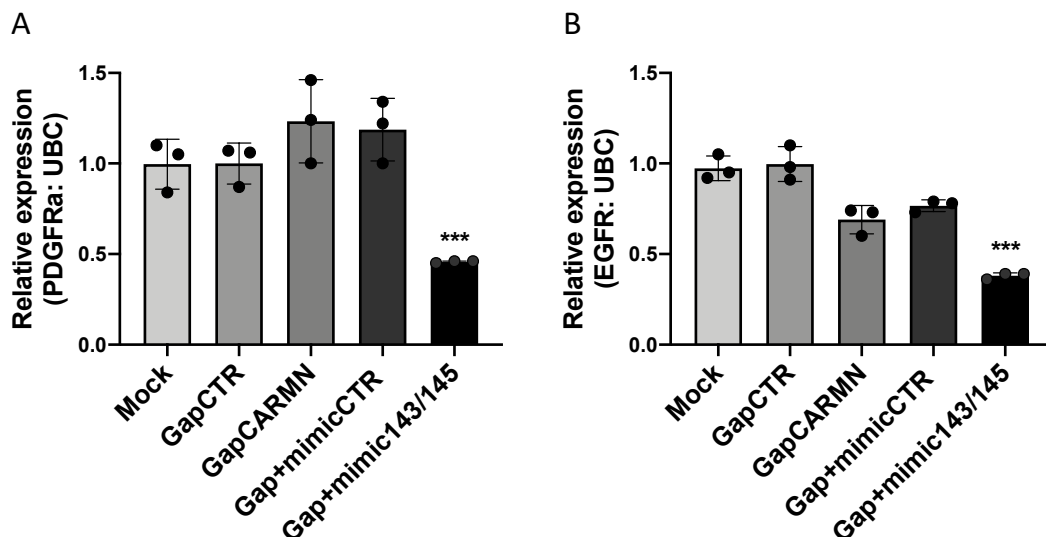


Figure 3.43 Expression of miR-143/145 targets following co-transfection with GapCARMN and mimics reagents.

A), B), qRT-PCR of PDGFRa and EGFR, respectively, in hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) transfected with GapCARMN and GapCTR and co-transfected with GapCARMN in combination with mimic control or mimics overexpressing miR-143 and miR-145. UBC was used as housekeeping gene. One-way Anova was used to assess *** P< 0.001. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

To understand if the increase of microRNA levels had an effect on *CARMN* expression, the level of the lncRNA were also assessed under the same conditions (Figure 3.44). Interestingly, the overexpression of the microRNA cluster significantly increased *CARMN* levels in GapCARMN + mimics143/145 versus control 3.5 $\pm$ 0.05 fold (Figure 3.44). These results suggest that the microRNA cluster might have a feedback loop effect on the activation of *CARMN* expression. However, the activation of the lncRNA expression is not enough to reinstate *CARMN* levels to those observed in basal conditions, remaining significantly downregulated versus GapCTR condition.

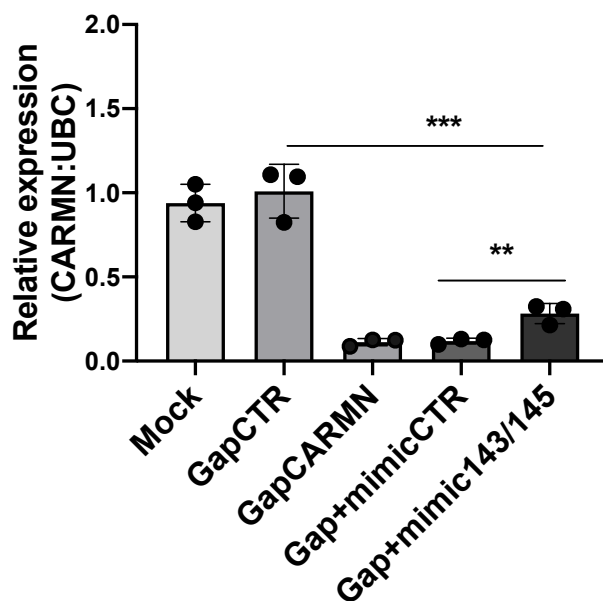


Figure 3.44 Expression of *CARMN* following GapmeR/mimics co-transfection in hCASMCS.

hCASMCS were plated, starved and co-transfected with 40nM of Gap CTR and Gap*CARMN* singularly or in combination with 5nM of miRNA mimic control (mimicCTR) or mimic upregulating miR-143 and miR-145 (mimic143/145). The graph represents qRT-PCR data of *CARMN* in hCASMCS (n=3 independent experiments each with technical triplicates, passage used 5 and 6) transfected with Gap*CARMN* and GapCTR and co-transfected with Gap*CARMN* in combination with mimic control or mimics overexpressing miR-143 and miR-145. UBC was used as housekeeping gene. One-way Anova was used to assess ** P< 0.01 and *** P< 0.001. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Having established efficient co-transfection conditions, the same conditions were used to test the capability of miR-143/145 overexpression to rescue the phenotypes observed following *CARMN* depletion.

The proliferation rate of hCASMCS at basal conditions was then interrogated following co-transfection with Gap*CARMN* and mimic143/145. In particular, co-transfected cells were induced to quiescence in starvation medium followed by treatment with EdU (10 μ M) in 0.2% FBS medium for 48 hours. Interestingly, the overexpression of miR-143/145 did not affect the phenotypic effect induced by *CARMN*-depletion and induced similar increases in hCASMCS proliferation rate, with 2.82 $\pm$ 0.9 fold increase compared to mimic control 2.42 $\pm$ 1 and Gap*CARMN* 2.40 $\pm$ 0.86 (Figure 3.45).

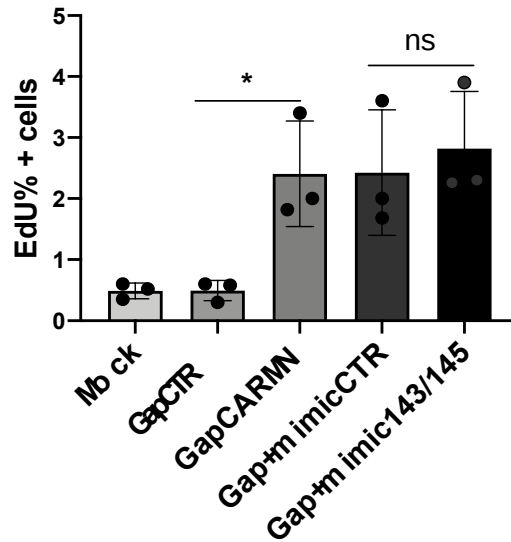


Figure 3.45 hCASM proliferation following overexpression of miR-143/145 in the presence of *CARMN* knock-down.

Percentages of positive hCASCs (n=3 independent experiments each with technical triplicates, passage used 4 and 5) to EdU incorporation obtained by FACS assay and analysed using FlowJo software. Statistics performed using One-way Anova for multiple comparisons. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

The depletion of *CARMN* was previously shown to induce a significant increase in the rate of hCASCs migration. Therefore, the effect of microRNAs overexpression was investigated in hCASCs knocked down for *CARMN*. Similarly to what was previously observed, the decrease in *CARMN* expression upregulated hCASC migration 1.65 ± 0.22 fold (Figure 3.46). Moreover, a 1.59 ± 0.21 fold increase was observed following co-transfection of Gap*CARMN* together with mimic CTR (Figure 3.46). However, this effect was rescued by the overexpression of miR-143/145 which reinstated the migration rate of hCASCs at basal conditions with 1.1 ± 0.02 fold decrease versus mimic control condition (Figure 3.46).

Figure 3.46 The effect of CARMN depletion on hCASC MC migration was rescued by miR-143/145 overexpression.

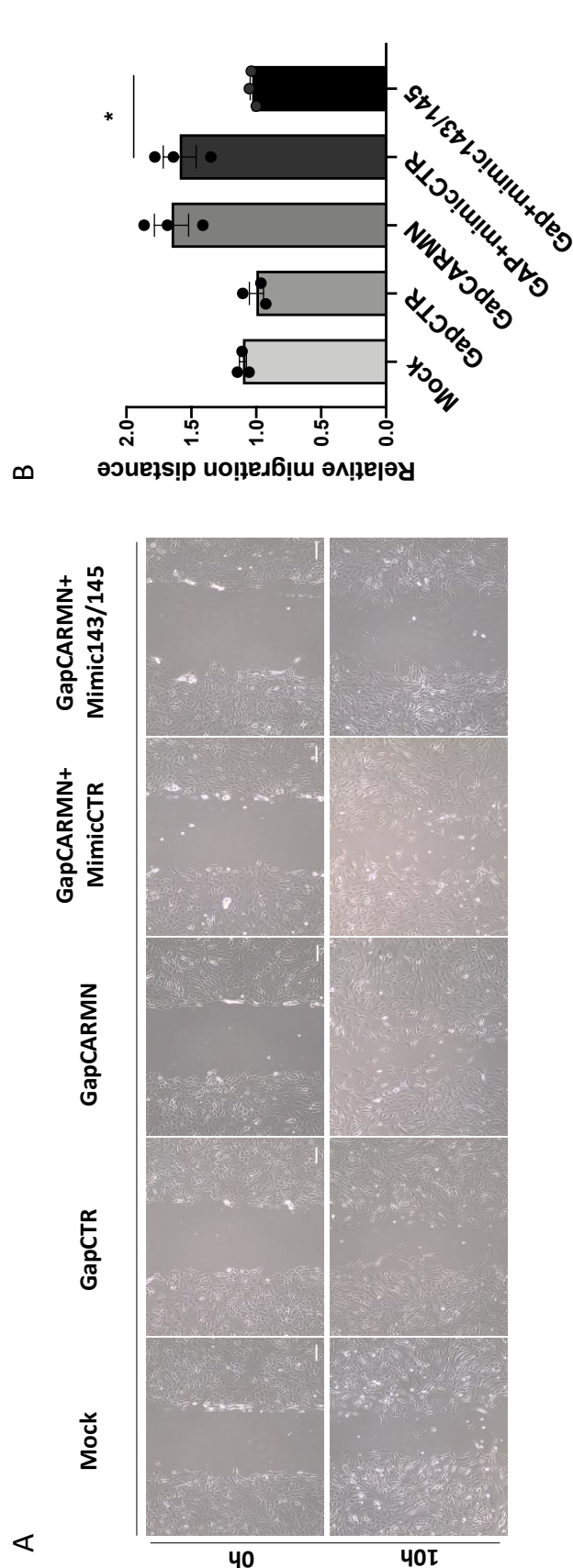


Figure 3.46 The effect of CARMN depletion on hCASC MC migration was rescued by miR-143/145 overexpression.

A) Representative micrographs of hCASC MC (n=3 independent experiments each with technical triplicates, passage used 5 and 6) following co-transfection with GapCARMN and mimics reagents induced to migrate by wound healing assay. Pictures were acquired at 0 and 10 hours post scratch with 100X magnification. Scale bars indicate 100µm. B) Quantification of migration distances relative to 0h control per each condition obtained with ImageJ tool. One-way Anova was used to assess * P< 0.05.

The effect of microRNAs overexpression was also investigated on the expression of SMC identity genes in unstimulated quiescent hCAsMCs. As previous experimental settings, hCAsMCs were initially co-transfected with Gap*CARMN* and mimics reagents followed by treatment in starvation medium. The expression of vSMC markers, ACTA2 and MYH11, was assessed as well as macrophage genes, LGALS3 and CD68. Interestingly, the expression of ACTA2 and MYH11 was confirmed downregulated of 11.1 ± 0.01 and 4.3 ± 0.1 respectively following transfection with Gap*CARMN*. However, the levels of both vSMC markers were reinstated and upregulated compared to their basal expression of 13.7 ± 1.6 and 48 ± 14.4 , respectively (Figure 3.47).

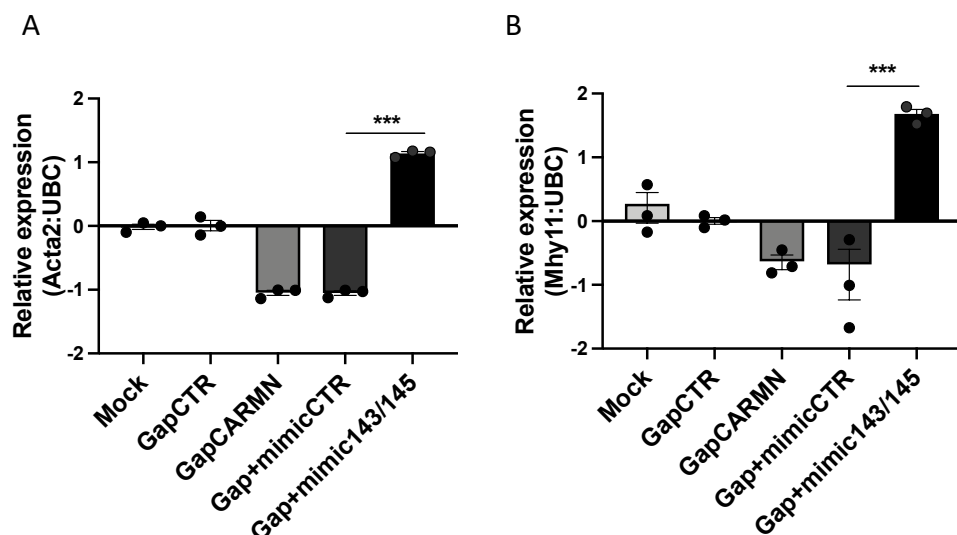


Figure 3.47 Expression of vSMC identity genes following the restoration of microRNAs expression.

A), B) qRT-PCR expression of ACTA2 and MYH11 following co-transfection of hCAsMCs (n=3 independent experiments each with technical triplicates, cell passage used 5 and 6) with Gap*CARMN* and mimic 143/145 or mimic control. UBC was used as housekeeping gene. One-way Anova was used to assess *** P < 0.001. Graphs are

shown as datapoints in logarithmic scale including the mean $\pm$ standard error of the mean.

The expression of markers for SMC de-differentiation towards a foam-cell like phenotypes were assessed in the same samples. In particular, CD68 was found increased in its expression of 2.64 ± 0.16 following *CARMN* depletion, while mimic-mediated miR-143/145 overexpression downregulated CD68 of 1.34 ± 0.08 compared to mimic control (Figure 3.48). Furthermore, the expression of LGALS3 resulted to be decreased following mimics transfection of 1.45 ± 0.13 (Figure 3.48).

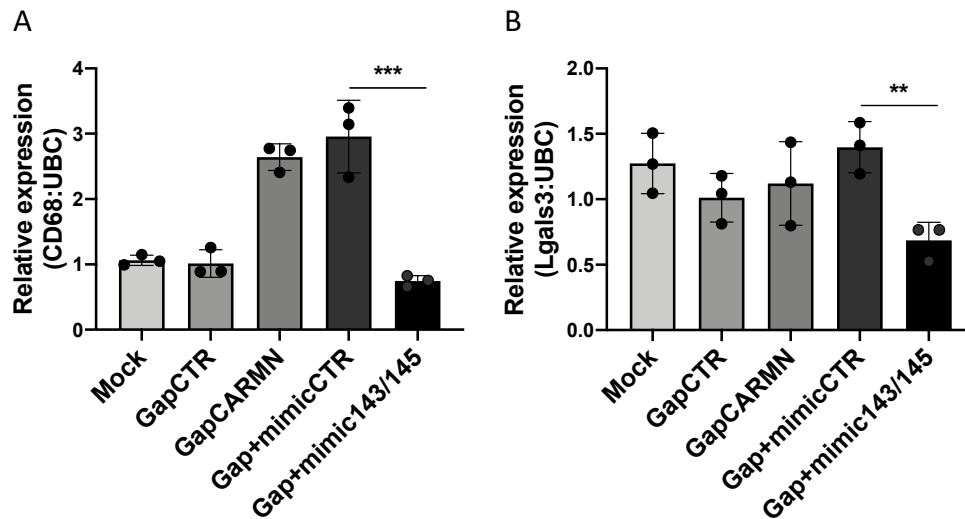


Figure 3.48 Activated macrophage makers CD68 and LGALS3 expression levels after overexpression of miR-143/145 cluster in hCASCs.

A), B) qRT-PCR expression levels of CD68 and LGALS3 respectively, following co-transfection with Gap*CARMN* and mimics in hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) at basal conditions. UBC was considered as housekeeping gene. One-way Anova was used to assess ** $P < 0.01$ and *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

3.3.9 The downregulation of *CARMN*/microRNAs leads to partial hCASCs de-differentiation phenotype.

Having established the contribution of microRNAs expression to the modulation vSMC identity genes *ACTA2* and *MYH11*, the expression of more markers was assessed in order to provide a more robust analysis of the de-differentiated state. In particular, the expression level of contractility markers, Transgelin (*TAGLN*)³⁰⁸ and Calponin1 (*CNN1*)³⁰⁹ was interrogated in hCASCs following treatment with CMBCD and in basal unstimulated conditions (0.2%BSA). In contrast to *ACTA2* and *MYH11*, *TAGLN* and *CNN1* are upregulated 1.38 ± 0.06 and 1.68 ± 0.19 fold versus GapCTR following downregulation of *CARMN* and consequently decrease in microRNAs levels in cells at basal conditions (Figure 3.49). Moreover, *TAGLN* and *CNN1* were upregulated of 2.22 ± 0.16 and 4.3 ± 0.65 in hCASC transduced with Gap*CARMN* under CMBCD treatment versus GapCTR (Figure 3.49). These data suggest that the *CARMN*/microRNAs-depletion, partially induces hCASC de-differentiation program by significantly reducing the levels of some vSMC markers.

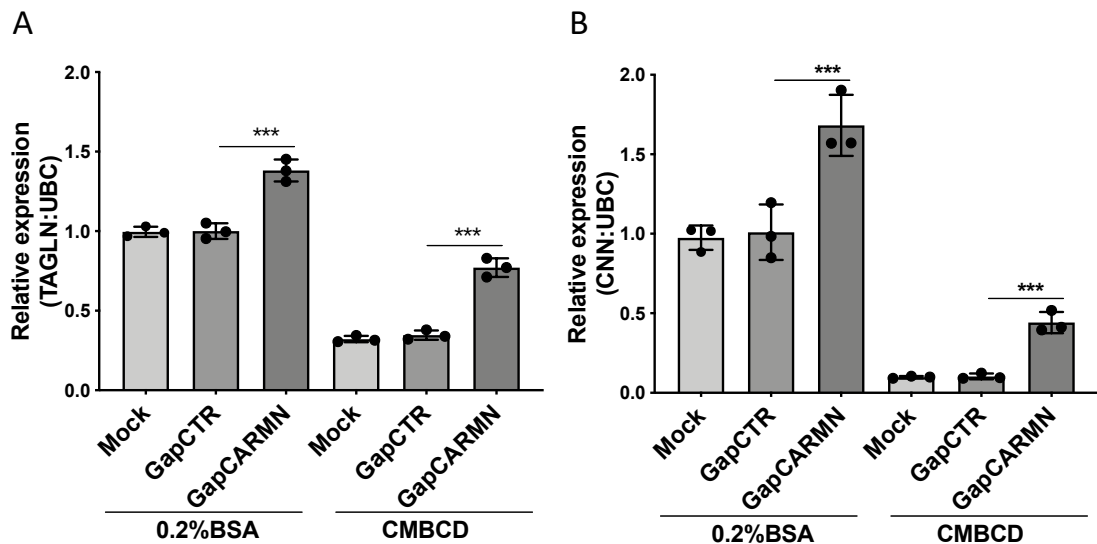


Figure 3.49 Expression of vSMC marker genes, TALGN and CNN1 during hCASC de-differentiation process.

A), B) TALGN and CNN1 expression levels detected by qRT-PCR in hCASC treated with CMBCD (10µg/ml) for 2 hours or 0.2% BSA control following transfection with Gap*CARMN* or GapCTR (n=3 independent experiments each with technical triplicates, passage used 5 and 6). UBC is reference housekeeping gene. One-way Anova was used to assess *** P< 0.001. Graphs are shown as datapoints including the mean ± standard error of the mean.

3.3.10 The manipulation of *CARMN* expression has effect on the *cis* neighbour gene: *CSNK1a1*

CARMN belongs to the group of long intergenic RNAs (lincRNAs), which includes lincRNAs autonomously transcribed with sequences not overlapping to protein-coding genes. LincRNAs can have role as local transcriptional regulators of neighbour genes. *CARMN* has been previously described as potential enhancer of gene expression in cardiomyocytes progenitor cells²⁸⁵. Active enhancer regions display very specific chromatin signatures characterised by methylation H3K4me1 or H3K27Ac acetylation³¹⁰. Chip-Seq is a technique which combines chromatin precipitation with DNA sequencing to gain information about the binding sites of proteins to the chromatin and characterise histone modifications for a specific locus³¹¹. To date there are no available publicly available Chip-Seq data on vSMC limiting our knowledge on histone modification profile on *CARMN* locus.

Therefore, to gain more understanding about the possibility for gene in proximity to *CARMN* locus to be modulated by *CARMN* depletion, expression of neighbour genes located in a region of 200kb to both sides of *CARMN* locus was checked in RNA-Seq results following knock-down of the lincRNA. Among the considered genes, Casein Kinase 1 Alpha 1 (*CSNK1A1*) was found to be significantly downregulated by 11.4 ±6 fold in the basal conditions included in the experimental design (proliferation,

migration and CMBCD loading) following *CARMN* depletion versus control (Figure 3.50).

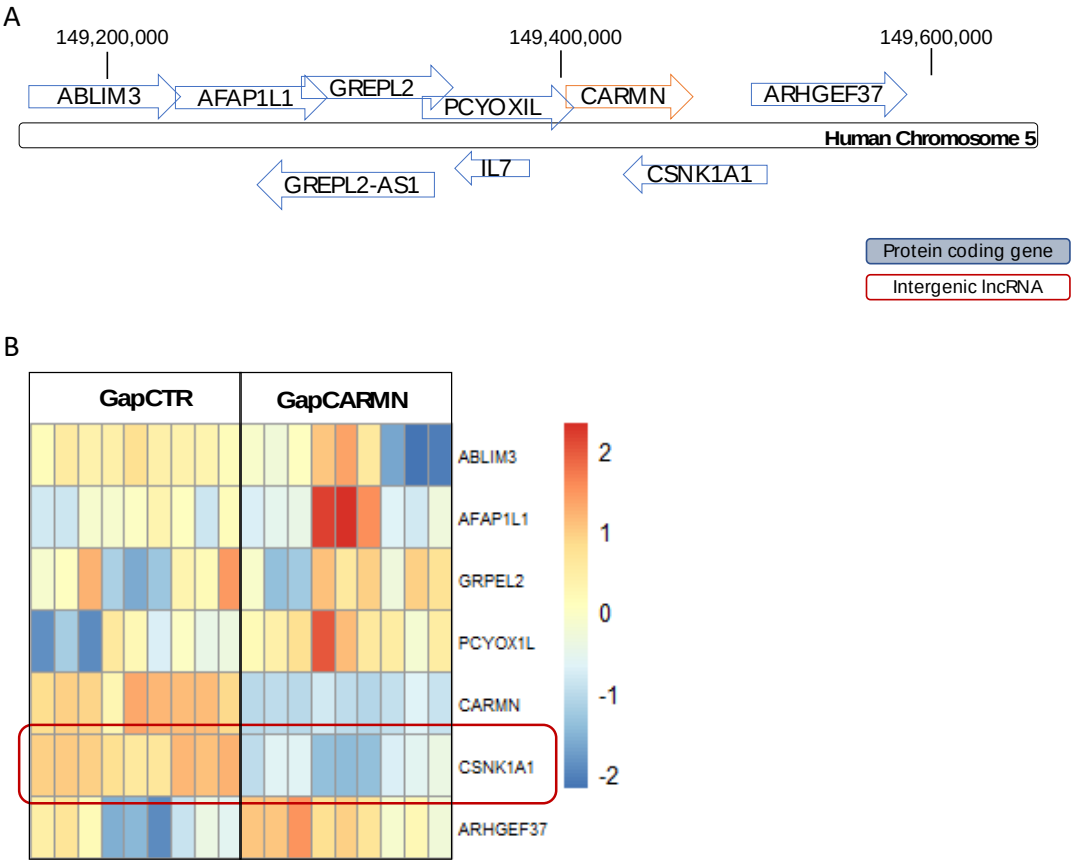


Figure 3.50 Expression of neighbour genes to *CARMN* locus in lncRNA-depleted hCASCs.

A) Schematic representation of *CARMN* locus in human Chromosome 5, spanning 200 kilobases in both directions. The sense of transcription of each gene is represented by arrows. B) Heatmap (expressed as z-score of $\log_2(\text{FPKM}+1)$) representing the expression of neighbour genes to *CARMN* locus following transfection with GapCARMN or GapCTR in hCASC basal conditions (0.2%FBS, 0.2%BSA, 0.2%FBS). CSNK1A1, dysregulated by GapCARMN versus control, is indicated by the red box. N=1 biological replicate with technical triplicates, cell passage 4.

Considering the above results, the expression of CSNK1A1 was validated in new samples generated. In particular, CSNK1A1 was found downregulated of 3.77 ± 0.15 fold in Gap*CARMN* versus GapCTR (Figure 3.51).

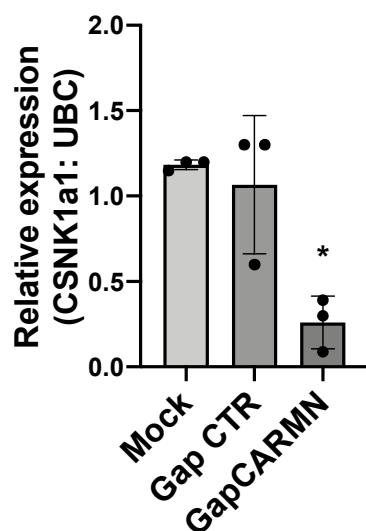


Figure 3.51 Expression of CSNK1A1 following *CARMN* depletion in hCASCs.

qRT-PCR data relative to CSNK1A1 expression in unstimulated hCASC (n=3 independent experiments each with technical triplicates, passage used 5 and 6) depleted for *CARMN* versus GapCTR. UBC was considered as housekeeping gene. One-way Anova was used to assess * $P < 0.05$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

CSNK1A1's role was previously linked with the regulation of cell proliferation in different cellular contexts ^{312–314}. Therefore, to explore whether the regulation CSNK1A1 expression could mediate the effect on hCASCs proliferation observed following *CARMN* downregulation, the expression of the casein kinase was assessed in the experimental settings of cell proliferation. To explore if the effect on CSNK1A1 is entirely mediated by *CARMN* depletion or linked with the modulation of microRNAs, CSNK1A1 expression was checked following co-transfection of Gap*CARMN* together with miR-143/145 mimics (Figure 3.52). CSNK1A1 was observed to be similarly decreased following Gap*CARMN* transfection or co-transfection with mimics reagents of 1.74 ± 0.09 and 2 ± 0.08 , respectively, indicating that the

upregulation of the microRNAs has no significant effect on the modulation of CSNK1A1 levels (Figure 3.52). Moreover, CSNK1A1 sequence did not display binding sites in its sequence assessed using online software (miRWalk and miRTarBase).

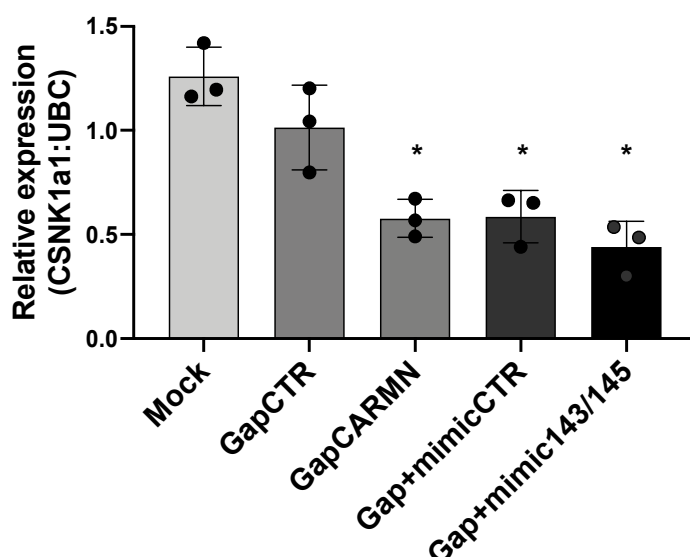


Figure 3.52 CSNK1A1 expression levels in co-transfected proliferating hCASCs.

qRT-PCR data relative to CSNK1A1 expression in hCASCs (n=3 independent experiments each with technical triplicates, passage used 5 and 6) transfected with GapCARMN singularly or in combination with mimic 143/145 or mimic control in basal conditions. UBC was considered housekeeping gene. One-way Anova was used to assess * $P < 0.05$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Having assessed the dysregulation of the casein kinase in the proliferation settings following CARMN depletion and having verified that the observed effect was not mediated through the modulation of microRNAs, the next step was to investigate whether the downregulation of CSNK1A1 affects hCASCs proliferation at basal conditions.

Dicer-Substrate short Interfering RNAs (DisRNAs) were used to interfere with the expression of CSNK1A1 in hCASCs. DisRNAs are double-stranded RNAs which after

being processed, bind the RNA Induced Silencing Complex (RISC) to target a specific region of mRNA sequences inducing strand-specific cleavage. In particular, three different DisRNAs (named S1, S2 and S3) have been designed (IDT Integrated Technologies online designing tool) and tested for their capability to affect the expression of the protein kinase. In addition, a disRNA scramble control (named NC) was used as negative control of this experiment. Two dosages of dsiRNA reagents have been included in the first test to assess the efficiency of knock-down, 40 and 80nM. The dsRNA control, of which the sequence was blasted against the human transcriptome to ensure no target off effect, did not affect the expression of the gene of interest confirming the absence of transfection effect. Remarkably, the three dsiRNAs tested affected the expression of CSNK1A1 at both concentrations, 40 and 80nM, of 15 ± 0.03 and 16 ± 0.05 fold respectively versus dsRNA control (Figure 3.53).

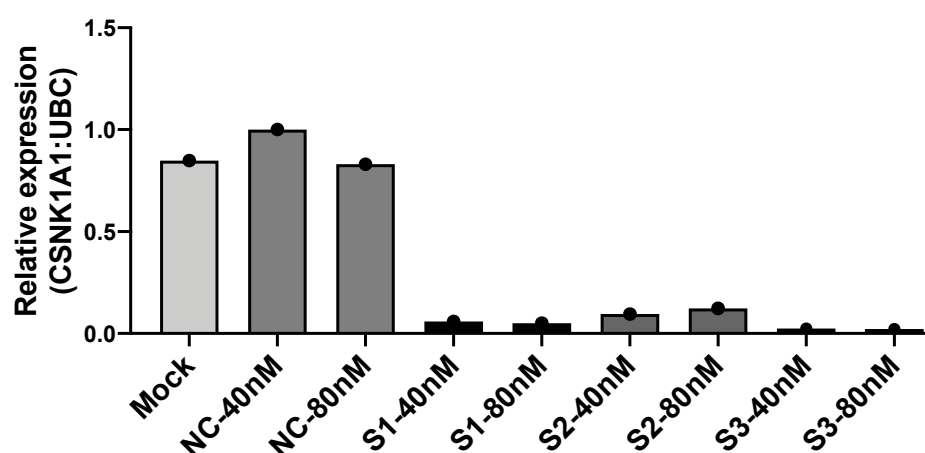


Figure 3.53 CSNK1A1 is downregulated by DsiRNA-mediated approach in hCASMCS at basal conditions.

qRT-PCR of CSNK1A1 in hCASMCS (n=1 independent experiment performed with technical triplicates, passage used 6) following transfection with three disRNA reagents (S1, S2, S3) and dsRNA control (NC) at two concentrations, 40 and 80nM in

hCASCs at basal conditions. UBC was used as housekeeping control gene. The graph is shown as datapoints.

Considering that the sequence of DsiRNA 1 (S1) was the highest in the ranking list produced by the online DsiRNAs designing tool (IDT), this was chosen as main DsiRNA to further investigate the consequence of CSNK1A1 downregulation on hCASCs proliferation. Therefore, hCASCs proliferation rate was assessed in the same experimental settings previously optimised. Cells were induced to quiescence for 48 hours following transfection with 40 or 80nM of DsiRNA 1. Cells were then treated for 48 hours in the presence of EdU in serum free medium (0.2%FBS) and EdU incorporation was verified using flow cytometry analysis. The experimental design included a control for cell proliferation to ensure the effective induction of cell proliferation in the presence of complete medium (15%FBS) versus control (0.2% medium). The knock-down of CSNK1A1 did not affect the proliferation levels of hCASCs at basal conditions (Figure 3.54), suggesting that the increase observed in *CARMN* depleted hCASCs, is not mediated through the regulation of CSNK1A1 casein kinase.

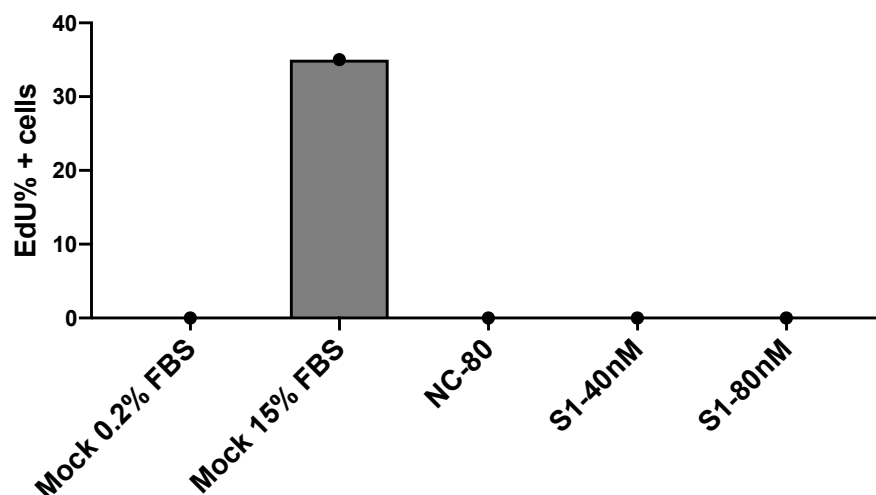


Figure 3.54 The knock-down of CSNK1A1 does not affect hCASCs proliferation at basal conditions.

Percentages of EdU positive hCASCs obtained by flow cytometry assay and analysed using FlowJo software. Cells were transfected with 40 or 80 nM of DsiRNA targeting CSNK1A1 (S1) and 80nM of negative control (NC) (n=1 independent experiment performed with technical triplicates, passage used 5). Following 48 hours of starvation, cells were treated with EdU in 0.2% FBS. In the case of Mock 15%FBS, cells were cultured in complete medium to verify the induction of cell proliferation. The graph is shown as datapoints.

3.4 Discussion

To summarise, in this chapter the expression and the potential role of *CARMN* axis was explored in unstimulated and phenotypically modulated hCASCs. *CARMN* as well as microRNAs were found downregulated in their expression following induction of proliferation and de-differentiation into macrophage-like cells. Loss-of-function experiments showed that GapmeR-mediated *CARMN* depletion affected the expression of miR-143/145 and dysregulated genes associated with vSMCs proliferation, migration, lipid metabolism and inflammation. Further functional validation confirmed that *CARMN* axis knock-down primed cells to a phenotypical switch by increasing proliferation and migration rate as well as induced a partial de-differentiation towards macrophage-like signature. Mechanistically, co-transfection experiments shown that *CARMN* regulated hCASCs proliferation rate independently from miR-143/145 regulation, which instead primarily control hCASCs migration and partial de-differentiation towards macrophage-like cells.

The evaluation of *CARMN* expression performed with transcript specific primers as well as pan primers indicated that this lncRNA is highly expressed in hCASCs, where also miR-143/145 are expressed at high levels. Ubiquitin C was used through the study as normaliser gene because of its stable expression displayed across the samples and treatments used in human cells and tissue. However, the use of another

lncRNA transcript, whose expression was not changing following the induction of SMC phenotypes, would have been a valuable approach to normalise *CARMN* expression. Nonetheless, considering the high cell-specific expression, the possible miss-annotation of transcriptional isoforms and the low levels of expression of many lncRNAs, the use of UBC was chosen as more stable option. For the purpose of this study, *CARMN* expression and function was explored in CSMCs, however previous reports have investigated the expression of *CARMN* in the heart as well as lung, where the microRNAs are expressed at high levels^{285, 286,275,276}.

Evaluating the expression of *CARMN* in the context of vSMC phenotypical transition gave the possibility to explore the potential involvement of *CARMN* axis in specific cellular processes dysregulated during the pathophysiology of atherosclerosis. *CARMN* and co-located microRNAs were found downregulated following pathological stimuli, and in particular treatment with the mitogen PDGF-BB and cholesterol loading, therefore indicating the expression of *CARMN* can be functionally involved in these pathways. While mechanical stimulation with scratch assay did not affect the expression of the locus, indicating that modulation of *CARMN* axis might not be directly caused by the induction of vSMCs migration.

The development of efficient knock-down strategy mediated by transfection with GapmeRs allowed to further proceed with loss-of-function studies. The GapmeRs used in this study include high-affinity ribosome modifications (LNA) surrounding a central DNA gap which allows them to be recognised and cut by RNase-H, with similar chemical modifications and mechanism of action of FDA approved ASOs, Mipomersen or Inotersen⁴²⁸. However, the possibility for off-target effect as result of complimentary binding of the GapmeR with non-specific exonic and/or intronic regions cannot be ruled out⁴²⁷. In-silico analysis to predict off-target effect can reduce the possibility of unwanted aspecific targeting. The GapmeR used in this study have been designed using in silico tools to check for antisense off-targets and select the most specific design in the spliced and un-spliced transcriptome. Moreover, the probe was blasted against ENSEMBL database to assess specificity for *CARMN*

transcripts. Throughout the study, a negative scrambled control was included in the experimental design, and no significant differences in the phenotypes studied in this research (proliferation, migration, de-differentiation) were observed with un-transfected mock control.

The GapmeR-mediated loss-of-function strategy allowed to study and identify the possible role of *CARMN* and, revealed the consequential downregulation of co-located miR-143/145. The positive correlation in the expression pattern of lncRNA and microRNAs might indicate that they might belong to the same transcriptional unit. However, there is not enough evidence to prove this hypothesis and is unclear to the literature whether the pri-microRNA precursor is transcribed from the same promoter shared with *CARMN* described in 2015²⁷¹ or it belongs to a promoter region downstream to *CARMN* transcripts as identified in 2009²⁷⁶. Therefore, it is difficult to establish if *CARMN* might play a cis regulation to the microRNA expression which is in turn negatively affected or the GapmeR-mediated strategy simply affects the primary transcript causing the decrease in microRNAs levels.

RNA sequencing performed in *CARMN*-depleted hCASCs and control has allowed the identification of more than 1000 genes dysregulated in basal conditions. Gene ontology analysis allowed to categorise these genes into: i) genes associated to positive regulation of cell proliferation (such as *ID1*, *CDC20*), ii) positive regulators of cell migration, iii) smooth muscle related genes (*ACTA2*, *MYH11*, *COL3A1*, *ELN*) iv) cell inflammation (*MMPs*, *CXCL1*, *IL1B*, *IL6*) and v) response to lipids (such as *ABCA1*, *ABCG1*, *CD68*, *LGALS3*). The wide effect of *CARMN* axis depletion on hCASCs suggests that this axis can contribute to the phenotypic transition towards synthetic de-differentiated and pro-pathogenic smooth muscle cells.

In particular, during the progression to a more advanced plaque, vSMCs in response to upregulation of pro-inflammatory cytokine, such as the mitotic Platelet-Derived Growth Factor (PDGF)-BB, or mechanical stimuli, exit from their quiescence state and

start proliferating³¹⁵. This is an extremely important reparative and protective mechanism during the first phases of vascular injury. However, during the progression to a more advanced plaque the imbalance of this process and the simultaneous increase in cell migration and phenotypic switch, leads to plaque instability during the later stages of human atherosclerosis¹⁰³. In this regard, the GapmeR-mediated knockdown of *CARMN* led to an increase in cell proliferation under PDGF-BB stimuli and importantly, stimulated quiescent cells to exit their G0 phase starting to proliferate in starvation medium (0.2% FBS). These results indicate that *CARMN* knock-down might be involved as a downstream regulator of the PDGF-BB pathway in synthetic vSMCs, but also *CARMN* loss might represent the root cause of vSMCs transition towards more proliferative phenotype. Of note, *in vitro* studies in monolayer of cultured cells do not entirely recapitulate the *in vivo* context therefore more studies are required to define the causal role of *CARMN* loss more clearly to vSMC phenotypes.

During their transition to synthetic phenotypes, vSMCs re-acquire the capability of migrating towards the innermost layer of vascular wall in response to mechanical stimuli, such as shear stress, and chemoattractant factors released in the injured environment. *In vitro* functional assays performed during the study, included the induction of hCAsMCs migration through scratch assay. The downregulation of *CARMN* axis induced hCAsMCs to increase their migratory rate and dysregulated genes involved with the positive regulation of cell migration, thus identifying a potential role for *CARMN* in initiating hCAsMCs cytoskeleton reorganisation and migration. Also, in this case it is important to underline that this technique has limitations which are mostly associated with the difficulty to relocate exact wound sites during time points and the irregularity of the edges following the scratch. Nevertheless, it remains challenging to study this process *in vivo* and the consequences of it during the pathogenesis of disease without lineage-tracing mice model.

During atherogenesis the vSMCs de-differentiation towards a pro-inflammatory foam-cell like phenotype has an important contribution to transition to synthetic stage. During this process, vSMC identity genes are downregulated while vSMCs acquire new abilities of uptaking toxic cholesterol molecules in excess, while decreasing the expression of typical markers regulators of vSMCs contractile stage²⁸. This phenomenon has been studied *in vitro* where *CARMN* and miR-143/145 cluster has been found significantly affected in hCASCs following treatment with water-soluble compound (CMBCD)³¹⁶, thus suggesting the involvement of the axis in the maintenance of vSMC identity which is lost concomitantly to *CARMN* depletion. Moreover, the GapmeR-mediated knock-down of the entire axis decreased the expression of vSMC identity genes, such as *ACTA2* and *MYH11*, while increasing the expression of macrophage-like genes, *ABCA1*, *ABCG1*, *CD68*. However, the effect observed on the regulation of vSMC identity genes was only partial as other markers, such as *CNN1* and *TALGN*, shown upregulation following *CARMN* knock-down.

The treatment with oxidised Low-Density Lipoproteins (ox-LDL) was previously described to induce the generation of lipids laden vSMC-derived foam cells *in vitro* and *in vivo*³⁰¹. Therefore, the effect of ox-LDL stimuli on *CARMN* expression was also explored. While *CARMN* was found downregulated in a dosage-dependent way versus control cells, the treatment with oxidised lipids did not affect the expression of microRNAs. This data might indicate the implication of *CARMN* during early response to pro-pathogenic pathways induced by ox-LDL exposure, where instead microRNAs might not be directly associated. Moreover, the treatment with modified LDL particles might activate pathways in SMCs that are different from the ones induced by their exposure to cholesterol⁹⁸, such as inflammatory pathways and apoptosis. These pathways, even though might act synergistically in de-differentiated SMCs, could involve *CARMN* but not directly miR-143/145 expression. However, whether the loss of *CARMN* axis is the causal factor which primes the loss of vSMC identity or if the decrease in *CARMN* axis enhances the effects already activated by extracellular pro-pathological factors is very difficult to clearly define. Nevertheless,

the broad spectrum of phenotypes that vSMCs are capable of simultaneously displaying enhances the difficulty of their identification *in vivo*.

GapmeR-mediated approach was instrumental to affect the expression of the entire *CARMN* transcript set, however the consequent downregulation of microRNAs does not allow to evaluate to which extent *CARMN* plays a functional role or whether the effect on microRNAs expression drives the observed phenotypes. This is one of the most challenging points of this project which required the development of efficient strategy to functionally dissect the two noncoding RNAs. The co-transfection with miRNAs mimics (mimic miR-143 and mimic miR-145) in the presence of GapmeR, restored and overexpressed the levels of miR-143/145 contemporaneously decreasing *CARMN* levels, allowing to investigate the contribution of *CARMN* to the observed phenotypes. However, the exogenous introduction of chemically synthesised microRNA mimics is not comparable to their physiological expression⁴²⁵. Moreover, the total level of transfected microRNA measurable by qRT-PCR could not accurately represent the levels of functional active microRNA⁴²⁶. The transfection of medium-low dosage of mimics (in this study 5nM were used to overexpress miR-143/145) can decrease the possibility of non-specific effect, and the assessment of validated targets following mimic transfection can indicate the efficiency of functional microRNA. However, considering the possibility of unwanted off-target effect, the usage of gene therapy mediated overexpression (for example using lentiviruses) would represent a more physiologically relevant approach to overexpress microRNAs. In this experimental design, the co-transfection of GapmeR control and miR-143/145 (Gap+miR-143/145) was not included. However, even though the GapmeR control used in this study did not have a significant effect on the capability of hCASMCM to proliferate, migrate, de-differentiate and did not affect the expression of *CARMN*, it is not possible to rule out the possibility of non-specific effects of miR-143/145. Albeit the possibility for non-specific effects, the results of this strategy indicate the possibility that the regulation of vSMC proliferation might possibly occur independently by miR-143/145. However, uncoupling effects of the

lncRNA from the microRNA cluster is a challenge to address experimentally due to the high complexity of this locus. Therefore, further studies would be required to understand the independent effects of the lncRNA from the co-located miRNAs for example using CRISPR editing approaches.

The induction of migration of unstimulated hCASCs at basal conditions was rescued by the overexpression of microRNAs revealing the fundamental regulatory role played of microRNAs. This is in line with previous findings which identified miR-143/145 down-regulation in the induction of podosome formation and migratory phenotype²⁷⁹. Nevertheless, these results do not exclude the possibility for *CARMN* dysregulation to contribute to the enhanced migration of synthetic vSMCs while strongly indicating the critical role played by microRNAs in the maintenance of vSMC contractile phenotype.

In relation to this, the overexpression of miR-143/145 showed a pronounced effect on the expression of vSMC identity genes in response to cholesterol-treated and unstimulated hCASCs. Previous findings shown the significant decrease in microRNA expression in vSMC laden with CMBCD compound³¹⁶. In this study, we expanded the analysis to *CARMN* expression which resulted to be decreased in a similar way. Furthermore, miR-143/145 overexpression allowed to identify their critical role in reverting the phenotypical changes induced by CMBCD stimuli. In particular, the decrease observed in vSMC genes (*ACTA2*, *MYH11*) with concomitant increase of CD68 and LGALS3 was attenuated by miR-143/145 expression.

Finally, the consequence of miR-143/145 overexpression was explored on the enhanced capability of hCASCs to proliferate as response to *CARMN* depletion in hCASCs. Interestingly, the expression of miR-143/145 cluster did not affect the observed increase hCASCs proliferation rate following *CARMN* knock-down suggesting that *CARMN* loss induces quiescent vSMCs to proliferate independently from the modulation of microRNAs expression. Nonetheless, these results do not

exclude the possibility for microRNAs to play a role in vSMCs proliferation which might be secondary to *CARMN* downregulation or in proliferating synthetic vSMCs.

Previous studies have identified the possibility for *CARMN* to play super-enhancer role in the regulation of genes in its proximity^{285,286}. Although currently there are no experimental validated data (such as Chip-Seq in vSMCs) which might reveal a super-enhancer signature in vSMCs, the expression of neighbour genes was interrogated following *CARMN* depletion in hCASCs at basal condition. Among all the genes included in the analysis (analysis included surrounding genes located within 200 kb in both orientations), Casein Kinase 1 Alpha 1 (*CSNK1A1*) resulted to be the only gene significantly dysregulated versus control. Moreover, the expression of *CSNK1A1* was not affected by the overexpression of miR-143/145, which excludes the possibility of being a direct potential target for the cluster. Interestingly, this protein was studied in the context of cell proliferation during pathology, as it regulates cell cycle progression and growth^{312–314}. However, although RNA interference strategy (DsiRNA-mediated) efficiently downregulated *CSNK1A1* in hCASCs, the knock-down of this casein kinase did not induce proliferation in quiescent hCASC thus suggesting that, although *CARMN* loss regulates the levels of the protein, the effect on hCASCs proliferation is not mediated through this process.

Collectively, the work performed *in vitro* has allowed to identify the function of *CARMN* axis in the pro-pathological switch of hCASCs and to functionally dissociate *CARMN* from miR-143/145. In particular, the evaluation of vSMCs response to *CARMN* loss in basal conditions identified *CARMN* as potential regulator of vSMCs proliferation independently from the modulation of miR-143/145, whose expression instead primarily modulate the capability of hCASCs to migrate and partially de-differentiate into macrophage-like signature.

Chapter 4 Examining the expression and function of *CARMN* axis in atherosclerosis.

4.1 Introduction.

One of the main characteristics of long noncoding RNA transcripts (lncRNAs), together with tissue and cell-specific expression is their poor level of conservation. In this regard, one of the cut off parameters to select, study and characterise the role and function of a lncRNA is often the presence of orthologs^{171,180}. Most recently, comparative analysis has shown that in the case of conserved lncRNAs, the sequence with higher conservation levels is their promoter region versus exonic or intronic regions³¹⁷. This is the case of the lncRNA *CARMN*, firstly discovered in human cardiomyocytes progenitor cells (CPCs) and further described by RNA-Sequencing (RNASeq) analysis in mouse embryonic tissue³¹⁸, and characterised by high levels of conservation in vertebrates, mainly in correspondence to the promoter sequence²⁸⁵.

The high conservation level in the case of some lncRNAs, has allowed the characterisation of their role with gain and loss of function experiments, the association of their differential expression patterns with human disease, and the identification of their clinical relevance using animal models. The use of efficient animal models has permitted extensive studies evaluating the lncRNA changes in expression levels during specific stages of disease, which have importantly guided further investigation on the regulatory function of these molecules. Atherosclerosis is a highly complex disease, characterised by different pathological stages finely regulated by interconnected molecular networks, difficult to be dissected and entirely understood³⁰. Several animal models such as mouse, rat, pig and primates have been used to study the pathophysiology of the disease. Among this range of options, mouse models have been widely used because of their small size, which makes them cost-effective models, the possibility to generate transgenic mouse lines and the topography of plaque development. In particular genetically modified mouse

models have proven to be useful in recreating partly the cascade of events that characterise the pathophysiology of the human disease. The genetic depletion of genes related to cholesterol homeostasis has been used to develop animal models of atherosclerosis. In particular, knock-out of receptor of Low-Density Lipoprotein (LDL), in charge of maintaining the appropriate concentration of LDL particles in the blood, or depletion of Apoprotein E, which is involved in the clearance of Very Low-Density Lipoprotein particles (VLDL) are two classic examples used for this purpose. The genetic alteration of these genes in combination with a high cholesterol diet for 15-20 weeks (HCD), favours the increase in cholesterol levels and the development of extensive atherosclerosis characterised by large necrotic core, pronounced inflammation, presence of calcification and loss of fibrous cap similarly to human pathology^{319–321}. Although they comprise valuable tools for the study of molecular mechanisms underlying the pathophysiology of disease, these mouse models suffer of major limitations including i) the topography of disease which in human occurs primarily in coronary arteries, while in mice usually occurs in the aortic trunk and carotid arteries³²², ii) the absence of early development of intimal thickening observed in human³²³, iii) the differences existing in cholesterol metabolism and genes responsible of cholesterol homeostasis³²⁴, the diverse inflammatory responses in response to pathological stress stimuli³²⁵ and the difficulty to observe spontaneous plaque instability and rupture in mice³²⁶. Previous studies have tried to overcome these differences with human plaques by long-term maintenance (>1 year) of APOE knockout mice (APOE^{-/-})^{327,328}, however the costs do not favour its usage. Instead, recently, the cross-breeding of APOE^{-/-} mice with Fibrillin1 heterozygous mice (Fbn1^{+/-}), a protein scaffold for elastin fibres deposition, revealed the ability to develop more similar plaques to human instable lesions characterised by presence of intraplaque haemorrhage and rupture³²⁹. However, breeding issues with this strain prevent large-scale usage.

Collectively, these models have been extensively used to investigate the involvement of a good number of conserved lncRNAs in atherosclerosis. Examples are the

endothelial enriched lncRNA SNHG12³³⁰, found downregulated in advanced versus early plaque whose knockdown increased atherosclerosis and cellular senescence of LDLR^{-/-} mice. Another example is represented by MALAT1, involved in the recruitment of leukocytes at the plaque sites whose partial loss of function (MALAT1^{+/-}) increased the formation of the plaque of APOE^{-/-} animals causing immune dysregulation²³⁸. In addition to this, knockout of lncRNA MIAT, found significantly upregulated in advanced atherosclerosis, promoted a decrease in the plaque area, increase in fibrous cap and plaque stability in APOE^{-/-} mice²⁵⁴. Furthermore, the adenoviral-mediated overexpression of the liver-expressed lncRNA LeXis in LDLR^{-/-} mice induced pronounced reduction of hepatic cholesterol and plaque formation³³¹. As crossing of a gene knock-out to an atherosclerosis-prone strain is costly and time-consuming, a mouse model based on the Adeno-Associated (AAV) mediated long-term overexpression of Pro-Protein Convertase Subtilisin/Kexin type 9 (PCSK9) was developed as a more rapid and cost-effective model for the development of advanced (but not ruptured) plaques³³².

Having chosen the most suitable mouse model, differential expression profiles of the lncRNA of interest can be evaluated with different techniques. Microarray technology^{333–337} and RNA-Sequencing^{244,338,339} analysis have greatly added to the knowledge of the cellular stages characterising atherosclerotic human and mouse plaques, identifying important mechanisms underlying the pathophysiology of disease. Recently, single cell RNA sequencing has allowed the study of lesions at single-cell resolution which extended previous knowledge of the contribution of each cell population in the disease progression, the identification of new sub-populations of inflammatory⁷⁰, as well as smooth muscle cells⁸⁹ populating the plaque microenvironment, and the characterisation of cell heterogeneity at different stages of the disease³⁴⁰. Collectively, these techniques have enabled the scientific community to comprehensively profile lncRNA expression in plaque stages, cell types, and correlate expression patterns with the progression of the disease.

Importantly, these strategies have provided the basics for future validation (through qRT-PCR and immunohistochemical techniques) and functional studies.

Atherosclerotic mouse models and gene expression strategies have been previously used to describe the profile of miR-143/145. In this regard, previous efforts have increased the knowledge about this cluster and revealed the complexity of the regulatory mechanisms controlling its expression. In fact, previous studies have shown opposite results and found miR-143/145 downregulated following vascular injury, while the adenovirus-mediated restoration of its activation was associated with reduced neointima formation following balloon injury²⁷⁷. At the same time, miR-143/145 knock-down correlated with decrease of neointima formation in response to carotid artery ligation²⁷⁸ and attenuated atherosclerosis in a mouse model of disease²⁸⁴. Collectively, these studies highlight how challenging the study of this locus is, whose genomic organisation includes the lncRNA *CARMN*. In this regard, few years later, *CARMN* was described as non-coding long intergenic RNA with potential roles in the differentiation of cardiomyocyte progenitor cells toward smooth muscle or cardiomyocyte lineage. However, the role of *CARMN* in relation to the pathology of vascular remodelling as well as the molecular interaction with microRNAs was not explored yet. Therefore, the study of the entire locus, lncRNA and microRNAs, during the events characterising the pathophysiology of the disease would potentially uncover its underlying mechanism of action and associated function.

4.2 Aims.

In this chapter the expression of *CARMN* axis was characterised in specific stages of plaque development and the hypothesis tested with the experiments herein described is:

CARMN and miR-143/145 are dysregulated in human and mouse atherosclerosis, and their expression is lost during the progression to more advanced plaque.

- To profile the expression of the *CARMN* and miR-143/145 in mouse plaques advancing from early stages to more complex phenotype.
- To profile *CARMN* axis levels and localise *CARMN* expression in early and advanced human atherosclerotic lesions.
- To identify cell types expressing *CARMN* in human plaques.

4.3. *CARMN* expression in mouse.

In a similar way to human *CARMN*, the first step to assess *CARMN* expression in mouse was to design a set of primers specifically amplifying each of the two isoforms annotated to the reference annotation (Ensembl 98) (Figure 4.1). Moreover, a unique set of primers, designed in a region common to both isoforms, was used to assess the levels of *CARMN* throughout the study.

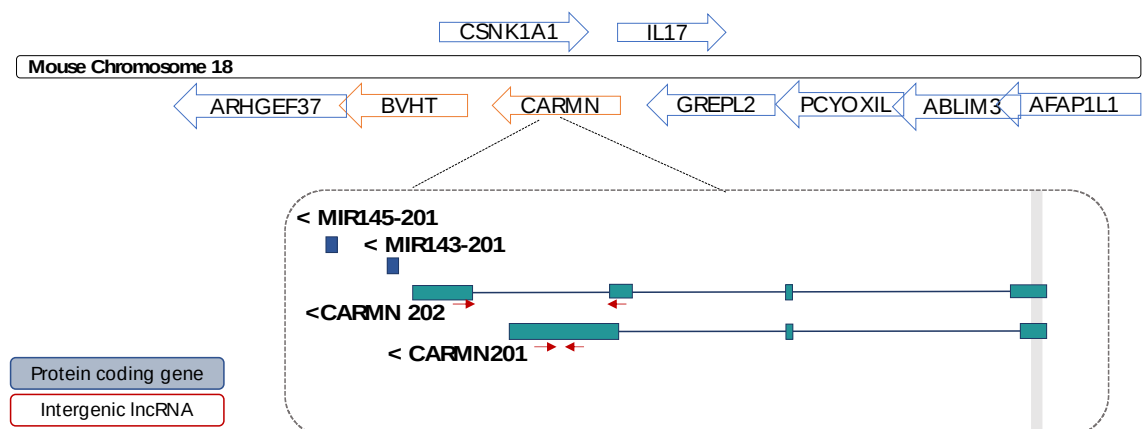


Figure 4.1 Schematic representation of mouse *CARMN* transcripts and pre-microRNAs miR-143 and miR-145 (GRCh38/hg38).

Mouse *CARMN* is intergenic lncRNA located in mouse chromosome 18, represented in this picture in orange. Protein-coding genes surrounding *CARMN* are included in the picture in light blue. The arrows indicate the sense of transcription of each gene. Braveheart is a lncRNA located in proximity and opposite to *CARMN* locus, in orange. *CARMN* transcripts (in green in the picture) annotated in Ensembl database are two,

201 and 202. The locus also includes miRNAs miR-143 and miR-145 (dark blue) downstream to *CARMN* isoforms. The red arrows indicate isoform-specific primer sets amplifying isoform 201 and 202. The grey box represents a common exonic region shared from 201 and 202 transcripts which is recognised by the common primer set.

Two transcript-specific primer sets, designed to amplify specifically each transcript, were validated through the evaluation of their melting curves obtained by qRT-PCR in isolated mouse aortic smooth muscle cells (ASMCs), to ensure only one amplification product (Figure 4.2). In addition, to confirm a single amplicon of correct size, amplification products were run on agarose gel and finally sequenced to confirm the nucleotide sequences of annotated isoforms (Figure 4.2). Nucleotide sequences obtained through sequencing were blasted against the mouse transcriptome to assess primer specificity.

Figure 4.2 Assessment of isoform-specific couple of primers amplifying mouse CARMN in ASMCs.

The two annotated isoforms, 201 and 202, were found expressed in unstimulated mouse ASMCs and their relative quantification together with expression levels obtained using a common primer set to both transcripts is reported in Figure 4.3. Of note, since the primer specificity might be different, it is not possible to compare the expression levels of the two transcripts. However, considering that this study aims at the wide characterisation of the locus, and not at the characterisation of one specific transcript, one primer set amplifying a region shared by both isoforms was designed and used throughout the study to identify *CARMN* expression levels *in vivo* (Figure 4.3). With regard to the expression in human cells, *CARMN* was found being expressed at similar levels in SMC at basal conditions (Ct value ~25-27) using primers amplifying the majority of the transcripts. However, considering that the set of primers amplifying the human and mouse transcripts might have different amplification efficiency, it is not possible to compare the results obtained in the two species.

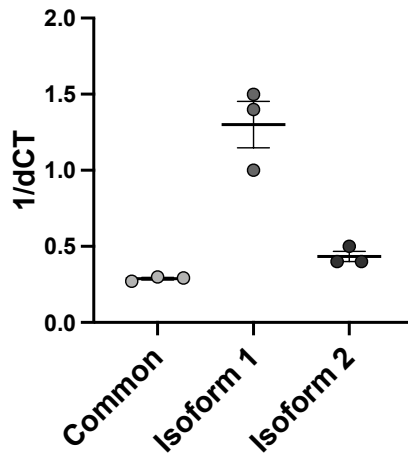


Figure 4.3 *CARMN* expression in mouse ASMCs.

Mouse ASMCs were isolated from aortas of wild type animals in order to perform gene expression analysis. The graph represents qRT-PCR data (n=3 independent experiments, each performed in technical triplicates; cell passage used 4 and 5) relative to the expression of *CARMN* isoform 1 (201 in Ensembl annotation) and 2 (202), together with common primer set amplifying both isoforms (designed targeting a shared sequence of exon 1). Data expressed as 1/dCT values. Cyclophilin (*Ppia*) was used as housekeeping control. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

4.3.1 *CARMN* is dysregulated in mouse atherosclerosis.

Having developed a robust strategy to characterise *CARMN* transcripts in mouse, the expression and function of *CARMN* in a mouse model of atherosclerosis was investigated. One of the most efficient and widely utilised mouse models of atherosclerosis involved the use of Low Density Lipoprotein receptor knock-out (*LDLR*^{-/-}) mice fed with Western-Type High Cholesterol Diet (HCD)³¹⁹. This model is based on the genetic depletion of one of the most important genes for cholesterol metabolism, LDL receptor (*LDLR*), which maintains the plasma levels of LDL particles

by favouring cholesterol-rich LDL particles to be up-taken and metabolised, thus avoiding their accumulation in the plasma. LDLR^{-/-} mice fed with HCD develop hypercholesterolemia and advanced atherosclerosis, while feeding with a normal diet (chow) leads to a moderate increase of plasma cholesterol levels versus wild type animals, and early atherosclerosis develops ³⁴¹. Therefore, the expression of *CARMN* was evaluated in both atherosclerotic stages, to assess whether *CARMN* profile changed during the development of more advanced plaques versus early lesions. The mouse study in this project was conducted in collaboration and under the direct supervision of Prof. Judith Sluimer's team (University of Maastricht).

8-10 weeks old male LDLR^{-/-} mice were divided into 2 groups (Figure 4.4):

- 1) Mice fed with normal chow diet for the entire experimental time (14 weeks) to develop an early lesion with aggregation of lipid laden macrophages (fatty streak).
- 2) Mice fed with High Cholesterol Diet (0.25% cholesterol) for 14 weeks to develop advanced plaques characterised by a pool of pro-inflammatory macrophages, foam cells, dysregulated smooth muscle cells, a large necrotic core and a thin fibrotic cap.

Mice were sacrificed following 14 weeks from the start of high fat diet and aortic roots were embedded in cryo-preserved solution (OCT) and sectioned (5µm), to assess plaque stages in the two groups by Haematoxylin and Eosin (H&E) staining (Figure 4.4). In particular, plaques in animals on normal chow diet developed early lesions characterised by fatty streak mostly populated by foam cells deposited at the site of the plaque formation and small fibrotic cap. Whereas histological examination of plaques from animals on high cholesterol diet revealed more complex plaques with large necrotic cores, deposits of cholesterol crystals, thin fibrotic cap and calcification inclusions.

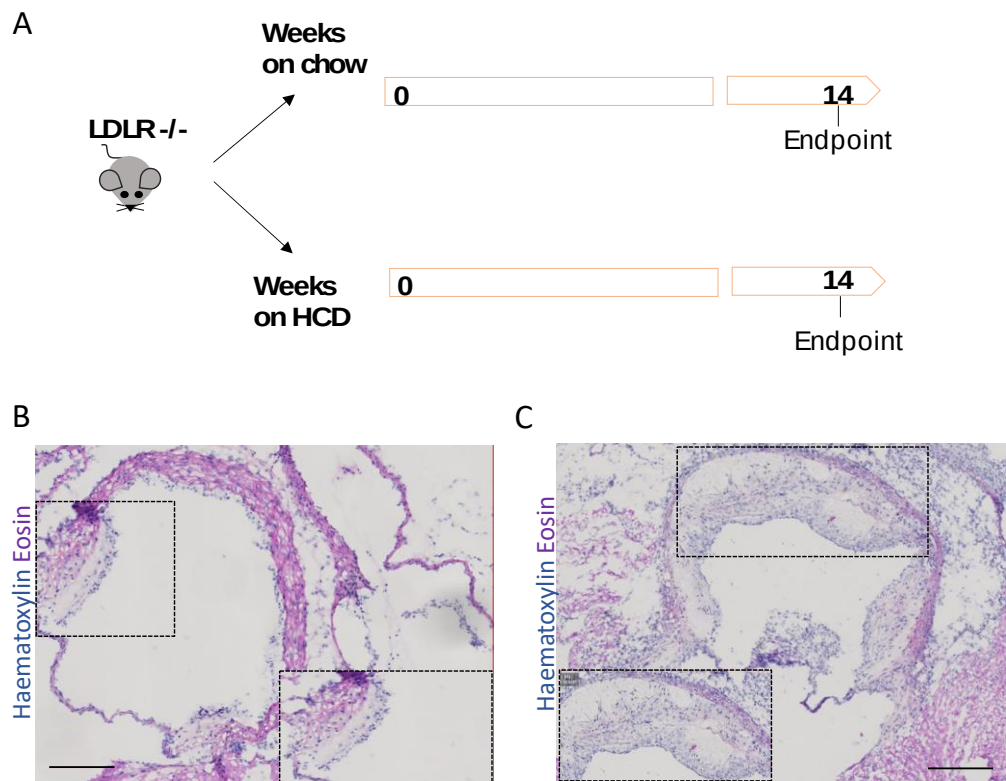


Figure 4.4 LDLR^{-/-} mouse model of atherosclerosis.

LDLR^{-/-} mice were fed for 14 weeks with normal chow diet or High Fat Diet to develop early fatty streak or advanced plaques, respectively. Aortic roots were then fixed on cryo-preservative solution (OCT) and sectioned to assess plaque stages in the two groups by Haematoxylin and Eosin (H&E) staining. A) Schematic representation of the experimental design used to induce LDLR^{-/-} to atherosclerosis by feeding with high cholesterol diet or normal chow diet for 14 weeks. B) Haematoxylin and Eosin staining in cross-sections of aortic roots from LDLR^{-/-} fed for 14 weeks with normal chow diet to develop early fatty streaks or C) fed with HCD (0.25% cholesterol) to developed advanced plaque. Pictures were acquired with 40X magnification, scale bar 200μm. The zoomed-in boxes indicate the atherosclerotic plaque tissue developed in aortic valves, sectioned and collected during the Laser Capture Microdissection (LCM) for gene expression analysis.

Moreover, to visualise the neutral lipids accumulated in the plaques of LDLR^{-/-} mice fed with normal or HC diet, Oil Red O (ORO) staining was performed in cross sections

of aortic roots. The staining revealed large atherosclerotic lesions and necrotic cores in the plaque of animals fed with HFD while smaller size plaques were observed in animals fed with normal diet (Figure 4.5).

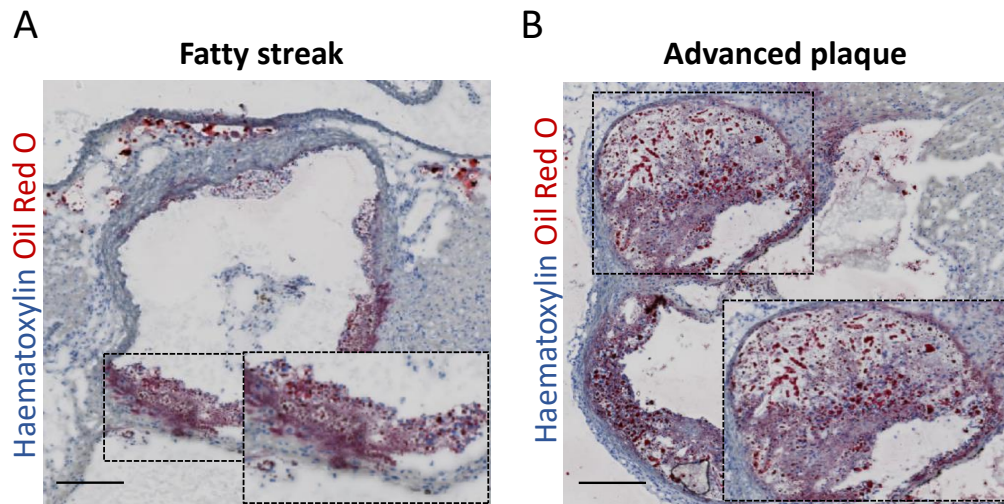


Figure 4.5 Visual assessment of intraplaque lipids in plaques of LDLR^{-/-} animals.

LDLR^{-/-} mice were fed for 14 weeks with normal chow diet A) or High Fat Diet B) to develop early fatty streak or advanced plaques, respectively. Aortic roots were then fixed on cryo-preservative solution (OCT) and sectioned to assess the presence of plaque lipids in the two groups by Oil Red O (ORO) staining. A), B) Cross sections from aortic roots stained with ORO staining for lipids (red) and Haematoxylin staining the nuclei (blue) in early fatty streak group (A) and mice developing advanced plaques (B). Pictures were acquired at 40X magnification, scalebar 200μm. The zoomed-in boxes represent the plaques isolated with Laser Capture Microdissection (LCM) for gene expression analysis.

The isolation of mouse plaques for downstream analysis is very challenging due to the microscopic size of the lesions accumulated at the aortic arch site. Therefore, to

investigate *CARMN* expression on these animals, Laser Capture Microdissection (LCM) was performed to efficiently isolate the plaque tissue from each aortic valve in the aortic root of LDLR^{-/-} animals. LCM is based on the selective isolation of the tissue of interest (plaque) by microscopic visualization (Palm Robo). Once the region of interest is selected, the tissue can be dissected using laser pulses and collected for downstream analysis of gene expression (Figure 4.6). In the case of atherosclerotic lesions, the area of the plaque, defined by the internal elastin lamina to the edge of the lumen, was included in the ROI selection. Moreover, in order to increase the RNA yield, multiple sections were processed per animal (18 sections) and collected for downstream gene expression analysis. This experiment was conducted in collaboration with Professor Tabas Ira's team at Columbia University (NY) following similar experimental set-up previously published²⁹².

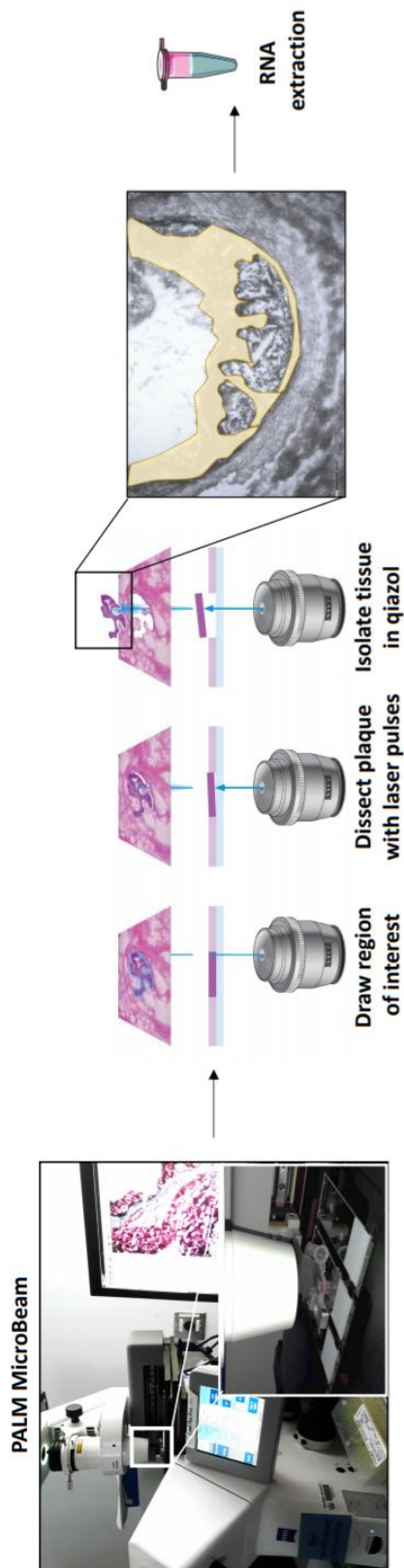


Figure 4.6 Workflow of Laser Capture Microdissection (LCM) of mouse plaque.

Aortic roots from LDLR^{-/-} fed with normal chow or high cholesterol diet (HCD) were isolated after 14 weeks of diet and fixed in cryo-preservative solution (OCT) and sectioned. Cross-sections of aortic roots (isolated from mice developing early fatty streak or advanced plaques) were used for LCM analysis performed with PALM MicroBeam microscope. The region of interest was first selected microscopically (the necrotic core was excluded from the tissue isolated in advanced plaques, as shown in the micrograph in this picture) and dissected from each section using laser pulses. 18 sections were used from each mouse. The detached tissue was then collected in Qiazol for following RNA isolation and quantitation of gene expression by qRT-PCR. The schematic of LCM procedure was adapted from [University of Gothenburg](#).

Figure 4.6 Workflow of Laser Capture Microdissection (LCM) of mouse plaque.

Following the isolation of early and advanced plaques, the samples integrity and RNA concentration was assessed by Agilent Bioanalyser. Plaque tissue is very heterogenous and processes such as oxidative stress, and release of detrimental substances in the tissue surrounding the atheromatous core and cholesterol crystals might decrease the quality of RNA³⁴². To reduce RNA degradation, frozen tissues were cut on the same day prior to be processed for LCM, and snap frozen immediately after their collection in Qiazol. Moreover, in order to exclude the region of major RNA degradation the necrotic core was not included in the dissected ROI from animals developing advanced plaques. The RNA yield obtained in the two groups following isolation was enough to perform downstream gene expression analysis (in the fatty streak group the average amount in the samples was 300pg/ul, in advanced lesions the average amount in the samples was 600pg/ul). RNA Integrity was reduced compared to RNA isolated from cells in culture but comparable between samples and in line with previous studies²⁹² (RIN obtained in samples was in a range of 3.8-5).

Following RNA isolation, samples were subjected to qRT-PCR analysis for *CARMN* and miR-143/145. Importantly, the average expression of two housekeeping genes (*Ppia* and 18S) was used for gene expression analysis to reduce sample variability and *CARMN* expression was then quantified relatively to the average obtained between the two housekeeping genes.

Interestingly, *CARMN* expression was downregulated by 2.7 ± 0.18 -fold in the advanced plaque versus the fatty streak lesions (Figure 4.7). Similarly, miR143 and miR-145 were found decreased by 2.7 ± 0.47 and 2 ± 0.6 -fold respectively, in the advanced versus early plaques, thus showing a negative correlation of the *CARMN* axis with the progression to more advanced mouse plaque (Figure 4.7).

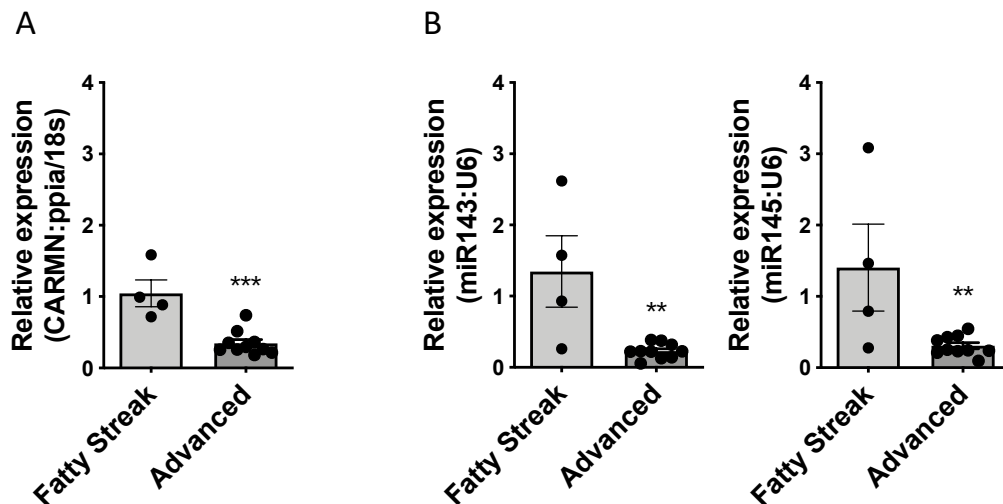


Figure 4.7 Expression of *CARMN* in mouse atherosclerosis.

Gene expression analysis were performed in plaque tissue isolated from aortic roots of *LDLR*^{-/-} isolated through Laser capture microdissection. Animals were fed for 14 weeks with normal chow diet to develop fatty streak or with high cholesterol diet to induce advanced plaque deposition. A), B) qRT-PCR results relative to *CARMN* and *miR-143/145* expression respectively, in plaques isolated from aortic roots of *LDLR*^{-/-} mice developing fatty streak (n=4 animals) or advanced plaques (n=11 animals). *Ppia*, 18S and U6, in the case of microRNAs, were used as housekeeping genes. Student's *t*-test was used to assess ** $P < 0.01$ and *** $P < 0.001$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

4.3.2 *CARMN* expression is lost in advanced versus early human plaques.

Considering the dysregulation of *CARMN* and the microRNAs in the advanced versus early fatty streak in mouse plaques, *CARMN* expression was assessed in RNA Sequencing, previously performed in our laboratory, in stable and unstable human carotid plaques²⁴⁴. Human plaques (n=4) were obtained using carotid endarterectomy from symptomatic patients. Each plaque was then macroscopically

dissected based on plaque features: “unstable” regions were identified by areas with a thrombus or intraplaque haemorrhage, and/or calcification. The adjacent, softer regions to the unstable area of the plaque, without obvious bleeding were considered as “stable” throughout the study as previously described²⁴⁴. (Figure 4.8). In this regard, it is important to underline that considering the high level of heterogeneity of atherosclerotic plaques and the simultaneous presence of cells at diverse de-differentiation and activation stages, regions defined as “stable” might still include pathologically advanced regions in microscopy. *Ex vivo* 18F-sodium fluoride emission tomography is an imaging technique which allows to detect the accumulation of the 18F-sodium fluoride in culprit lesions, as it localises specifically to calcium crystals. Studies have shown that the positive correlation between calcium and 18F-sodium fluoride emission can be used to establish plaque vulnerability in symptomatic patients³⁴³ and this method was used as validation for the identification of more unstable regions. RNA Sequencing was performed in both plaque regions and transcriptomic analysis to interrogate differential gene expression was performed (Figure 4.8). Interestingly, *CARMN* levels, expressed as Fragments per Kilobase of Transcripts per Million Mapped Reads (FPKM), was downregulated significantly from FPKM ~100 in the stable area to FPKM ~30 in unstable region (Log Fold Change -1.72, adjusted p-value(padj) 1.72 E-07) (Figure 4.8).

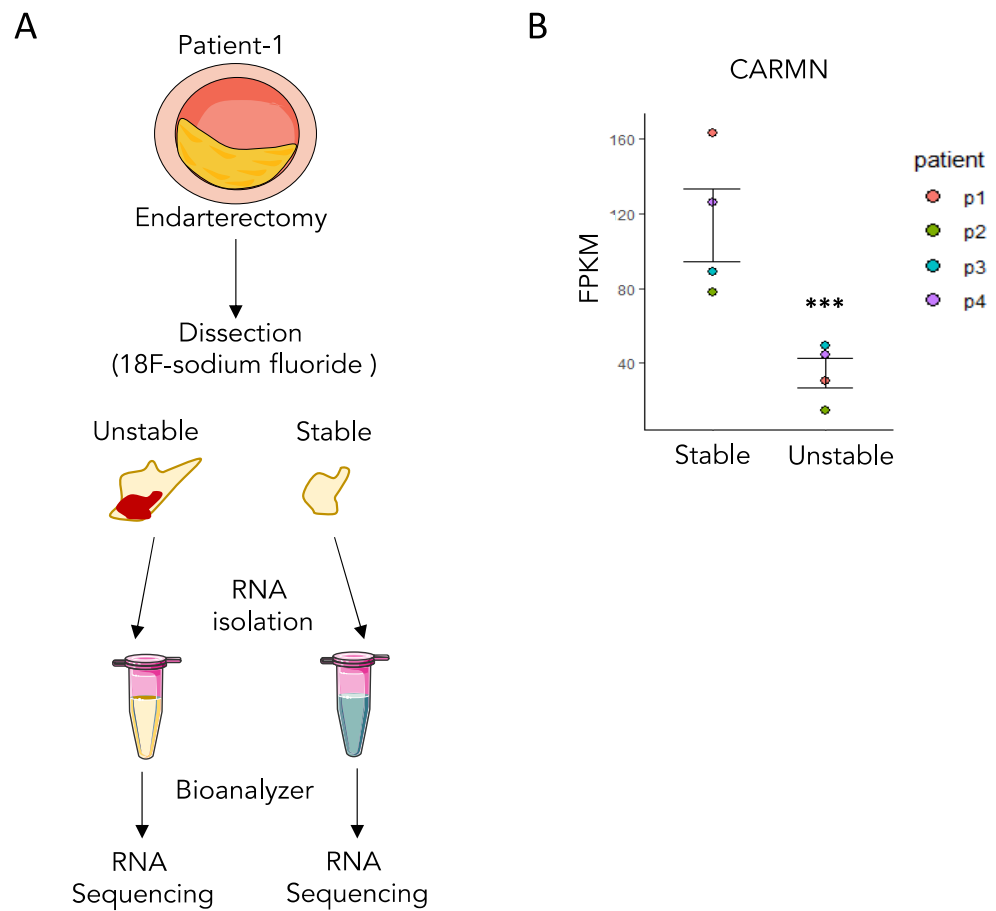


Figure 4.8 *CARMN* expression in human stable and unstable atherosclerotic plaques.

A) Schematic illustration of the workflow used to assess differential gene expression in human plaques. Plaques were obtained through endarterectomy from symptomatic patients. Each plaque was macroscopically dissected into two regions defined in this study as: “unstable” and the less pathological adjacent area, “stable” region. As described above, considering the high level of heterogeneity of atherosclerotic plaques and the simultaneous presence of cells at diverse de-differentiation and activation stages, regions defined as “stable” might still include

pathologically advanced regions in microscopy. 18F-sodium fluoride was used to validate the dissection. RNA was isolated from each region and its purity was assessed using bioanalyzer. RNA-Seq was then performed in both regions. B) *CARMN* expression as FPKM in stable versus unstable regions from 4 patients. P value and statistics were obtained based on the raw read counts using DESeq2 method. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Having assessed the expression of *CARMN* in RNA Sequencing analysis performed in human plaques, *CARMN* expression was then validated by qRT-PCR in samples isolated from other symptomatic patients, following the same protocols used for the preparation of samples for RNA-Seq analysis. Importantly, *CARMN* expression was downregulated 5 -fold in unstable versus stable region, validating the previous RNA-Seq results (Figure 4.9). Moreover, microRNA expression was also examined in the same samples. MiR-143 and miR-145 expression were concomitantly downregulated 10 and 19-fold respectively, in unstable plaques, therefore confirming the positive correlation between the expression of *CARMN* and microRNAs in human atherosclerosis (Figure 4.9).

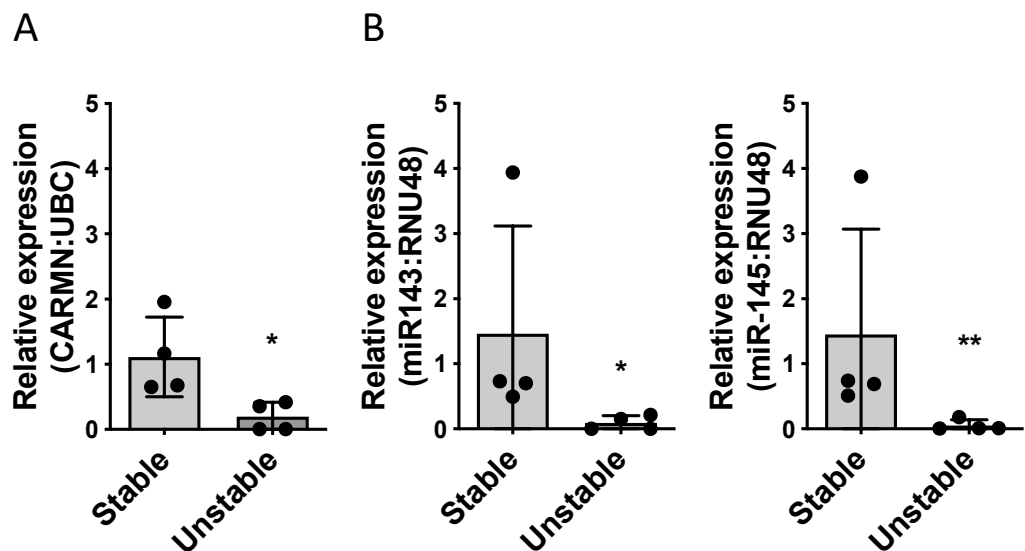


Figure 4.9 Expression of *CARMN* axis in human plaques.

Human plaques were isolated from symptomatic patients (experienced vascular stroke) by endarterectomy. Following collections, samples were macroscopically dissected into unstable region, displaying plaque rupture, calcification and intraplaque haemorrhage, and adjacent portions characterised by with less pathological phenotype, referred to as stable region. RNA was extracted from both regions for gene expression analysis. A), B) qRT-PCR data of *CARMN* and microRNAs miR-143 and miR-145 respectively, in human stable (n=4) and unstable (n=4) plaques UBC for gene expression and RNU48 for microRNAs were used as housekeeping control genes. Mann Whitney was used to assess * $P < 0.05$ and ** $P < 0.01$. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

4.3.3. *CARMN* localised in human atherosclerotic plaques.

Considering the heterogeneity of the plaque microenvironment, *CARMN* expression and location was investigated in cells within the lesions. In-situ hybridization (ISH) is a powerful technique to specifically localise gene expression within histological sections. This technique exploits the use of highly specific single stranded nucleotide RNA probes (22-23nt) designed to hybridise with target transcripts. The probes are double labelled with digoxigenin at both ends (5' and 3') to increase the detection of low-abundance targets in tissues. In the case of *CARMN*, a customised probe was designed in the first exon to target most of the *CARMN* transcripts using the Exiqon (Qiagen) designing tool which considers different parameters (secondary structures, DNA and RNA melting temperature, GC content). The customised designed probe was also tested for its specificity to recognize *CARMN* transcripts by blasting the sequence to the human transcriptome using Primer Blast software. Moreover, a scramble probe specifically designed to not target any RNA transcript was used as negative control for the experiment. Atherosclerotic plaques can be very challenging tissues

to be stained due to their high degree of tissue degradation. Therefore, the efficiency of ISH protocol was first tested in human plaques using a positive control probe targeting Ubiquitin C (UBC) human transcript (Figure 4.10). UBC expression localised mostly in the cytoplasmic compartment of cells.

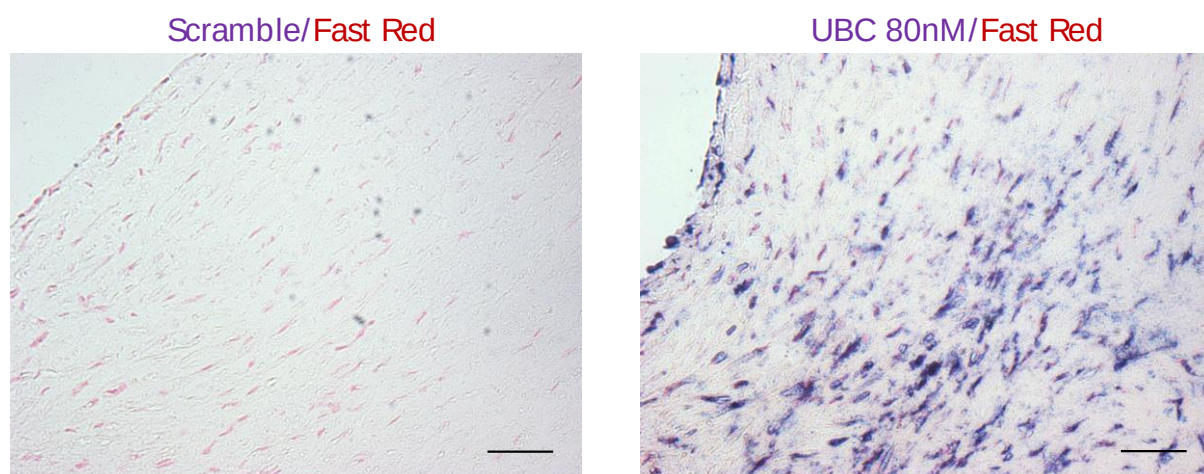


Figure 4.10 Localisation of UBC in human plaques.

Human carotid plaques were fixed following isolation from symptomatic patients and sectioned into 5 μm sections used for histological examination. This picture includes images obtained through In-situ Hybridization of UBC, considered a positive control for the experiment, and scramble negative control in human stable plaques. Nuclear fast red was used for nuclear staining. Scramble (80nM) and UBC (80M) positive control probe are visualised in purple. Pictures were acquired at 63X magnification, scale bar 200 μm .

Having optimised the protocol for the detection of UBC transcript in human plaques, the localisation of *CARMN* was assessed in human plaques. After assessing the specificity of the probe by blasting the probe sequence to ENSEMBL, two different probes concentrations, 50 and 100nM, were tested to detect *CARMN* expression in plaques. Over the two tested concentrations, 100nM was selected to proceed with further experiments as it provided a signal while the concentration at 50nM did not

detect the expression of *CARMN* in the tested sections (Figure 4.11). Considering that lncRNA expression can be tissue-specific, multiple sections belonging to different patients have been assessed. In particular, *CARMN* signal was detected in cells localising close to the arterial lumen, presumably in the fibrotic cap, generally composed of dysregulated SMCs. Also, *CARMN* was present in cells surrounding the necrotic core, which is known to include inflammatory cells such as macrophages (Figure 4.11 B).

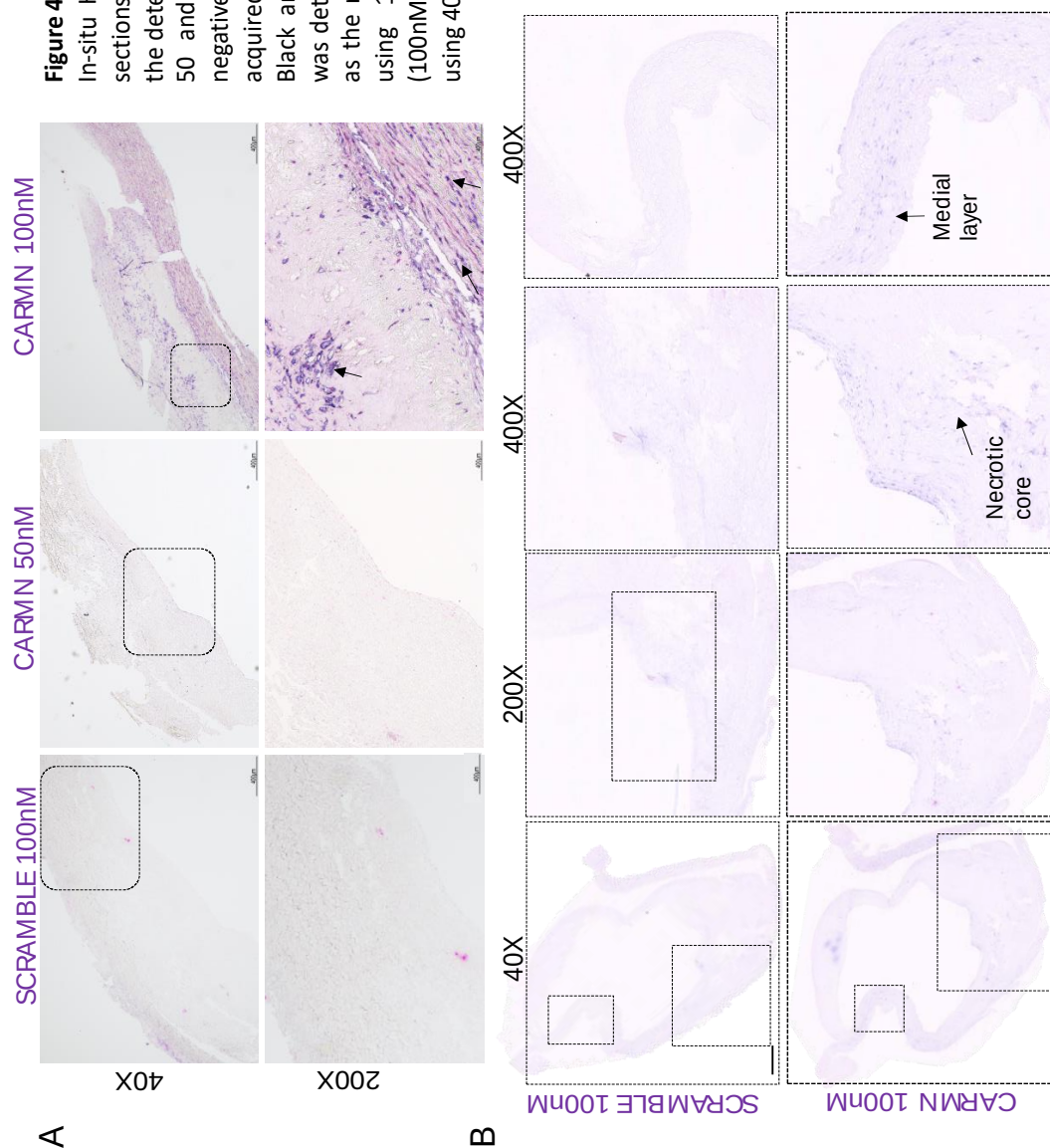


Figure 4.11 CARMN localisation in human plaques.
In-situ Hybridization of CARMN (purple signal) in cross-sections of human plaque. A) Panel of pictures illustrating the detection of CARMN using two probe concentrations, 50 and 100nM, and Scramble (100nM) was used as negative control probe in an adjacent section. Pictures acquired at 40-200X magnification. Scale bar 400µm. Black arrows indicate CARMN positive cells. B) CARMN was detected in different regions of human plaques such as the necrotic core and the medial layer (black arrow) using 100nM probe concentration. Scramble probe (100nM) was used as negative control. Pictures acquired using 40-200-400X magnification, scale bar 200 µm.

Figure 4.11 CARMN localisation in human plaques.

4.3.4. *CARMN* co-localises with alpha-SMA and CD68 markers.

Considering that *CARMN* expression was detected in different regions of the human plaque, the next step was to interrogate which cell type populating the lesion express *CARMN*. In particular, vSMCs and activated macrophages are the most abundant cell types inhabiting the plaque, therefore *CARMN* expression was specifically investigated in both cell types. Previous results indicated that *CARMN* expression was found downregulated in unstable plaques, therefore stable plaques were used to address whether *CARMN* co-localised with alpha-smooth muscle actin (α SMA), typical marker of vSMCs and Cluster of Differentiation 68 (CD68), marker for activated infiltrated macrophages. To this end, ISH technique was exploited to detect *CARMN* in combination with Immunohistochemistry (IHC) assay for visualization of marker genes. Importantly, experimental controls for both techniques (scramble and IgG) were included in the experimental design. Therefore, following optimisation of each staining singularly, co-localisation of *CARMN* and α SMA or CD68 was performed in plaque cross-sections. Importantly, *CARMN* was found co-localising with α -SMA signal, confirming its enrichment in vSMCs (Figure 4.12).

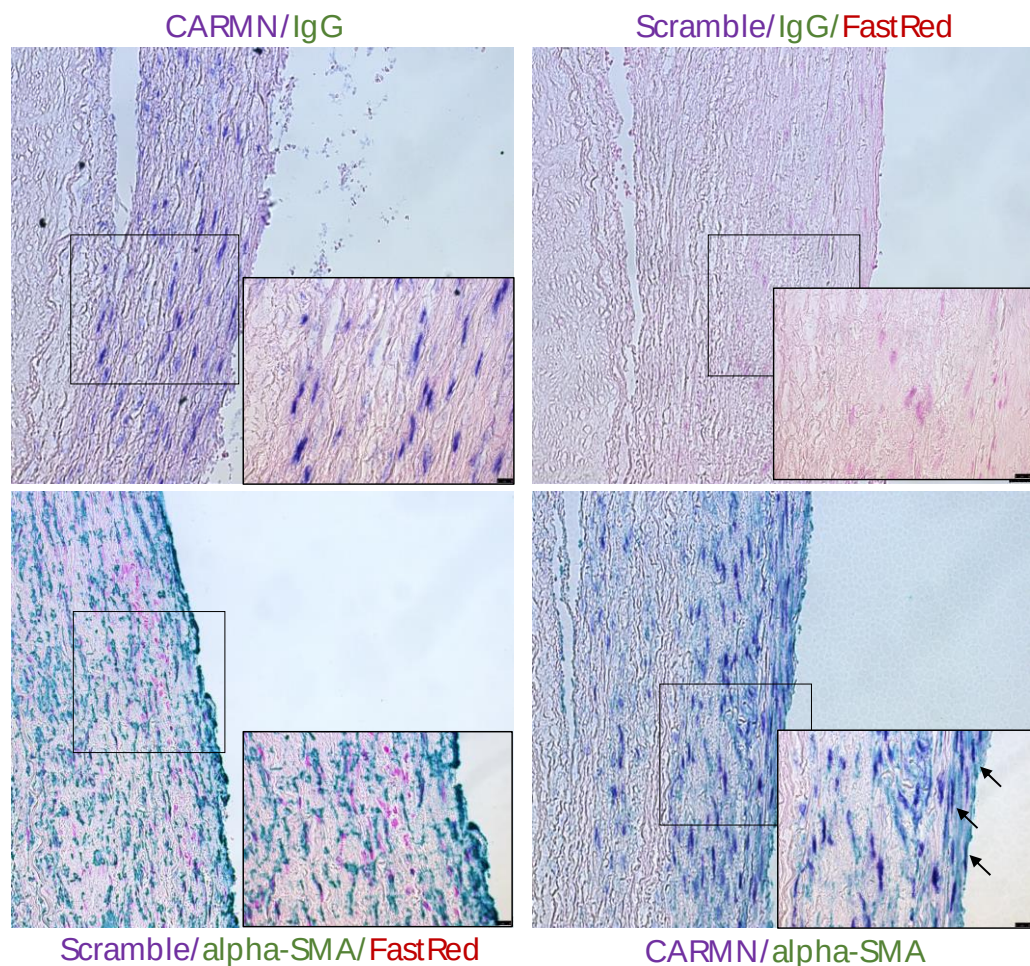


Figure 4.12 *CARMN* co-localised with α SMA in human stable plaques.

Co-localisation of *CARMN* or Scramble probe at 100nM (in purple) with α SMA or IgG negative control (green-blue signal) in adjacent cross-sections of stable plaques from symptomatic patients. Nuclei are stained in red. Pictures were acquired at 400 and 600X (bottom right zoomed in picture in each condition, black arrows indicate co-localisation signal), scale bars 10 μ m.

Further co-localisation experiments indicated that *CARMN* localised with a marker for inflammatory macrophages within the plaque, CD68 (Figure 4.13). Considering that dysregulated vSMCs retain their ability of migrating towards the inner atheromatous core and expressing pro-inflammatory markers, it would be interesting to investigate whether the pool of CD68-expressing cells positive for *CARMN* might

include de-differentiated macrophage-like vSMCs, such as by using lineage-tracing mice to identify the origin of CD68 positive cells, whether immune or smooth-muscle cell derived.

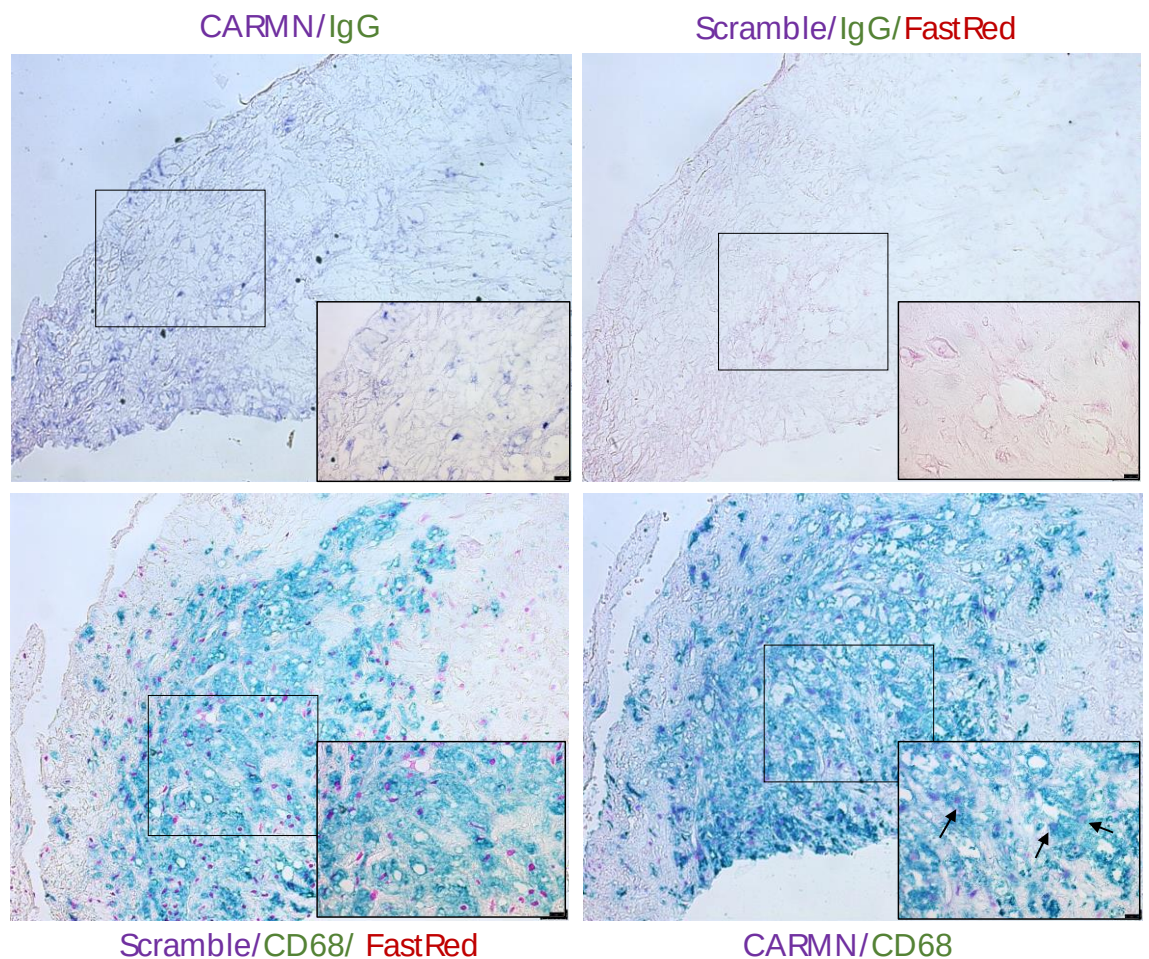


Figure 4. 13 *CARMN* expression co-localised with CD68-positive cells within human plaques.

Co-localisation of *CARMN* or Scramble probe at 100nM (in purple) with CD68 or IgG negative control (green-blue signal) in adjacent cross-sections of stable plaques from symptomatic patients. Nuclei are stained with nuclear fast red. Pictures were

acquired at 400 and 600X (bottom right zoomed in picture in each condition, black arrows indicate co-localisation signal), scale bars 10µm.

4.4 Discussion

In this chapter the expression profile of *CARMN* and miR-143/145 was explored in mouse and human atherosclerosis and revealed a downregulation of the entire *CARMN* axis in human and mouse early to advanced plaque phenotype. In-situ hybridisation performed in human plaques also allowed to detect *CARMN* expression in different locations of human plaques and showed that *CARMN* co-localises with two of the main cell types comprising the plaque, smooth muscle cells and macrophages.

CARMN belongs to the few lncRNAs well conserved across species, which is an important characteristic, and provides the opportunity to study and characterise the role of this lncRNA *in vivo*. Of note, also in the case of the mouse genome, the annotation of lncRNAs might be incomplete. Therefore, the expression levels of *CARMN* transcripts were interrogated in isolated mouse aortic smooth muscle cells (ASMCs). The latest Ensembl annotation (GRCh38/hg38 genome assembly) includes two transcripts belonging to *CARMN* locus, hence isoform-specific primers were designed spanning the exon-exon junctions. In addition, a primer set amplifying a common region to both isoforms was included in the design. Experimental primer validation confirmed the expression of both isoforms in mouse cells. Of note, this strategy does not allow comparative analysis of transcripts abundances, as the designed primers were not tested for their efficiency of amplification. However, considering that the purpose of this study is to widely characterise *CARMN* locus in human and mouse atherosclerosis, similarly to what was previously described in Chapter 3 for *in vitro* studies, a common set of primers was used throughout the study to assess *CARMN* expression.

Previous results obtained *in vitro* through RNA-Sequencing and functional validation in hCASCs, indicated the possibility for *CARMN* to play a role in the pathophysiology

of atherosclerosis. In previous Chapter 3, *CARMN* axis was described modulated in response to pro-pathological processes involved in the disease progression, such as loading of hCASCs with cholesterol or following the treatment of hCASCs with pro-pathogenic cytokine stimulation. Herein, the expression levels of *CARMN* axis were tested in LDLR^{-/-} mouse model of atherosclerosis. To characterise the levels of *CARMN* in the transition process from early to advanced plaque, two groups of animals developing fatty streak or more advanced plaques were included in the experimental design. As previously mentioned in the introduction of this chapter, studies have shown that the genetic depletion of the receptor for LDL particles favours the accumulation of cholesterol in plasma and subsequent formation of foam laden cells deposited at different sites of mouse vascular system. This effect is enhanced by the association of a diet high in cholesterol (HCD), which favours progression to advanced plaques characterised by large necrotic cores, cholesterol crystals deposition and calcification. The feeding of LDLR^{-/-} mice with HCD for 14 weeks, produces hypercholesterolemia and advanced atheroma. Atherosclerosis-prone regions of the vascular bed where atherosclerosis develops first in mice are the aortic root, arch, and brachiocephalic artery. In consideration of the small size of the brachiocephalic trunk and the difficulty of tissue isolation, only the aortic trunk was used for gene expression analysis. Laser Capture Microdissection is nowadays a well-established technique to isolate and characterise specific regions in the context of tissue heterogeneity³⁴⁴. This powerful technique, previously used for atheroma isolation^{292,337,345,346}, was used to micro-dissect the plaque area underlying the aortic valves of LDLR^{-/-} animals developing early and advanced lesions, using laser microscopy, and RNA was isolated for downstream analysis of gene expression profile. However, plaques are characterised by a different cellular composition with different vSMC and macrophage content during the transition from early to advanced lesions. Therefore, it is not possible to clearly determine whether the decrease in *CARMN* expression is the result of a change in plaque composition or is the result of a differential expression within de-differentiated cells expressing *CARMN*. Albeit the limitation associated with the study of the whole tissue, gene expression analysis by

qRT-PCR, revealed downregulation in the levels of *CARMN* and miR-143/145 in the plaques isolated from animals developing advanced lesions compared to early fatty streak. In this regard, further assessment of marker genes, such as *Acta2* or *CD68*, could be included to assess the cell composition of early and advanced plaques. The microdissection of early and late plaques following staining with these two markers could also be a good indicator during the LCM technique, however this strategy results very challenging as the integrity of the tissue and RNA might be affected. Nonetheless, to understand the cause/consequence effect of *CARMN* modulation in the progression to late phases of plaque stages, would require more interventional approaches *in vivo*.

The correlation of *CARMN* with mouse atherosclerosis opened more questions on whether the differential expression profile observed in mouse also characterises human atherosclerosis. Therefore, these observations guided the next steps of investigation leading to the assessment of *CARMN* expression in human RNASeq analysis, previously performed in the laboratory in human stable and unstable plaques. In particular, previous studies, aiming at defining the stages of atherosclerosis, described that human plaques can advance to reach instability and plaque rupture^{347,41}. Morphological studies using serial cross-sections have shown that these plaques are characterised by intraplaque haemorrhage, accumulation of hydroxyapatite aggregates, large necrotic core and thin smooth muscle fibrotic cap⁴⁶. Among these features, macroscopically visible intraplaque haemorrhages and calcification (assessed through 18F-sodium fluoride emission) were considered in this study to macroscopically dissect the stable region from unstable regions of lesions isolated from patients following acute neurovascular event (symptomatic). RNASeq analysis performed in both regions allowed to compare gene expression profiles and the expression of *CARMN* was found significantly downregulated in the unstable and advanced plaque region compared to the early plaques, confirming the observation in mouse plaques. Moreover, *CARMN* expressed transcripts were found highly expressed in the stable lesions (FPKM~100) thus indicating the possibility for *CARMN*

to be expressed by multiple cell sub-populations located within the plaque. *CARMN* levels were further validated by qRT-PCR analysis in other samples of stable/unstable plaques, obtained using same experimental conditions from other patients. The expression of *CARMN* and microRNAs was observed decreased in unstable versus stable plaque, suggesting that also in humans the downregulation of the lncRNA associates with the progression from early to latest stages of the disease. With regards to the functional role of *CARMN* in human atherosclerosis, it might be possible that the decrease observed in the expression of *CARMN* and miR-143/145 correlates with a decrease in SMC number during the advancement to a more vulnerable stage versus stable plaque. Similarly to the experiments performed in mouse, the analysis of the whole tissue does not allow the clear identification *CARMN* expression at a cellular level. The quantification of marker genes (*ACTA2*, *MYH11*, *CD68*) alongside with *CARMN* expression, might allow to assess the correlation between *CARMN* decrease and SMC content in plaque. However, considering the possibility of co-expression of these markers by de-differentiated SMCs, this approach might be limited by the possibility of co-expression of these markers in different cell types included in early and advanced plaques. Histological analysis performed in stable plaques showed that *CARMN* co-localises with smooth muscle cells within the plaque and infiltrated macrophages, indicating that *CARMN* is also expressed in immune cells. In this regard, *in vitro* and *in vivo* studies have shown that SMCs can de-differentiate towards pro-inflammatory activated macrophages, able to co-express SMC and immunity-like markers^{315,348}. However, to verify if the pool of *CARMN*/CD68 double positive cells are of smooth muscle cell origin, would require more studies with SMC reporter mice to correlate *CARMN* expression with SMCs plasticity and heterogeneity in the context of plaque formation.

In summary, the expression profile of *CARMN* and miR-143/145 was comprehensively characterised in human and mouse atherosclerosis, where both noncoding RNAs were found negatively affected in their expression towards more complex and advanced plaque phenotype. Therefore, these results suggest the possibility for

CARMN axis to be involved in the key events characterising the transition between early to advanced stages of plaque development. Localisation experiments have also confirmed the expression of *CARMN* in the nucleus of vSMCs populating the plaque, and its association with CD68 expressing cells. Therefore, these results lead to the next question aiming to understand the functional and molecular consequences of *CARMN* axis loss to the development of disease *in vivo*.

Chapter 5. Characterisation of *CARMN* function in a mouse model of atherosclerosis.

5.1 Introduction

Genome editing tools have revolutionised the approach to studying lncRNA functions, facilitating the manipulation of their expression in a highly specific and efficient way. Several approaches to genetically modulate lncRNAs expression, through silencing or activation, have been developed for *in vivo* and *in vitro* studies. One example is the use of Zinc Finger nucleases (ZFNs), artificial structures composed by zinc finger binding proteins combined with the endonuclease FokI. In particular, this strategy allows the production of double-strand DNA breaks to splice out a specific portion of DNA which is then replaced with a desired sequence³⁴⁹. Although this technology was mostly used to study protein coding genes³⁵⁰, it was used for the study of lncRNA function, for example in the case of the lncRNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1)^{238,351,352}. The lncRNA MALAT1, a nuclear lncRNA was initially described as being associated with lung cancer³⁵³. Subsequently MALAT1 was studied in the context of atherosclerosis, where its expression was found to be downregulated in plaques from atherosclerotic patients versus controls²³⁸. Loss-of-function of MALAT1 was performed by ZFN insertion of a poly-adenylation site used as RNA destabilizing element to silence the MALAT1 transcript. This approach resulted in advanced atherosclerosis in MALAT1^{-/-} animals versus wild type controls^{238,354}. Another example of genome editing technology exploiting engineered endonucleases is Transcription activator-like effector nucleases (TALEN)³⁵⁵. The principle of action of this strategy is very similar to ZFN, where the FokI endonuclease is combined with a DNA-binding protein domain named TAL. However, the TALEN system has enhanced specificity due to single base recognition of TALE-DNA instead of the triplet-confined ZFN system. This technology has allowed the study and characterisation of the mechanism of action of the lncRNA ANRIL. ANRIL is located in the human chromosome 9.21.3 region, a locus characterised as one of the most important risk factors for the development of

CVDs³⁵⁶ and atherosclerosis^{357–360}. To understand the consequences of 9.21.3 risk haplotype, TALEN was used to produce homozygous and heterozygous deletion of the entire locus in human iPS cells differentiated towards vSMC lineage. Importantly, the deletion of the risk haplotype restored the vSMC phenotype, while the transcriptional profile resembling the non-risk haplotype and overexpression of ANRIL partially induced vSMCs to the risk-haplotype phenotype²⁴⁰. However, although these technologies have allowed the study and characterisation of the molecular interactions of coding and non-coding transcripts, the prediction of their specificity can be challenging. Clustered regularly interspaced short palindromic repeat/CRISPR associated 9 (CRISPR/Cas9) technology represents an efficient alternative gene editing strategy to the previously developed restriction-nuclease based strategies (ZFN, TALEN). Unlike previous genome editing methods, CRISPR/Cas9 technology is more versatile, specific, and easily applicable for *in vitro* and *in vivo* studies. The CRISPR/Cas9 system exploits the *Streptococcus pyogenes*' defensive mechanism against foreign DNA. It requires two components: the endonucleases Cas9, able to introduce double-strand DNA breaks, and a short single guide RNA (sgRNA) complementary to the target site. The sgRNA pairs to the DNA target, guiding the Cas9 to the desired DNA region^{361,362}. The double-strand breaks generated are blunt-ended at the level of the target region, which are then repaired by Non-homologous End Joining (NHEJ) repair pathway. This mechanism favours the insertion of indel mutations resulting in the inactivation of the gene of interest. Otherwise, the insertion of a new portion at the site of cut can be obtained by providing a repair template homologous to the cut site³⁶³. The advantages of this strategy allowed its application in a wide spectrum of physiologic and pathologic contexts. In particular, CRISPR/Cas9 offers the possibility to reduce off-target effects due to the site-specificity of sgRNA design³⁶⁴. Moreover, the simplicity of its design decreases the time and costs associated with the applications of this technology³⁶⁵. Furthermore, the versatility of this approach allows to concurrently modulate multiple loci by using multiple sgRNAs for large-scale screenings^{361,366}. In addition to this, the possibility to use multiple sgRNAs facilitates the regulation of very complex

gene loci expression, such as the *CARMN* locus which includes multiple transcriptional isoforms and transcriptional start sites, modulating the entire panel of transcripts with the same efficiency, therefore resulting a more efficient option than RNA interference³⁶⁷. Therefore, CRISPR/Cas9 was chosen as the most suitable strategy to functionally characterise *in vivo* the consequences of *CARMN* depletion during the progression of atherosclerosis. Importantly, CRISPR-mediated genome editing has been applied in the context of atherosclerosis, not only to modulate atherogenic-associated genes such as PCSK9^{368,369}, but also to characterize lncRNA function and expression-associated phenotypes. This is the case of the lncRNA ANRIL whose linear transcript expression was associated with increased risk of atherosclerosis³⁷⁰. However, the expression of the circularised form of this lncRNA, circANRIL, was demonstrated to positively correlate with non-risk haplotype revealing atherosclerosis-protective role²⁴¹. In particular, CRISPR technology was exploited to genetically deplete circANRIL to produce knock-out cell lines and study the effect of this modulation on cell apoptosis and proliferation²⁴¹. The application of CRISPR/Cas9 to study lncRNA functions during the development of vascular disease *in vivo* is very powerful due to all the above-mentioned features of this strategy, however it requires the generation and crossing of multiple genetic modified mouse lines. This includes the modulation of the lncRNA of interest and the generation of atherosclerotic mouse model using the traditional ApoE^{-/-} or LDLR^{-/-} mice), which increases the time and costs. To solve this issue, gene therapy approach is a very useful approach to avoid germline genetic engineering for the generation of hyperlipidaemic mouse model in a rapid and cost-effective way. In particular, Adeno-associated viral vectors (AAV) have been characterised as excellent tools to effectively modulate gene expression in a safe, highly specific and long-term effective way³⁷¹. Indeed, AAV were used as valid alternative to efficiently induce atherosclerosis in mice by the overexpression of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9)^{372,373}. PCSK9 belongs to the subtilisin family, mainly expressed in the liver, with a function in cholesterol homeostasis³⁷⁴. In particular, the circulating PCSK9 binds to the Low-density lipoprotein receptor (LDLR) favouring the

internalisation of PCSK9-LDLR complex by hepatocytes, thus decreasing the hepatic uptake of LDL, which then accumulate in the systemic circulation. Previous studies have correlated dysregulation of PCSK9 functions due to gain-of-function mutations with substantial increase of LDL particles in blood, while disruption of its activity was found correlating with decrease in circulating cholesterol levels³⁷⁵. Therefore, recombinant AAV particles (AAV8) were injected once to constitutively overexpress PCSK9 levels in the liver of mice. Moreover, the combination with high cholesterol diet led to pronounced hyperlipidaemia and allowed the formation of advanced plaques following 18 weeks post injection³⁷⁶. Therefore, utilising this model was an ideal option for the study of the phenotypic consequences of CRISPR/Cas9 mediated knock-out of *CARMN* on the development of atherosclerosis.

5.2 Aims

In this Chapter, *CARMN* role was explored in the pathophysiology of atherosclerosis *in vivo*, and the hypothesis that is proposed to be tested is

The genetic depletion of CARMN axis contributes to the progression to pronounced advanced phenotype.

Therefore, the aims of this chapter are:

- To explore the consequences of *CARMN* depletion on the expression of microRNAs miR-143 and miR-145 *in vivo* using CRISPR-Cas9 mediated approach.
- To investigate the effect of *CARMN* loss on the development of atherosclerotic plaques in AAV8-PCSK9 mediated mouse model.
- To characterise the cellular composition of the lesions developed in aortic roots and brachiocephalic arteries of *CARMN*-depleted atherosclerotic mice.

5.3 Results

5.3.1 Generation and validation of CRISPR/Cas9-mediated *CARMN* knock-out *in vivo*.

To study the consequence of *CARMN* modulation in mouse atherosclerosis, the deletion of the *CARMN* locus achieved by CRISPR/Cas9 genome editing. To date, many different variants of this technique have been validated and widely used to study the effects of gene manipulation on specific physiologic and pathologic processes. In particular, *CARMN* knock-out (*CARMN*^{-/-}) and wild type (*CARMN*^{+/+}) animals were purchased by TACONIC Biosciences and the following parameters were considered for the design of CRISPR/Cas9-mediated *CARMN* constitutive depletion. As previously mentioned, two *CARMN* transcripts exist in mouse genome (chromosome18) and similarly to human, miR-143 and miR-145 are located downstream of the *CARMN* isoforms (Chapter 4). The knock-out strategy was applied using two single guide RNAs (sgRNAs) designed in the portion surrounding the *CARMN* Transcriptional Start Site (TSS). The proximal sgRNA was designed at 4.8 kilobases upstream to the TSS while the distal sgRNA targeted exon one, common to *CARMN* transcripts (in red in Figure 5.1 A, B). The sgRNAs were selected based on the position and the low number of predicted off-targets based of GRCm38/mm10 assembly (2011). Therefore, this strategy allowed the deletion of exon 1, leaving exons 2/3 and the miRNA stem loops intact. Constitutive *CARMN* knock-out mice were generated through the co-injection of Cas9 mRNA and proximal and distal sgRNAs into the cytoplasm of C57BL/6NTac zygotes.

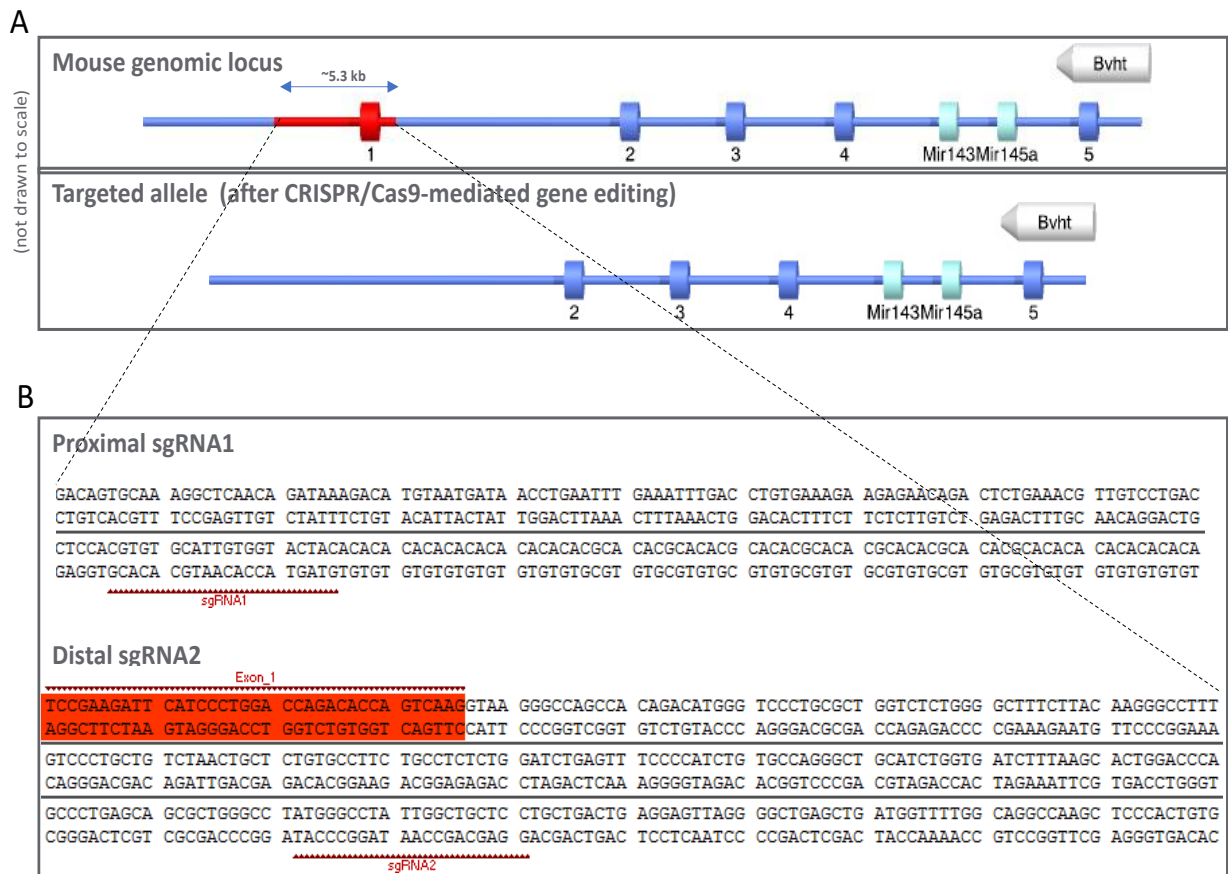


Figure 5.1 CRISPR/Cas9 strategy used to delete *CARMN* locus in mouse chromosome 18.

CRISPR/Cas9 was used to constitutively knock-out *CARMN* loci in mouse. This strategy is based on the use of two sgRNAs which guide the endonuclease Cas9 to the selected target sequence. In the case of loss-of-function experiment, the two sgRNAs are designed before and after the portion of interest. In the case of *CARMN*, two sgRNAs targeting the portion of genome before exon one (promoter) and after (intronic portion). A) Schematic representation of the longer *CARMN* transcript in dark blue, microRNAs miR-143 and miR-145 in light blue and Braveheart lncRNA in grey, before and after the excision of the promoter + 1st exon. B) Sequence details of the sgRNA design targeting a proximal region to *CARMN* promoter (proximal, sgRNA1) and 4.8 kilobases upstream the TSS, (distal, sgRNA2). In total, 5.3 kilobases including lncRNA promoter and the first exon were removed from *CARMN* locus.

In order to assess the efficiency of this strategy, the aortic arch and thoracic aorta were collected from homozygous *CARMN*^{-/-} F2 generation (C57Bl/6N background) adults and littermate controls *CARMN*^{+/+}, for gene expression analysis (Figure 5.2). In particular, *CARMN* expression was evaluated in both genotypes using common primer set (details of design and localisation in Chapter 4) targeting exon 1 of *CARMN* isoform 201 and 202. *CARMN* expression was found undetectable in the aortas of *CARMN*^{-/-} (n=6) versus animals *CARMN*^{+/+} (n=4) animals (Figure 5.2).

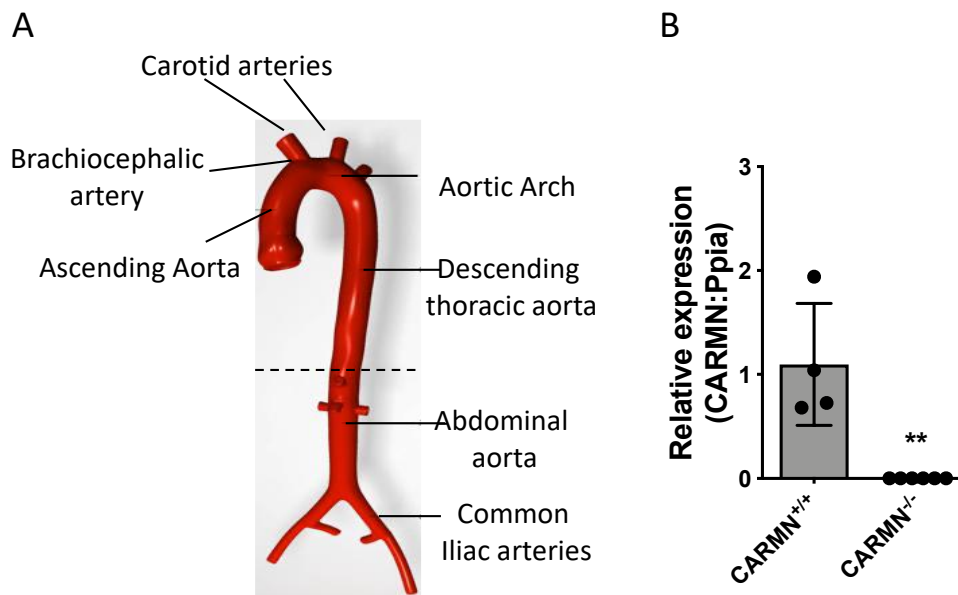


Figure 5.2 Expression of *CARMN* in aortas isolated from *CARMN*^{-/-} versus wild type animals at baseline.

Adult 8-weeks old *CARMN*^{-/-} and wild type mice were sacrificed and aortic vessel (from the base of the heart until the descending portion) was isolated and snap frozen. The tissue was then processed for RNA extraction and downstream gene expression analysis through qRT-PCR. A) Anatomical detail of the mouse aorta after isolation. The aortic trunk and descending aorta were collected from each animal. The discontinue line indicates approximatively the portion of tissue collected for downstream gene expression analysis following RNA extraction. B) qRT-PCR data of *CARMN* expression in aortas isolated from *CARMN*^{-/-} (n=6) and *CARMN*^{+/+} (n=4)

animals. Cyclophilin (Ppia) was used as housekeeper gene. Mann Whitney was used to assess significance. ** $P < 0.01$. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

Since two *CARMN* transcripts exist, their expression was assessed in the same samples, using isoform-specific primer sets. In particular, isoform 201 and isoform 202 showed a 6.65 ± 0.13 and 11.3 ± 0.06 fold downregulation, respectively, in knock-out versus wild type animals (Figure 5.3 A, B). However, although the levels of the two transcripts were significantly decreased, the expression of both transcripts was detectable ($C_t \sim 22$ at baseline to ~ 27 in knock-out animals). Considering that the primer sets, specifically amplifying each transcript, were designed targeting the exons downstream of exon 1, these results might indicate the existence of truncated transcripts or un-annotated isoforms with a different TSS from *CARMN* isoform 201 and 202.

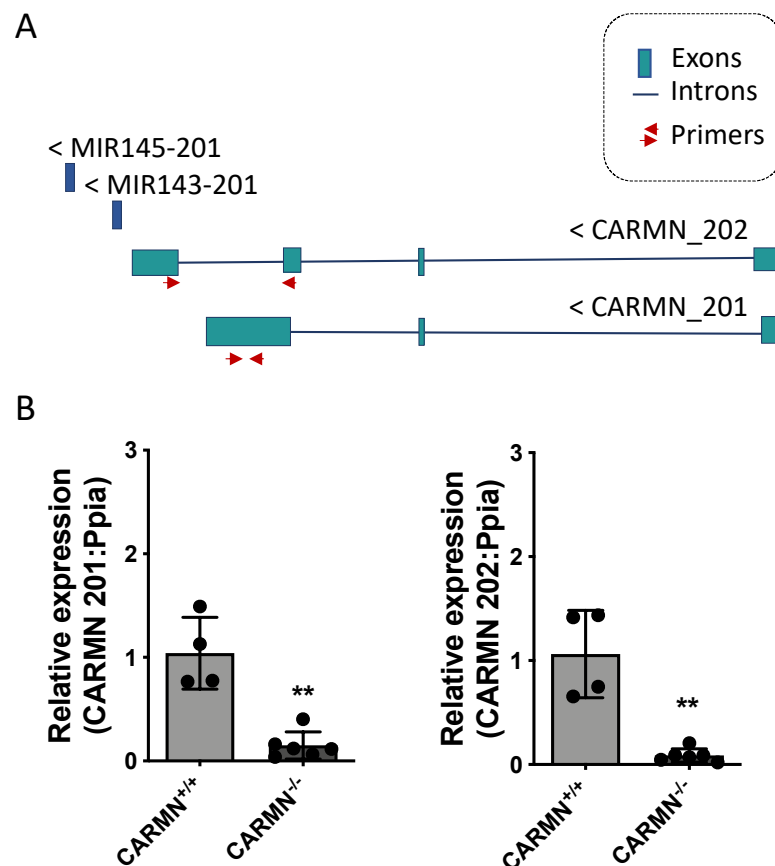


Figure 5.3 Expression of *CARMN* transcripts using isoform-specific primers in aortas from *CARMN*^{-/-} and wild type animals at baseline.

Adult 8-weeks old *CARMN*^{-/-} and wild type mice were sacrificed and aortic arches from the base of the heart until and including the descending aorta) were isolated and snap frozen. The tissue was then processed for RNA extraction and downstream gene expression analysis through qRT-PCR. A) Schematic representation of mouse *CARMN* isoforms located in chromosome 18, 201 and 202 (light blue) and the pri-microRNAs miR-143 and miR-145 (dark blue). The red arrows indicate the location of isoform-specific couple of primers used to amplify isoform 201 and 202. The black arrows next to the transcripts name indicate their sense of transcription. B) qRT-PCR data of *CARMN* transcript 201 and 202 respectively, obtained with transcript-specific primer sent in aortas isolated from *CARMN*^{-/-} (n=6) and *CARMN*^{+/+} (n=4) animals. Cyclophilin (Ppia) was used as housekeeper gene. Mann Whitney was used to assess significance ** P< 0.01. Graphs are shown as datapoints including the mean ± standard error of the mean.

Since the structural composition of the locus includes the two microRNAs downstream of the *CARMN* transcripts, similarly to human, the effect of *CARMN* knock-out strategy on the levels of the microRNAs was interrogated and the expression of microRNAs miR-143 and miR-145 was assessed in the same samples. In particular, miR-143 and miR-145 expression levels were decreased to 4.5 ±0.05 and 4.8 ±0.07 fold respectively in *CARMN* knockout mice versus wild type controls (Figure 5.4). This effect may be due to the direct regulation of the pre-microRNA transcript by the CRISPR/Cas9 strategy. While, whether the modulation of microRNAs is consequential and modulated by *CARMN* remains an open question and would require more studies to dissect the cause/consequence cascade of events.

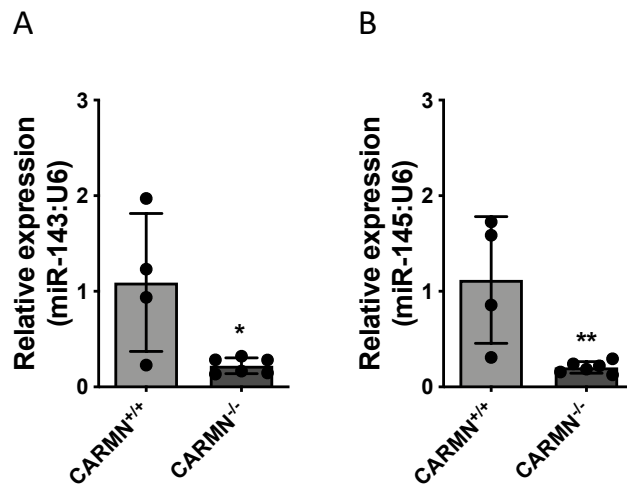


Figure 5. 4 Expression of miR-143 and miR-145 in aortas from *CARMN*^{-/-} and wild type animals at baseline.

Aortas were isolated from *CARMN*^{-/-} (n=6) and *CARMN*^{+/+} (n=4) animals and analysis of microRNAs expression was performed. A), B) qRT-PCR data relative to miR-143 and miR-145 respectively, U6 was used as housekeeper gene. Mann Whitney was used to assess * P< 0.05 and ** P< 0.01. Graphs are shown as datapoints including the mean ± standard error of the mean.

The characterisation of the locus included the detection of the expression of Braveheart (BVHT), a mouse-specific lncRNA, antisense to *CARMN*, located approximately 18 kilobases downstream to *CARMN* exon 1. This lncRNA was previously described to be highly expressed in the heart with important function during the mesodermal differentiation towards the cardiac fate³⁷⁷. Moreover, BVHT was found to be silenced in *CARMN* knock-down in mouse cardiomyocytes²⁸⁵. Therefore, BVHT expression was investigated following *CARMN* modulation, however, no significant change in the levels of this lncRNA was observed in aortas from *CARMN*^{-/-} and *CARMN*^{+/+} animals (Figure 5.5).

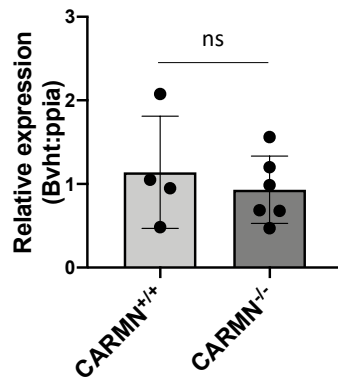


Figure 5.5 Expression of Braveheart in *CARMN* knockout mice at baseline.

qRT-PCR data relative to Bvht assessed in the aortas of *CARMN*^{-/-} (n=4) and *CARMN*^{+/+} (n=6) animals. Cyclophilin (Ppia) was used as housekeeper control gene. Mann Whitney was used to assess statistical significance. ns = not significant. Graph is shown as datapoints including the mean $\pm$ standard error of the mean.

5.3.2 PCSK9-mediated model of murine atherosclerosis

Atherosclerosis was induced in mice through the overexpression of the proprotein convertase subtilisin/kexin type 9 (PCSK9) delivered by recombinant Adeno-associated viral vector serotype 8 (AAV8), combined with high cholesterol diet (HCD-0.25% cholesterol) for 18 weeks following AAV8-PCSK9 injection, in line with what was previously validated³⁷². In order to assess preliminary efficiency and appropriate dose of the AAV to induce hypercholesterolemia and sufficient atherosclerosis in our lab, an initial pilot study was undertaken with a small group of animals. In this study n=5 C57BL/6NTac 8-weeks old male mice were injected i.p with recombinant AAV8-PCSK9 obtained through collaboration with Prof. Mauro Giacca (AAV Vector Unit, ICGEB, Trieste). In particular, this experiment was part of a dosage dependent experiment performed in collaboration with other laboratories in Maastricht (Prof. Sluimer team) and Glasgow (Prof. Maffia team), where 1×10^{11} (dosage chosen in Glasgow), 3×10^{11} (dosage chosen in Maastricht) and 1×10^{12} vg/mouse (dosage chosen

in this preliminar study) where included. Systemic cholesterol levels were compared between different dosages and observed that 1×10^{12} vg/mouse induced more robust increase in cholesterol levels²⁹¹, comparable to previous studies, therefore this dosage was chosen to be used in the study. One week post AAV8-PCSK9 injection, mice were subjected to high-cholesterol diet for 18 weeks (Figure 5.6). During the experimental period, blood was collected from animals at 0, 6 and 10-weeks post injection to assess circulating cholesterol levels in plasma. The weight of animals was noted every week to check for any difference within the group (Figure 5.6). Circulating cholesterol levels increased by 17 ± 4.7 fold following 6 weeks versus baseline and 24 ± 5 fold by 10-weeks post injection, indicating effective induction of hyperlipidaemia in line with previous studies³⁷². Body weight did not change among animals within their assigned group.

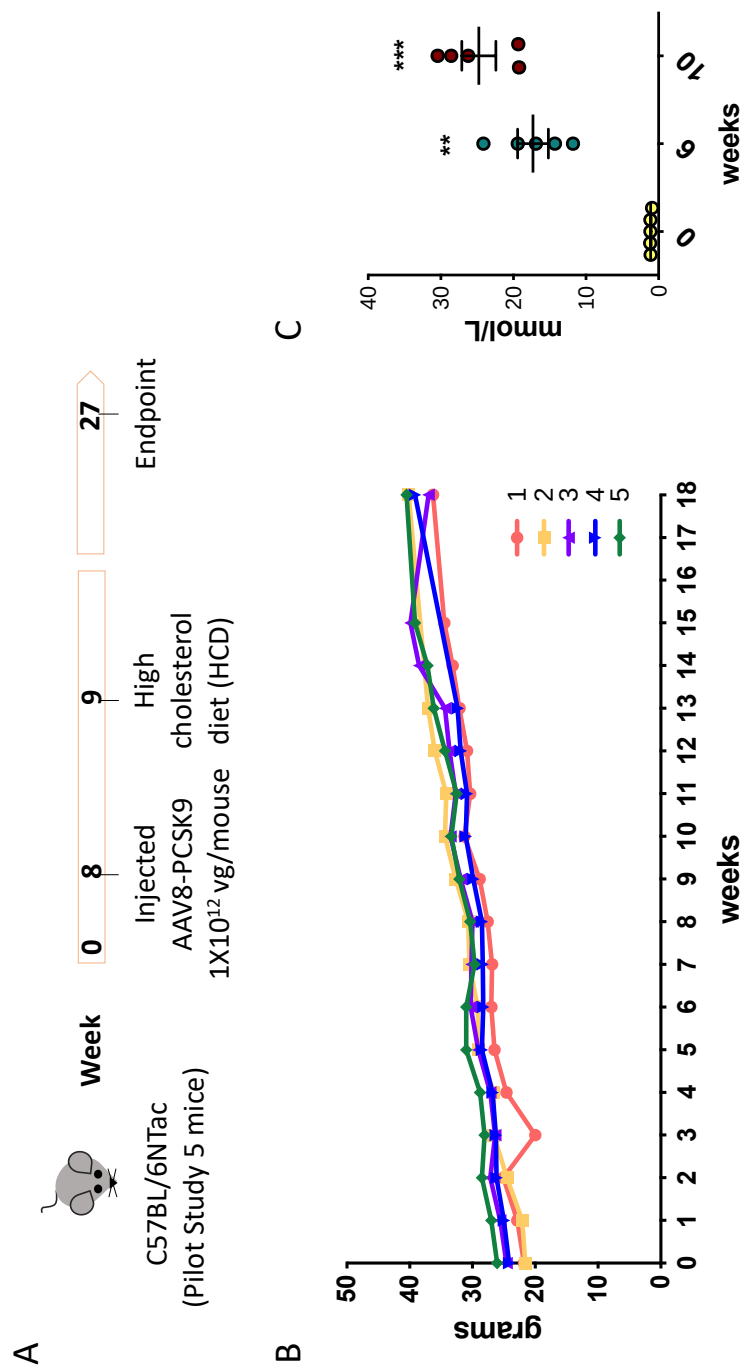


Figure 5.6 AAV8-PCSK9 mediated model of atherosclerosis in mice.

Wild type animals C57BL/6NTac (n=5, 8-weeks old) were injected intraperitoneally once, with recombinant viral particles AAV8 overexpressing proprotein convertase subtilisin/kexin type 9 (PCSK9) with Multiplicity Of Infection = 1×10^{12} vector genome/mouse. Mice were fed with High Cholesterol Diet (HCD) at one week post injection for 18 weeks. Details of the workflow in A). B) Graph representing the weight (grams) of the 5 animals included in the pilot study during the experimental period. C) Circulating cholesterol levels (mmol/L) at baseline (prior to inject mice with AAV8) and 6, 10-weeks post injection. One-way Anova was used to assess ** $P < 0.01$ and *** $P < 0.001$.

Figure 5. 6 AAV8-PCSK9 mediated model of atherosclerosis in mice.

Animals were harvested at 18 weeks post injection and histological analysis of their plaques was performed. Considering the variability in the development of the the plaque at different anatomical sites^{378,379,380}, one of the important decisions during the evaluation of plaque formation in the study of atherosclerosis *in vivo*, is the vascular bed to be examined. In this regard, the region most widely considered is the aortic root, however atherosclerosis may vary with each vascular bed and performing analysis of plaque formation only in this site can thus be limiting. Therefore, the brachiocephalic artery (or innominate artery) was also included in this study. In particular, the aortic roots and brachiocephalic arteries were isolated from each mouse and tissues were then fixed in 4% paraformaldehyde, sectioned (4 μ m) and stained with Haematoxylin and Eosin (H&E) (Figure 5.7). All the animals developed visible plaques at both sites considered, brachiocephalic artery and aortic root with visible necrotic core, infiltrated cholesterol crystals and formation of fibrotic cap (Figure 5.7 A, B).

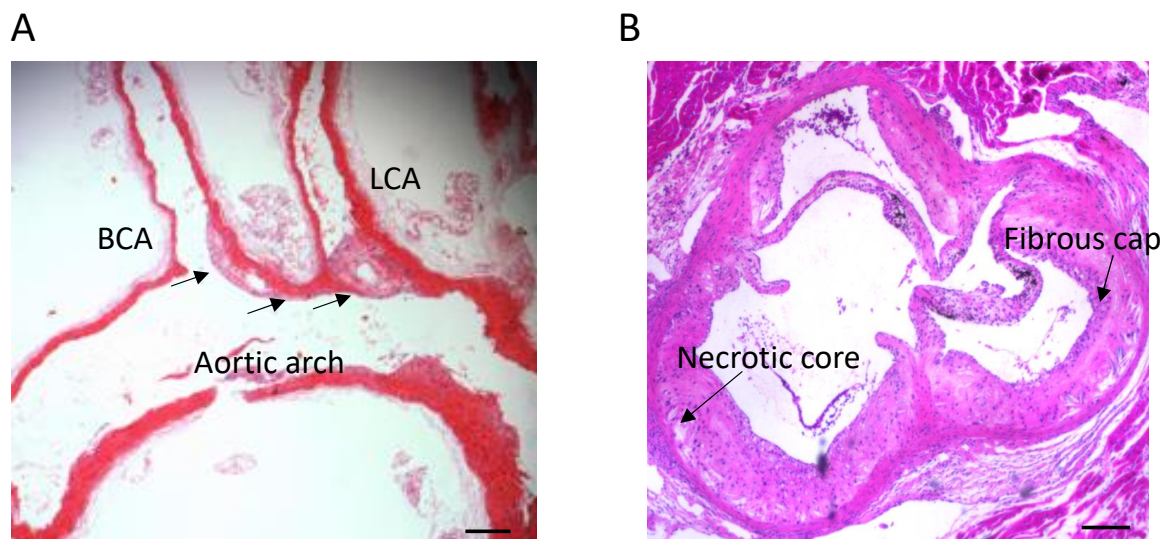


Figure 5.7 Histological analysis of aortic arch and aortic root from atherosclerotic mice.

Wild type mice (n=5 males C57BL/6NTac 8-weeks old) were injected once intraperitoneally with AAV8-PCSK9 (1×10^{12} vg/mouse) and fed with HCD for 18 weeks to develop atherosclerosis. Aortic roots and brachiocephalic arteries were harvested, fixed (4% PFA) and sections stained with H&E. A) Longitudinal sections of aortic trunk isolated from atherosclerotic mice and stained with H&E. In the picture it is possible to see the aortic arch, Left Carotid Artery (CCA) and Brachiocephalic Artery (BC). Left subclavian artery in this section is not yet visible. Plaque tissue is indicated with black arrows. B) Cross-sections of aortic root with visible aortic valves. Necrotic core and fibrous cap are indicated with black arrows. Pictures acquired at 4X, scale bar 100µm.

5.3.3 Evaluation of circulating plasma cholesterol and body weight of *CARMN*^{-/-} and *CARMN*^{+/+} animals.

Having assessed the efficiency of AAV8-PCSK9 mediated induction of atherosclerosis through the pilot study, a large-scale study using *CARMN*^{-/-} and *CARMN*^{+/+} animals was undertaken. In particular, considering the variability in the development and distribution of plaque formation that might exist within the groups, the determination of the number for downstream statistical analysis represents a critical step during the design of the *in vivo* study. In consideration of AHA ATVB council scientific statement recommendations about groups size where more than 10 animals per group are advised for the study of atherosclerosis *in vivo*³²¹, male littermates (8-weeks old) n=19 *CARMN*^{+/+} and n=16 *CARMN*^{-/-}, were included in the study. All animals were injected once *i.p.* with AAV8-PCSK9 (1×10^{12} vg/mouse) and fed with HCD for 18 weeks. In this study, blood was collected at 0, 6 and 10-weeks post injection and body weight was annotated every week. Moreover, in the pathological setting of atherosclerosis, abnormal proliferation of vSMCs^{315,26} and resident macrophages^{68,381} represents a key feature of developing plaques. Therefore, in order to analyse the proliferation of cells within the plaque, 24 hours previous harvesting, animals were injected once *i.p.* with 200mg/kg of sterile 5'-Ethylnyl-2'-deoxyuridine (EdU). EdU dosage and timing was chosen according previous

studies^{382,383}. Animals were checked regularly for their body weight and no differences were observed in the two groups (Figure 5.8 A). Circulating cholesterol levels of both wild type and knock-out animals at 6-weeks post injection increased to ~28 and ~31 mmol/L, respectively, and to ~38 and ~31 mmol/L, respectively, at 10-weeks following injection indicating the pronounced induction of hyperlipidaemia in both genotypes (Figure 5.8 B). The levels of cholesterol detected in wild type animals were higher than the measurement obtained in the pilot study, which can be the result of the use of different cohorts of animals, diet, lots of kit used for the detection. Albeit these factors of variability, the animals in the two independent experiments displayed comparable levels of cholesterol within each condition (baseline, 6-weeks and 10-weeks) and confirmed the increase in cholesterol levels following the infection with AAV-PCSK9 and high cholesterol diet, as previously described^{429,430}. Non-responder mice were identified (n=3 in total, n=2 *CARMN*^{+/+} and *CARMN*^{-/-} n=1), in which no change in plasma cholesterol was observed compared to baseline, therefore they were excluded from the analysis. Plasma cholesterol levels of hyperlipidaemic mice showed no significant differences between *CARMN*^{-/-} and *CARMN*^{+/+} animals, suggesting that the knock-out of *CARMN* did not functionally affect the development of hyperlipidaemia at systemic level (Figure 5.8 C).

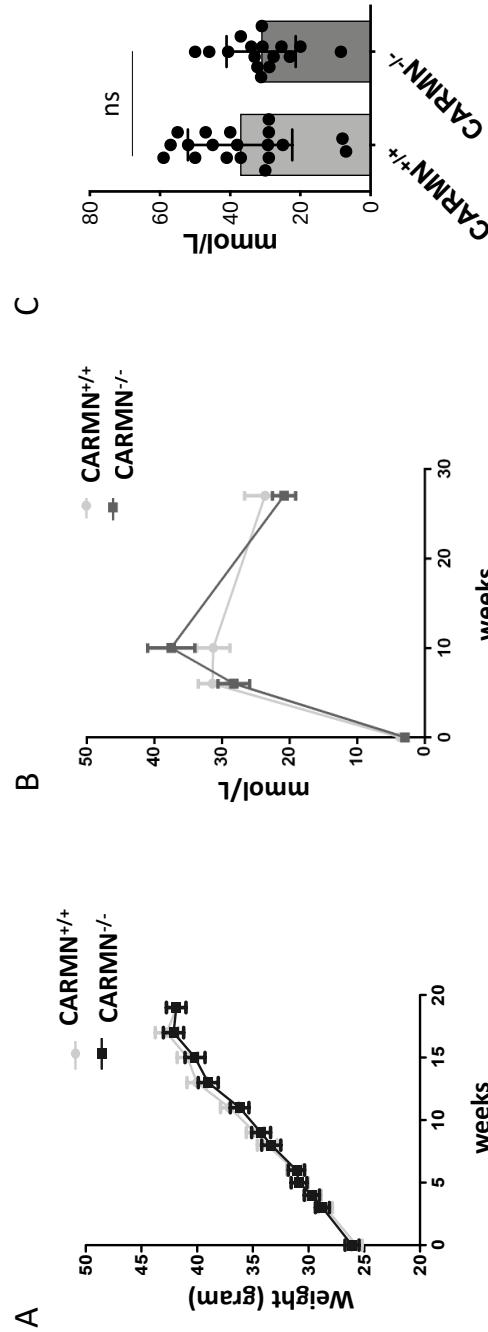


Figure 5.8 Assessment of biochemical parameters in CARMN^{-/-} and CARMN^{+/+} animals.

CARMN knock-out (n=16) and wild type (n=19) animals were induced to atherosclerosis and hyperlipidaemia through AAV8-PCSK9 overexpression in combination with HCD for 18 weeks. All mice were checked weekly for their body weight and cholesterol levels (0, 6, 10-weeks post injection and at sacrifice). A) Graph indicating the body weight (expressed in grams) of CARMN^{-/-} and CARMN^{+/+} animals assessed weekly for the experimental period. B) Circulating cholesterol levels (expressed as mmol/L) of CARMN^{-/-} and CARMN^{+/+} mice assessed prior injection (baseline, week 0), at 6 and 10-weeks post injection and at sacrifice (week 27). C) Plasma cholesterol levels of CARMN^{-/-} and CARMN^{+/+} mice at 10-weeks post injection. Student's t-test was used to assess statistical significance. Ns= not significant

Figure 5.8 Assessment of biochemical parameters in CARMN^{-/-} and CARMN^{+/+} animals.

5.3.4 Evaluation of plaque area and volume in atherosclerotic plaque from *CARMN*^{-/-} and *CARMN*^{+/+} animals.

The measurement of plaque area is a common method to explore the effects that genetic manipulation, drugs or alteration of diet can have to the development of lesions. The variability of plaque size and distribution throughout the arteries in response to fluid dynamics and stress might affect the consistency of results³⁸⁴. Therefore, this method includes the analysis of five sections of the aortic root area isolated at equally spaced intervals through the aortic root, from the origin of aortic valves till the ascending aorta³⁸⁵. Using the average value system controls for variation in plaque size, the plaque area of *CARMN*^{-/-} and *CARMN*^{+/+} was measured in the aortic root of atherosclerotic animals by H&E staining of cross-sections (Figure 5.8 A), revealing increase from $348066 \pm 127994 \mu\text{m}^2$ in *CARMN*^{+/+} group to $427630 \pm 123413 \mu\text{m}^2$ in *CARMN*^{-/-} mice (Figure 5.9 B). Assessing the area of necrotic core can be important predictor of plaque stages and progression towards more advanced phases³²¹. Therefore, the histological examination of *CARMN*^{-/-} and *CARMN*^{+/+} plaques, included the evaluation of necrotic core size. However, no differences were observed in the two groups (Figure 5.9 C).

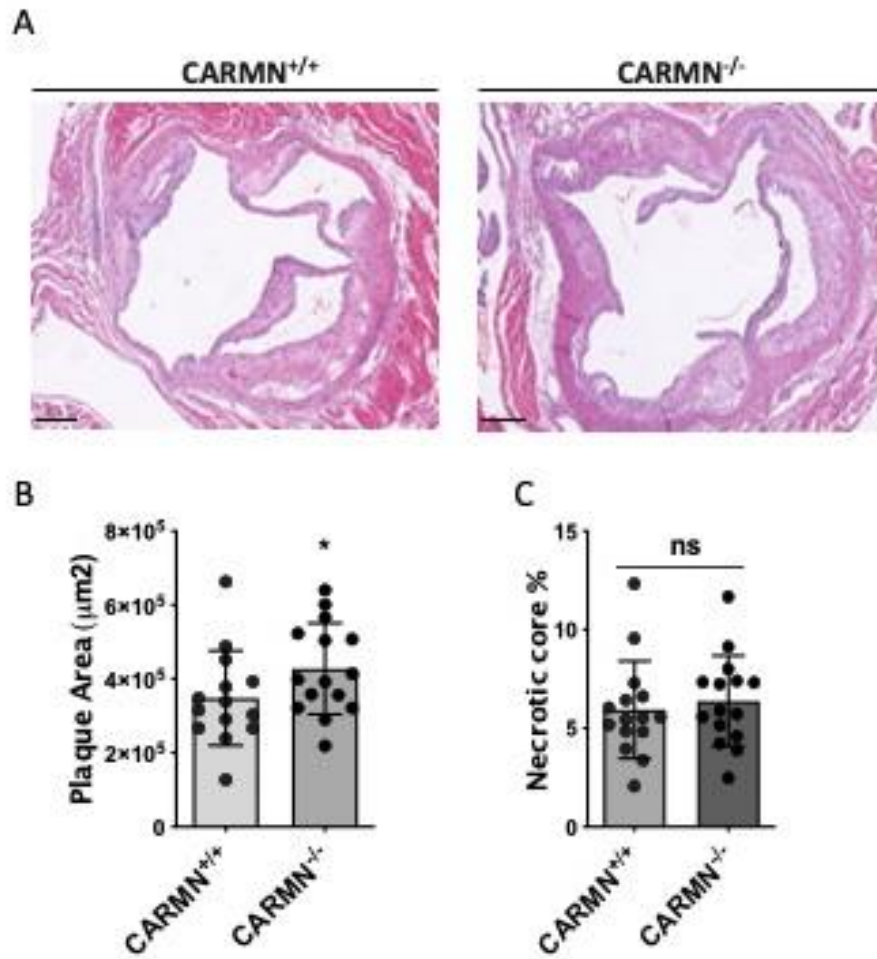


Figure 5.9 Assessment of plaque area and necrosis in aortic roots of *CARMN*^{-/-} and *CARMN*^{+/+} mice.

CARMN^{+/+} and *CARMN*^{-/-} mice were induced to atherosclerosis using AAV8-mediated overexpression of PCSK9 protein, in association with HCD feeding for 18 weeks. Mice were then harvested, and aortic roots were fixed (4% PFA) and sectioned (4 μm) to be stained with H&E. Plaque area and necrotic cores were then measured using QuPath (v0.2.3) software. A) Representative pictures of cross-sections of aortic roots isolated from *CARMN*^{+/+} and *CARMN*^{-/-} animals and stained with H&E staining. Pictures were acquired with 4X magnification, scale bar 100μm. B) Quantification of plaque area in aortic valves from *CARMN*^{+/+} (n=14) and *CARMN*^{-/-} (n=15) animals (data obtained with QuPath software and expressed as μm²). C) Quantification of necrotic cores in plaques from *CARMN*^{+/+} (n=15) and *CARMN*^{-/-} (n=15) animals (data obtained

with QuPath software and expressed as necrotic core %). Student's *t*-test was used to assess * $P < 0.05$. ns= not significant. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Histological evaluation of plaque in *CARMN*^{+/+} and *CARMN*^{-/-} animals was performed in the brachiocephalic artery of animals undergoing the study. In addition to the standard 2-dimensional histological evaluation of the lesions, 3-dimensional quantitation of atherosclerotic plaque volume by Optical Projection Tomography (OPT) was included in the study. This technique records the emission of light and opacity from a semi-translucent sample (aortic arch processed as described in Methods, 2.31) following excitation at specific wavelength, and acquires 400 images as the sample rotates²⁹³. The reconstruction of the images acquired (performed by CTan software), allows the quantification of the plaque volume. The brachiocephalic arteries of *CARMN*^{+/+} and *CARMN*^{-/-} animals were subjected to OPT analysis which revealed an increase in the percentage of plaque volume of *CARMN*^{-/-} animals 11% $\pm$ 4, versus 6% $\pm$ 2.8 in *CARMN*^{+/+} mice (Figure 5.10 A, B). The brachiocephalic arteries undergoing OPT analysis were subsequently subjected to histological evaluation of plaque area by H&E staining. The data obtained revealed an increase in percentage of maximum plaque area of *CARMN*^{-/-} animals 57 $\pm$ 28 versus 31 $\pm$ 25 in *CARMN*^{+/+} animals (Figure 5.10 C, D), confirming the results obtained with OPT analysis.

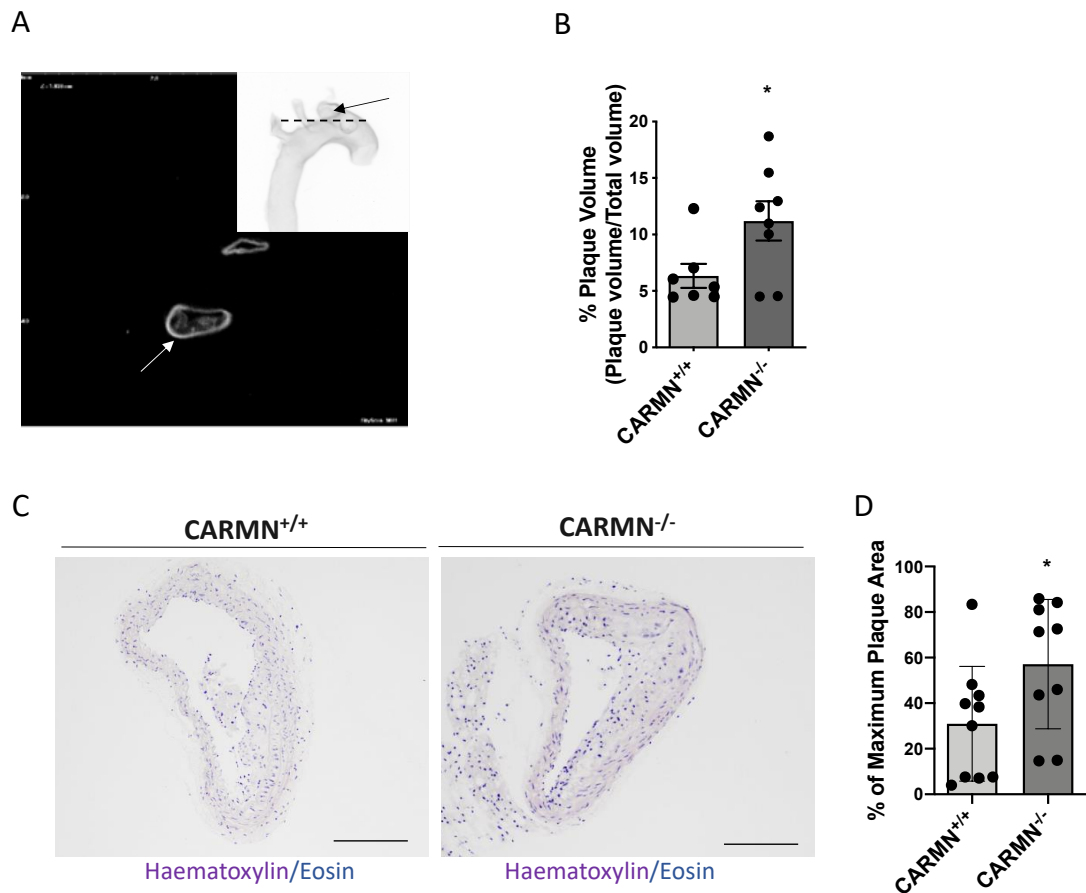


Figure 5.10 Assessment of plaque volume and area in brachiocephalic arteries from *CARMN*^{-/-} and *CARMN*^{+/+} mice.

CARMN^{+/+} and *CARMN*^{-/-} mice were induced to atherosclerosis using AAV8-mediated overexpression of PCSK9 protein, in combination with HCD feeding for 18 weeks. Mice were harvested, and aortic arches were fixed (4% PFA) and processed for OPT analysis. Following OPT, aches were sectioned (5 μ m) and stained with H&E. Plaque area was measured using ImageJ software. A) Representative picture of tomographic reconstruction obtained with OPT analysis of aortic arch. The arrows indicate the brachiocephalic artery. B) Quantification of plaque volumes assessed in brachiocephalic arteries isolated from *CARMN*^{+/+} (n=7) and *CARMN*^{-/-} mice (n=8) (data expressed as percentage of plaque volume). C) Representative H&E performed in cross-sections of brachiocephalic artery from *CARMN*^{+/+} and *CARMN*^{-/-} mice. Pictures acquired at 10X magnification, scale bar 200 μ m. D) Quantification of plaque area in

cross-sections of brachiocephalic artery from *CARMN*^{+/+} (n=10) and *CARMN*^{-/-} (n=9) mice (data expressed as percentages of maximum plaque area). Student *t*-test was used to assess * *P* < 0.05. Graphs are shown as datapoints including the mean ± standard error of the mean.

5.3.5. Assessing the cellular composition of atherosclerotic plaque from *CARMN*^{-/-} and *CARMN*^{+/+} animals.

The evaluation of cellular composition of atherosclerotic plaques is a fundamental step towards the identification of mechanisms underlying the pathophysiology of atherosclerotic disease. Vascular SMCs and inflammatory macrophages are among the cell types forming the plaque and constitute determining factors of plaque stages by the regulation of inflammation and pathological remodelling³⁰. Therefore, vSMC and macrophage content was assessed by immunohistochemistry in cross-sections of both vascular sites, brachiocephalic arteries, and aortic roots from *CARMN*^{+/+} and *CARMN*^{-/-} mice. In particular, Alpha Smooth Muscle Actin (αSMA) and Myosin Heavy Chain 11 (MYH11) were used as markers¹⁸ to assess the content of smooth muscle cells localising in the plaque area of brachiocephalic arteries and aortic roots. Analysis of αSMA and MYH11 content did not show differences in *CARMN*^{-/-} versus *CARMN*^{+/+} mice plaques (Figure 5.11 A, B, C). These results do not confirm the data obtained *in vitro* in SMCs and might indicate the possibility that different mechanism of action and pathways are activated and contribute to the mouse phenotype. At the same time, it is important to mention that the culturing of isolated cells *in vitro* in a controlled environment it is not comparable with an *in vivo* model of disease, where multiple factors might interact and contribute to the phenotype. The study of CARMN function in mouse ASMCs depleted for CARMN, or the use of a lineage-tracing mouse model displaying inducible SMC-specific knock-out of CARMN, such as using Myh11-Cre-ER^{T2} mouse model⁴³¹.

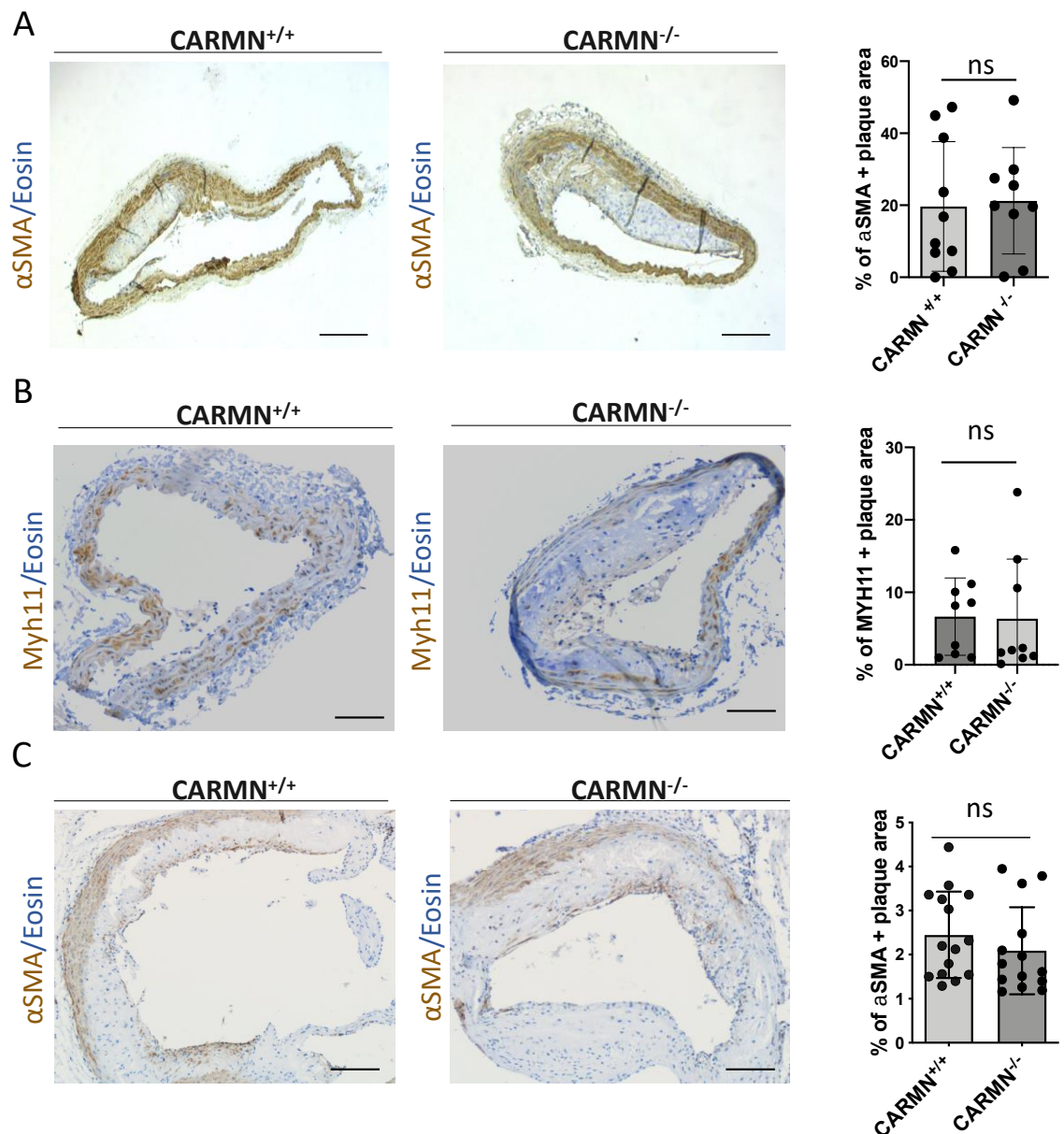


Figure 5.11 Evaluation of vSMC content in plaques from *CARMN*^{+/+} and *CARMN*^{-/-} mice.

Brachiocephalic artery and aortic roots were isolated from atherosclerotic *CARMN*^{+/+} and *CARMN*^{-/-} mice, fixed (4% PFA) and sectioned to perform immunostaining to detect α SMA and MYH11 content. A) Representative images and quantification of α SMA staining performed in cross-sections of brachiocephalic artery isolated from *CARMN*^{+/+} (n=10) and *CARMN*^{-/-} (n=9) mice. B) Representative images and quantification of MYH11 in cross-sections of brachiocephalic artery isolated from *CARMN*^{+/+} (n=9) and *CARMN*^{-/-} (N=9) mice. C) Representative images and

quantification of α SMA signal in cross-sections of aortic root isolated from *CARMN*^{+/+} (n=14) and *CARMN*^{-/-} (n=14) mice. Pictures were acquired with 10X magnification, scale bar 100 μ m. Student *t*-test or Mann-Whitney test were performed to assess statistical significance. ns= not significant. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Inflammatory macrophages are main contributors of inflammatory response and are activated during the plaque progression. In particular, following an initial period where infiltrated macrophages mediate lipids clearance, lipid-laden macrophages (transforming to foam-cells) secrete pro-inflammatory mediators and extracellular matrix (ECM) proteases which drive the progression of the plaque to more advanced stages⁶⁶. Galectin3 (*Lgals3*) is a marker for activated macrophages and/or SMC-derived foam cells³¹⁵, found in human³⁸⁶ and mouse³⁸⁷ plaques, which regulates chemotaxis and inflammation. The expression of this marker was explored in *CARMN*^{+/+} and *CARMN*^{-/-} plaques. Analysis of LGALS3 content within the plaques developed in the brachiocephalic artery shown increase from 2 ± 1.31 in *CARMN*^{+/+} mice to 8 ± 7 in *CARMN*^{-/-} animals (Figure 5.12 A, B). However, the quantification of LGALS3 signal performed in the aortic root of the same group of animals did not reveal significant change in *CARMN*^{-/-} versus wild type controls (Figure 5.12 C). In addition, the expression of CD68, a myeloid lineage marker found localising the plaques, was evaluated in *CARMN*^{+/+} and *CARMN*^{-/-} plaques. The content of this marker was not affected by *CARMN* axis depletion at the site of aortic root (Figure 5.12 D), while the staining for this marker was not technically feasible in the brachiocephalic arteries probably due to the processing to which brachiocephalic arteries undergo during optical projection tomography before to be used for histological analysis (details of the procedure can be found in the Methods paragraph 2.31).

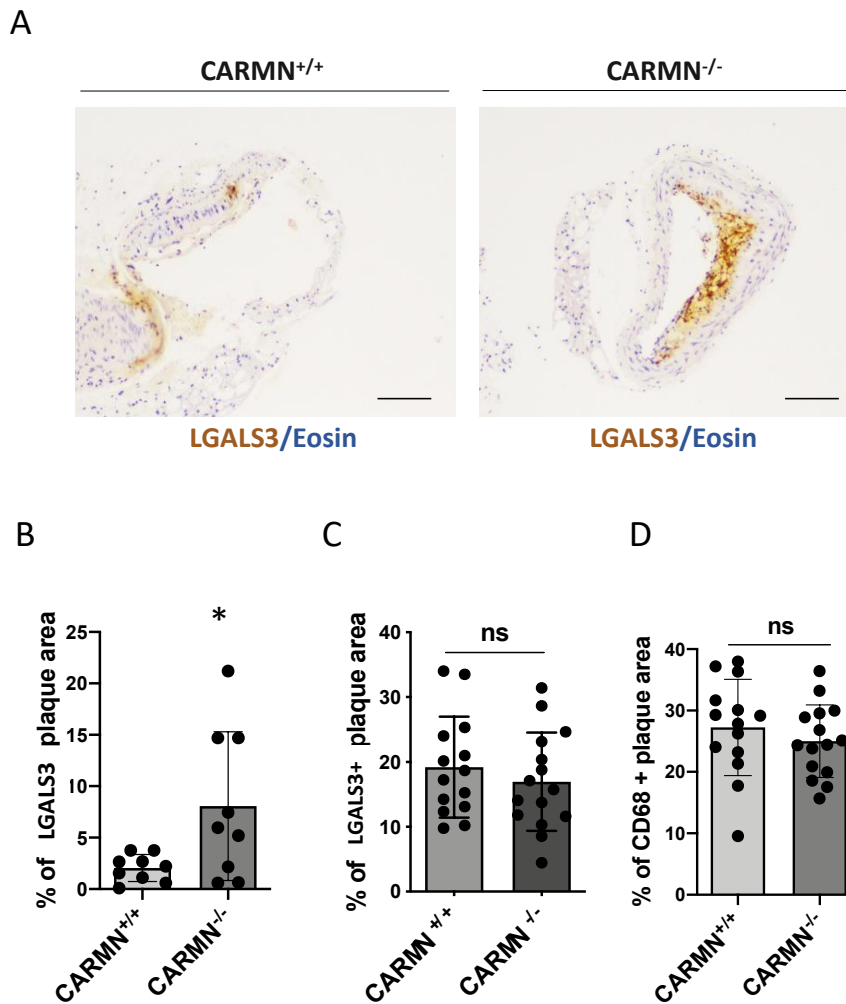


Figure 5.12 Quantification of LGALS3 and CD68 positive cells in plaques from *CARMN*^{+/+} and *CARMN*^{-/-} mice.

Brachiocephalic artery and aortic roots were isolated from atherosclerotic *CARMN*^{+/+} and *CARMN*^{-/-} mice, fixed (4% PFA) and sectioned to perform immunostaining to detect LGALS3 and CD68 content. A) Representative immunostaining detecting LGALS3 expression in cross-sections of brachiocephalic arteries displaying atherosclerotic plaques from *CARMN*^{+/+} and *CARMN*^{-/-} mice. B) Quantification of the staining to detect LGALS3 performed in brachiocephalic arteries of *CARMN*^{+/+} (n=9) and *CARMN*^{-/-} (n=9) mice using QuPath software. C) Quantification of LGALS3 staining in aortic roots from *CARMN*^{+/+} (n=14) and *CARMN*^{-/-} (n=15) mice using QuPath software. C) Quantification of CD68 staining in aortic roots from *CARMN*^{+/+} (n=14) and

CARMN^{-/-} (n=15) mice using QuPath software. Data are expressed as percentage of plaque area. Pictures were acquired with 10X magnification, scale bar 100µm. Student *t*-test was performed to assess statistical significance. * *P* < 0.05 and ns = not significant. Graphs are shown as datapoints including the mean ± standard error of the mean.

5.3.6 Assessment of cellular proliferation and collagen secretion in plaque from *CARMN*^{-/-} and *CARMN*^{+/+} animals.

Cellular proliferation is one of the processes involved in the pathophysiology of atherosclerotic disease. In the context of plaque formation, *in vitro* and *in vivo* studies have extensively demonstrated that vSMCs re-acquire the ability to proliferate to form the stabilizing fibrous cap as well as to generate the plaque core, therefore contributing to the propagation of inflammation and disease progression^{97,104,108,388}. In addition to this, the rapid turnover of plaque resident macrophages through their local proliferation, has also been described as contributing factor to pathological remodelling and plaque progression towards the latest stages^{389–391}. Therefore, cellular proliferation was explored in the plaque developed in *CARMN*^{+/+} and *CARMN*^{-/-} mice. Analysis of EdU incorporating cells, resident within the plaque developed in the brachiocephalic artery, showed an increase from 3.74 ± 2.7 observed in *CARMN*^{+/+} controls to 8.9 ± 6 in *CARMN*^{-/-} animals (Figure 5.13 A, B). In contrast plaques that developed in the aortic arches of *CARMN* knock-out mice did not show significant change in cellular proliferation versus wild type controls (Figure 5.13, C). Vascular remodelling during atherogenesis is characterised by profound changes of the composition of eECM, mainly caused by reduced secretion of ECM components (collagen, fibronectin and elastase) with concomitant enhancement of metalloproteases production³⁹². Significant change in the composition of ECM, such as proteolysis of collagen fibres, affects the stability of the plaque and induces advancement to the latest stages of the disease. Quantitative evaluation of collagen content was performed in *CARMN*^{-/-} mice versus wild type

controls using picosirius red staining and showed a decrease from 4.7 ± 2.2 in *CARMN*^{+/+} to 2.3 ± 1.6 fold of *CARMN*^{-/-} in the plaques of the brachiocephalic arteries (Figure 5.13 D, E). Analysis of collagen detected in the plaques developed at the aortic root site, did not show significant differences when the two groups were compared (Figure 5.13 F). The differential response observed at the two vascular locations analysed, aortic root and brachiocephalic artery, can be due to different site-specific responses influenced by diversity in haemodynamic stimuli and intrinsic cellular factors. Studies in male mice have shown as the aortic root might be characterised by cells more susceptible to the development of the plaque than the brachiocephalic artery, which might be caused by different haemodynamic stimuli³⁹⁶. Therefore, the time required for both sites to respond to pro-atherogenic stimuli and develop atherosclerosis might differ³⁹⁷. These observations might indicate that the development of advanced plaques at the aortic root reached a stage of plateau and differences in plaque composition, such as cell proliferation or collagen deposition, were not identifiable.

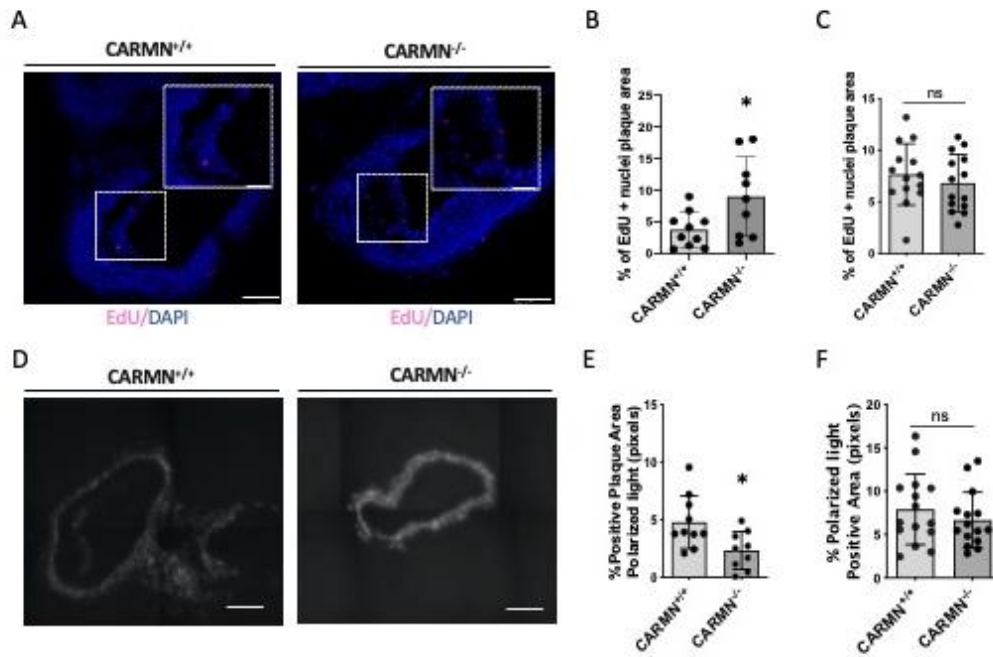


Figure 5.13 Quantification of cellular proliferation and evaluation of collagen content in plaques from *CARMN*^{+/+} and *CARMN*^{-/-} mice.

Brachiocephalic artery and aortic roots were isolated from atherosclerotic *CARMN*^{+/+} and *CARMN*^{-/-} mice, fixed (4% PFA) and sectioned to perform EdU and picrosirius red staining to detect cellular proliferation and collagen content. A) Representative pictures of brachiocephalic artery stained with EdU (pink) to detect cell proliferation. Nuclei are stained with DAPI (blue). B), C) Quantification of EdU positive cells in the plaque area of *CARMN*^{+/+} and *CARMN*^{-/-} brachiocephalic artery (WT n=10, KO n=9) and aortic roots (WT n=14 KO n=14), respectively. D) Representative images of collagen content detected using picrosirius red staining in brachiocephalic artery of *CARMN*^{+/+} and *CARMN*^{-/-} mice using polarised light microscopy. E), F) Quantification of collagen content in brachiocephalic artery (WT n=10, KO n=9) and aortic roots (WT n=15 KO n=15), respectively. Quantification of EdU positive cells and collagen content was obtained with ImageJ software. Pictures were acquired at 10X magnification, scale bar 100µm. Student's *t*-test was performed to assess * $P < 0.05$, ns= not significant. Graphs are shown as datapoints including the mean $\pm$ standard error of the mean.

Adaptative and innate immune responses represent an important factor determining the stages of atherosclerosis. The inflammatory cascade modulated through the secretion of immune modulators (such as inflammatory cytokines and chemokines), influences the plaque formation and stability^{393,394}. Therefore, to understand whether *CARMN* depletion would have affected the subsets of immune cell types, the abundance of lymphocytes subsets was assessed in blood, spleen and bone marrow isolated from *CARMN*^{+/+} and *CARMN*^{-/-} mice. Flow cytometry analysis showed a similar distribution of cells and subtypes between genotypes in all the organs included in the analysis, therefore indicating that *CARMN* depletion does not affect the abundances of the different immune cell subsets (Figure 5.14, 5.15, 5.16, 5.17).



Immunocyte subsets were quantified in blood from CARMN^{+/+} (n=10) and CARMN^{-/-} mice, using flow cytometry and absolute cell counts were obtained using TruCount. Blood was subjected to erythrocyte lysis prior to antibody labeling. Different antibodies were used to identify leukocytes (CD45), B-cells (B220), T-helper cells (CD3), cytotoxic T-cells (CD3/CD8), NK cells (CD8/NK1.1) NKT cells (CD3/NK1.1), monocyte subsets (Ly6C) and eosinophils (SiglecF/CD11b). A) Gating strategy used for the identification of blood lymphocytes subsets. B) Quantification of blood lymphocytes. Data were acquired and analyzed using FACSCanto II and FACSdiva software (BD Bioscience). Student's *t*-test or Mann-Whitney was used to assess statistical significance. Outliers identified using ROUT test (prism 9) were removed from the analysis.

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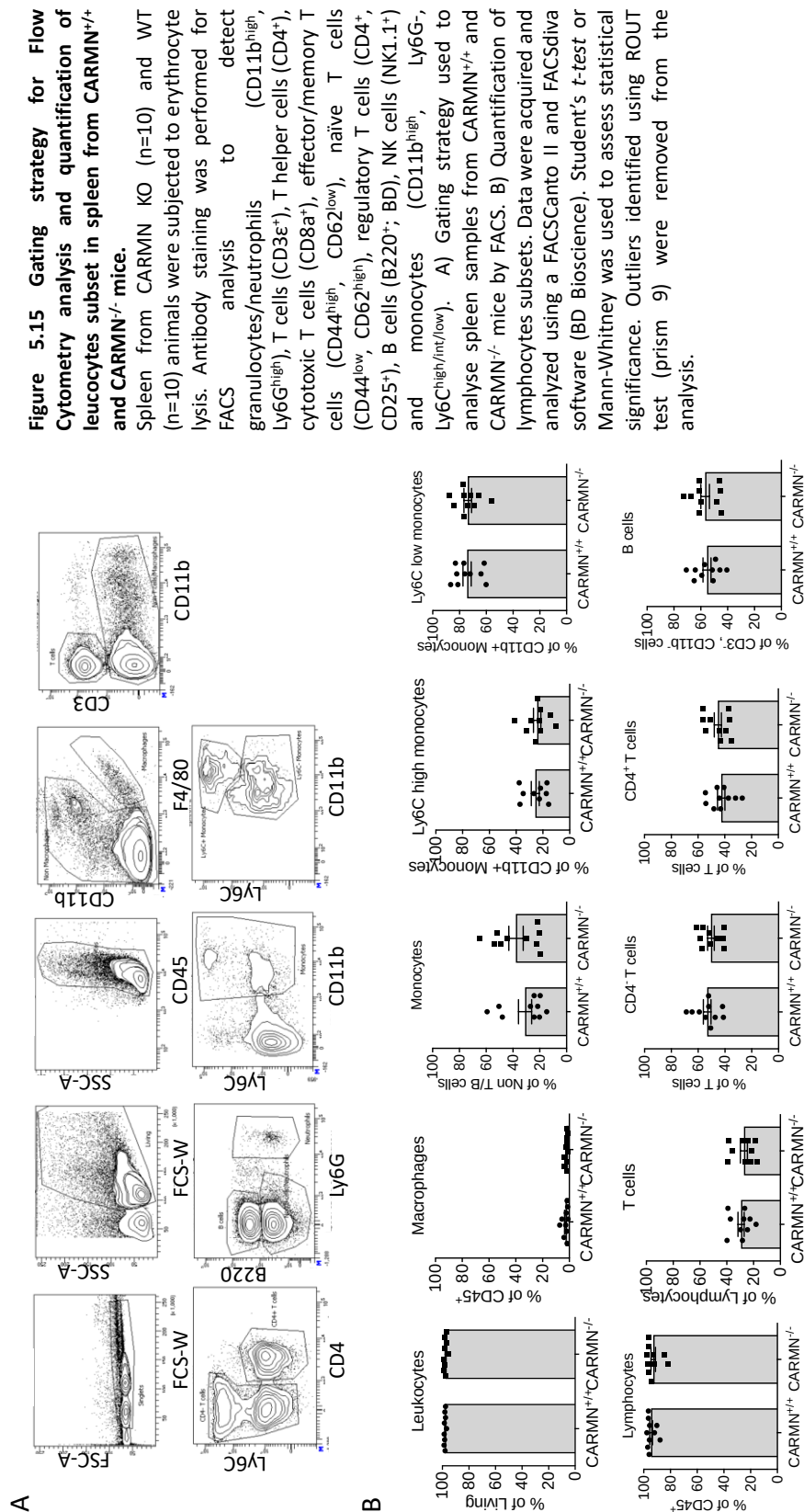


Figure 5.15 Gating strategy for Flow Cytometry analysis and quantification of leucocytes subset in spleen from CARMN+/+ and CARMN-/- mice, represented as datapoints with SEM.

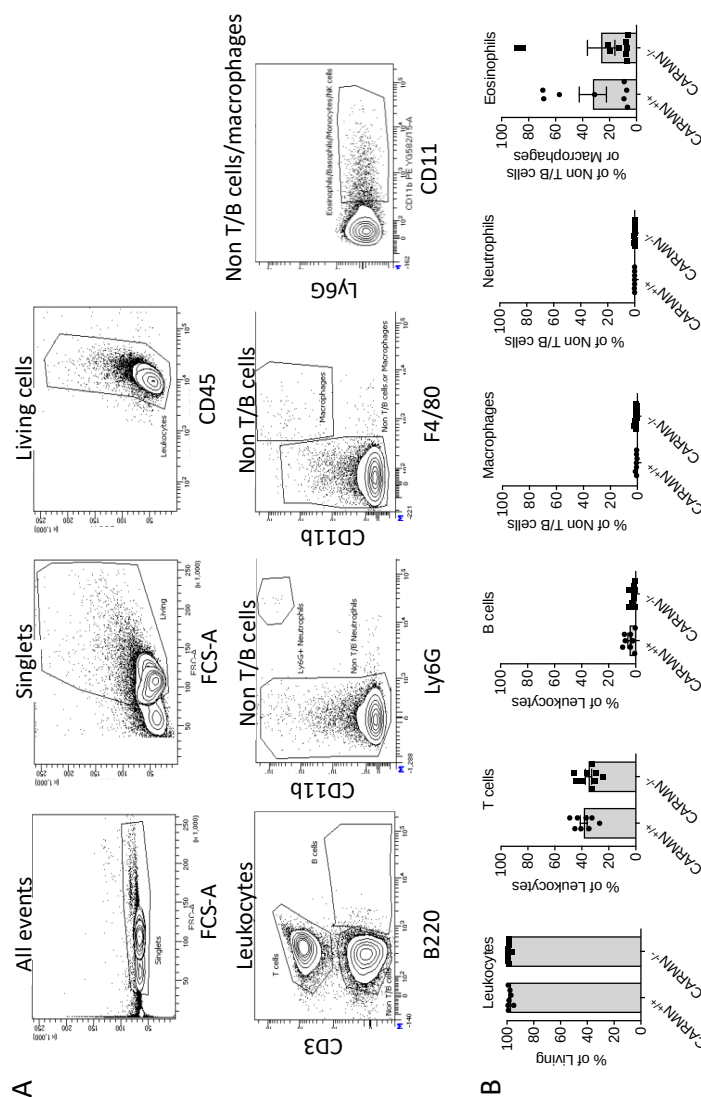


Figure 5.16 Gating strategy for Flow Cytometry analysis and quantification of leucocytes subset in lymph nodes from CARMN^{+/+} and CARMN^{-/-} mice.

Lymph nodes from CARMN KO (n=8) and WT (n=10) animals were subjected to erythrocyte lysis. Antibody staining was performed for FACS analysis to detect granulocytes/neutrophils (CD11b^{high}, Ly6G^{high}), T cells (CD3⁺), T helper cells (CD4⁺), cytotoxic T cells (CD8a⁺), effector/memory T cells (CD44^{high}, CD62^{low}), naive T cells (CD44^{low}, CD62^{high}), regulatory T cells (CD4⁺, CD25⁺), B cells (B220⁺, BD), NK cells (NK1.1⁺) and monocytes (CD11b^{high}, Ly6G⁻, Ly6C^{high/inter/low}). A) Gating strategy used to analyse lymph nodes from CARMN^{+/+} (n=10) and CARMN^{-/-} mice by FACS. B) Quantification of lymphocytes subsets in lymph nodes from WT and KO animals. Data were acquired and analyzed using a FACScanto II and FACSDiva software (BD Bioscience). Student's t-test or Mann-Whitney was used to assess statistical significance. Outliers identified using ROUT test (prism 9) were removed from the analysis.

Figure 5.16 Gating strategy for Flow Cytometry analysis and quantification of leucocytes subset in lymph nodes from CARMN^{+/+} and CARMN^{-/-} mice, represented as datapoints with SEM.

Figure 5.17 Gating strategy for Flow Cytometry analysis and quantification of leucocytes subset in bone marrow from CARMN^{+/+} and CARMN^{-/-} mice, represented as datapoints with SEM.

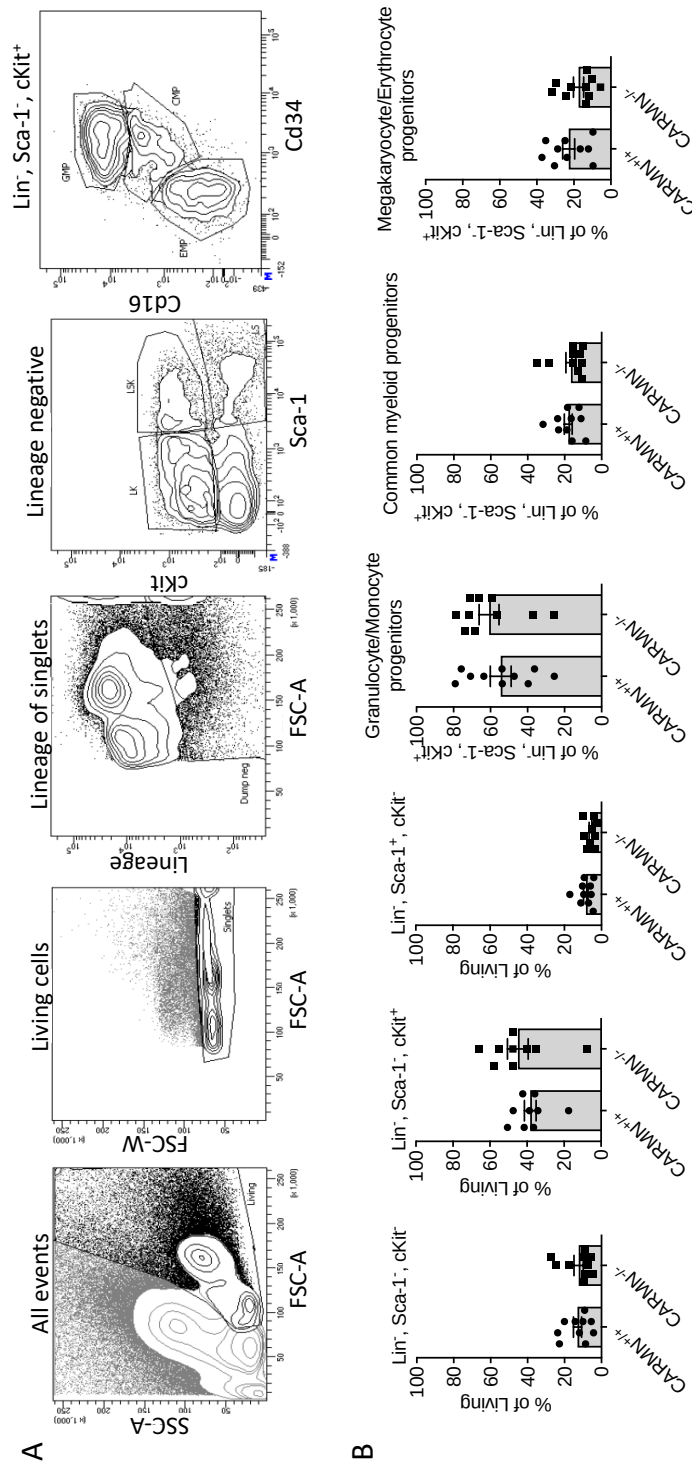


Figure 5.17 Gating strategy for Flow Cytometry analysis and quantification of leucocytes subset in bone marrow from CARMN^{+/+} and CARMN^{-/-} mice. Tibia and fibula from CARMN WT (n=10) and KO (n=10) animals were flushed and processed in order to obtain bone marrow cells. Cells were then subjected to erythrocyte lysis. All lineage negative cells (CD3⁻, B220⁻, CD11b⁻, Ly6G⁻, NK1.1⁻ and Ter-119⁻) were analyzed further for bone marrow stem cells (lin/Sca-1/c-kit), common myeloid progenitors (CD16/32^{int}, CD34^{int}), granulocyte-macrophage progenitors (CD16/32^{high}, CD34^{high}), erythrocyte-megakaryocyte progenitors (CD16/32⁻, CD34⁻). A) Gating strategy used to analyse bone marrow cells from CARMN^{+/+} and CARMN^{-/-} mice by FACS. B) Quantification of lymphocytes subsets in bone marrow from WT and KO animals. Data were acquired and analyzed using a FACSCanto II and FACSdiva software (BD Bioscience). Student's t-test or Mann-Whitney was used to assess statistical significance. Outliers identified using ROUT test (prism 9) were removed from the analysis.

5.4 Discussion

This chapter focussed on the characterisation of the phenotypical consequences of *CARMN* modulation, mediated by CRISPR/Cas9 approach, in the process of atherosclerotic plaque formation using an atherosclerotic mouse model. The deletion of *CARMN* lncRNA (*CARMN*^{-/-}) affected the expression of the co-located microRNAs, miR-143 and miR-145 versus controls. The use of AAV8-mediated overexpression of PCSK9 induced atherosclerosis in conjunction with HCD in *CARMN*^{-/-} and wild type controls. Histological analysis of plaque volume and area performed in the brachiocephalic artery and aortic root, showed an increase in the knock-out mice versus wild type genotype. Examination of plaque composition indicated increased LGALS3 positive cell content in *CARMN*^{-/-} (KO) versus *CARMN*^{+/+} (WT) animals, while no change was observed in α SMA and MYH11 comparing the two genotypes. Analysis of cellular proliferation shown increased EdU positive cells in the lesion of KO versus WT mice, together with decreased collagen secretion in the lesion of *CARMN* depleted animals thus suggesting a more advanced plaque phenotype at the site of brachiocephalic artery.

In order to study the role of *CARMN* *in vivo*, the lncRNA expression was modulated using CRISPR/Cas9 genome editing strategy. A portion of the promoter and the first exon common to the two *CARMN* transcripts (201 and 202) were specifically excised using multiple sgRNAs targeting the region of interest. This approach resulted in the knock-out of the first exon of *CARMN* lncRNA and the knock-down of downstream exons amplified using isoform-specific primers for 201 and 202 thus indicating the possibility of truncated transcripts or the existence of unannotated isoforms. In this regard, to have a complete knowledge of *CARMN* transcripts and the structure of the locus in the aortic tissue, the use of different experimental techniques such as RACE or long-reads sequencing, is required. Similarly to the human locus, *CARMN* locus in mouse includes the hosted microRNAs, miR-143 and miR-145. Therefore, the expression of this cluster was assessed following *CARMN* depletion at baseline. qRT-PCR data demonstrated that the expression of both microRNAs was affected by the

CRISPR-mediated depletion of *CARMN* promoter and first exon. The lack of complete knowledge on the promoter of the two microRNAs, whether it is shared with *CARMN* or not, limits the interpretation of these results. Therefore, the downregulation of miR-143/145 could be due to the deletion of portion of the primary transcript or could be a secondary effect caused by the existence of a regulatory network where *CARMN* modulates in cis the miRNAs levels. Considering that both are valuable possibilities, the interpretation of *CARMN*-axis dependent phenotype *in vivo* requires a wider prospective and cannot solely be ascribed to either the knock-down of *CARMN* nor the downregulation of microRNAs.

To characterise the role of *CARMN* *in vivo* and evaluate the effect of the genetic depletion of *CARMN* axis on the development of atherosclerosis, 8-week-old *CARMN*^{-/-} and *CARMN*^{+/+} mice were induced to atherosclerosis through overexpression of PCSK9 by single injection of the recombinant AAV8 viral particles expressing the protein. The serotype 8 was chosen for its high tropism for hepatocytes³⁹⁵, where PCSK9 plays regulatory cholesterol homeostasis associated functions. To induce the formation and progression to advanced plaques, animals were also fed with high cholesterol diet for 18 weeks following AAV8-PCSK9 injection. Hyperlipidaemia and atherosclerosis were confirmed with increased circulating cholesterol levels and formation of plaques in two of the most common sites where atherosclerosis develops in mice, aortic roots, and brachiocephalic arteries in both groups of mice. The increase in the levels of cholesterol in the plasma of the animals included in the study was used as parameter to confirm the efficiency of the model to induce hypercholesterolemia, and the increase obtained following 6- and 10-weeks post infection was in line with previous studies^{429,430}. The evaluation of plasma cholesterol and body weight did not show significant difference in the two genotypes, thus suggesting that lipid or inflammatory systemic changes might not be involved in the observed phenotypes, while changes in plaque area or composition might be due to local tissue factors. Atherosclerotic plaques were firstly assessed through histological analysis which showed an increase in plaques developed at both sites,

brachiocephalic artery and aortic roots of knock-out versus wild type animals. The increase in the plaque area observed in this study was comparable to previous studies analysing plaque formation in AAV8/PCSK9-induced mouse model⁴²⁹. It is important to mention that the size of the plaque, as well as the levels of hypercholesterolemia developed in mouse models of atherosclerosis are variable when comparing different genotypes commonly used for the study of atherosclerosis, such as ApoE or LDLR knock-out animals⁴³⁰. The evaluation of plaque volume, performed using Optical Projection Tomography of the aortic arches, indicated an increase in the volume of lesions in *CARMN*-depleted mice which might suggest that the loss of *CARMN* correlates with a more complex phenotype. To investigate if the observed increase in volume and area was associated with a change to the plaque composition, vSMC and inflammatory cell content was evaluated within the plaques. In particular, α SMA and MYH11 were used as smooth muscle cell markers and their expression was assessed by immunostaining. However, quantification of α SMA and MYH11 positive cells did not show significant change comparing the two genotypes. It is important to consider that vSMCs are capable of switching their phenotype when exposed to vascular injury and stress conditions, which results in the loss of typical markers (such as α SMA and MYH11) and the expression of inflammatory genes (such as LGALS3) both *in vitro* and *in vivo*^{97,108}. Therefore, it is difficult to clearly identify and quantify phenotypically dysregulated vSMCs within the plaque and necrotic core area. In addition to this, the content of inflammatory cells was evaluated in the lesions of KO and WT animals. In particular, LGALS3 positive cell content was found increased in the plaques developed in the brachiocephalic artery of knock-out mice versus controls, whereas no changes were observed in the expression of LGALS3 and CD68 in the aortic roots of *CARMN*^{-/-} mice versus wild type controls. Also in this case, considering that LGALS3 can be expressed not only by inflammatory macrophages infiltrated within the plaque, but also by plastic vSMCs, it is not possible to unambiguously define the origin of LGALS3 positive cells, whether this is from immune cells or if they belong to the pool of activated vSMCs expressing pro-inflammatory markers. Immune cell subsets in blood, spleen, bone marrow and

lymph nodes were analysed, and no significant difference were observed between the two genotypes. However, some of the cell subsets analysed shown a differential trend in *CARMN* knock-out versus wild type animals, indicating the possibility that *CARMN* deficiency might affect immune cell subtypes. The analysis of these parameters in a larger group of animals could have confirmed or not the trend observed. However, increasing the number of animals analysed would have been technically challenging. Cellular proliferation was also observed in plaque as result of clonal expansion of a subset of plastic vSMC¹⁰⁴ and local proliferation of lesional macrophages accumulated in the plaque⁶⁸. Therefore, proliferation was evaluated in the lesions of WT and KO animals through EdU incorporation in cell composing the plaque. Quantification of EdU positive cells indicated an increase of proliferating cells in the plaque of *CARMN*-depleted mice versus controls, therefore suggesting that the loss of *CARMN* might affect the cellular proliferation rate during the progression of the plaque. Considering that the collagen content influences the stability of plaques, the assessment of collagen synthesis was included in the evaluation of the plaque composition⁷⁶. The quantification of collagen secreted within the plaque of mice, indicated a decrease in KO versus WT mice which might be caused by a decrease in its synthesis or an increase in its breakdown. However, cellular proliferation and collagen content were found unchanged in the plaques developed in the aortic arches of mice which might be explained by site-specific or temporal differences in the processes leading to plaque development at brachiocephalic artery and aortic roots³⁹⁶. In particular, it was observed that haemodynamic stimuli together with intrinsic factors within cells populating different vascular walls, influence the severity of atherosclerosis in human and mice^{397,398}. It was observed that plaques in mice develop first in the aortic roots of atherosclerotic mice and only later appear at the level of brachiocephalic artery³⁹⁷. These considerations might indicate the possibility that the development of advanced plaques reaches a stage of plateau where differences in plaque area were not easily identifiable comparing the aortic roots of the two genotypes, or the existence of distinct intrinsic characteristics at diverse locations (such as cell origin and response to stimuli), which are differently regulated

in response to *CARMN* loss. The phenotyping of *CARMN*^{-/-} and *CARMN*^{+/+} did not include other parameters, such as blood pressure or glucose levels, as they do not fall within the scope of this study, however these can be assessed to investigate the presence of comorbidities even though it is not confirmed their correlation with the development of atherosclerosis in mice³²¹.

Collectively the results obtained *in vivo* suggest that the loss of *CARMN* in atherosclerotic mice might contribute to the development of more complex plaques characterised by increased cellular proliferation, enhanced inflammatory Lgals3 expressing cells and decreased collagen content, therefore influencing the progression to a more advanced phenotype versus controls.

Chapter 6. Discussion

6.1 General discussion

This PhD project investigated the expression and function of the long noncoding (lncRNA) host gene *CARMN*, and the two co-located microRNAs (miRNAs) miR-143 and miR-145 during the phenotypic switch of vSMC, characterising the pathophysiology of vascular remodelling *in vitro*, and during the development of atherosclerosis *in vivo*.

6.1.1 Assessing *CARMN* expression in vSMCs

One of the first steps towards the identification of a possible functional role of noncoding molecules is to assess their expression in the biological context of interest. This is particularly important for long noncoding RNA (lncRNA) molecules whose expression was observed to be highly tissue- and cell-specific. In this project, the expression and function of the long intergenic noncoding RNA *CARMN* was investigated in vascular smooth muscle cells, and specifically in coronary arterial smooth muscle cells (CASMCS). The rationale behind the choice of this cellular type includes: i) *CARMN* is a host gene lncRNA for miR-143/145, central modulators of vSMCs' physiological processes whose dysregulation was associated with the pathophysiology of atherosclerosis; ii) CASMCS constitute the coronary arterial vascular bed, which is one of the main sites where atherosclerosis develops in human. On account of the above-mentioned reasons, it is also important to consider the selection of vSMCs as the main cell type to study *CARMN*, however, we could not rule out the possibility that *CARMN* might have a function in other cell types involved in the pathophysiology of the disease, such as cells composing the architecture of the vascular wall or cells regulating systemic and local inflammation. Profiling the expression of lncRNA can be a challenging task considering the high complexity of lncRNA loci which sometimes share portions of their exonic/intronic sequences and the lack of tools to unambiguously identify their structural organisation. The

expression of *CARMN* was explored using different tools including qRT-PCR analysis, RNA sequencing, *in situ* hybridisation and subcellular fractionation. Each of these techniques can provide useful information in the path to understand and characterise this lncRNA locus, however they also have limitations that need to be taken into consideration in the global interpretation of the results. One example is represented by qRT-PCR analysis performed using Sybr Green primers, which in the case of *CARMN* were designed in exon-exon junctions where possible, amplifying transcript-specific sequences. However, the design of primers requires a previous knowledge of the locus which sometimes can be incomplete due to the transcript's mis-annotation. *CARMN*'s annotation was affected by changes during the years in which this project was conducted, and the latest updated annotation includes 19 isoforms identified by long-read sequencing, which more comprehensively covered the *CARMN* locus discovering un-annotated transcripts and confirming transcripts' ends. This technology overcomes the limits of characterising by new generation sequencing, including possible misalignment of the sequenced short length region to the reference genome, also giving the opportunity of transcript discovery analysis. Furthermore, in this case, the lower read accuracy and the coverage bias towards^{399,400} one of the transcript ends can influence its characterisation and quantification. Therefore, the combination of different strategies is the most efficient approach rather than relying only in one of them. Herein the expression of *CARMN* was assessed using a global approach with pan-amplifying primers targeting a region shared by most of *CARMN* transcripts. Isoform-specific primers also were used to ascertain the efficiency of *CARMN* manipulation during functional assays. *CARMN* axis expression was explored in vSMCs at basal conditions, where the lncRNA resulted to be expressed at high levels (Ct ~ 26 in vSMC at basal condition) with mostly nuclear localisation, but it was also investigated in relation to vSMCs response to pro-pathogenic stimuli. Of note, each of these stimuli separately can be used as a tool to investigate gene function, but the use of a single cell population stimulated in an artificially controlled way does not fully recapitulate the cell-cell crosstalk and the physiological processes characterising the *in vivo* contexts. Moreover, the

progressive de-differentiation phenomenon to which primary cells undergo when cultured *in vitro* needs to be considered when cell culture is used as a tool for gene function assessment. vSMCs were stimulated with PDGF-BB to proliferate, induced to migrate by scratch assay and loaded with cholesterol to promote the formation of lipid laden foam cells. The expression of miR-143/145 was previously observed reduced in dysregulated vSMCs following treatment with the pro-proliferative cytokine PDGF-BB and studies have identified a potential mechanism of action mediated by p53 regulating vSMCs' podosome formation during PDGF-BB-induced migration²⁷⁹. Moreover, miR-143/145 expression was found downregulated in the context of *in vitro* foam cell formation in response to cholesterol loading¹¹³. In this study the expression of the entire *CARMN* axis, including the lncRNA and miR-143/145, was found downregulated in proliferating and de-differentiating vSMCs following stimulation with PDGF-BB and water-soluble cholesterol. Water-soluble cholesterol was described as a good *in vitro* tool to explore the effects of cholesterol loading on gene expression⁹⁸, however in the pathological context of plaque formation vSMCs are exposed also to modified LDL particles in the sub-endothelial layer²⁸. Therefore, the effect of ox-LDL treatment to *CARMN* axis expression was explored. *CARMN* was found downregulated in a dosage dependent manner following 24 hours of treatment (dosage and timing were chosen in accordance with previous studies³⁰¹), while the expression of miR143/145 cluster did not change in response to stimulation. This result indicates that the miRNAs might not be involved in the early response to vSMCs' ox-LDL uptake, but it does not exclude the possibility for miR-143/145 to be implicated in the pathways regulating ox-LDL metabolism and homeostasis. In addition, the expression of *CARMN* following induction of migration by scratch assay in quiescent vSMC was evaluated, however this stimulus did not induce any change in the expression of *CARMN* axis. Also in this case, the complexity of mechanical stresses involved in the stimulation of vSMC medial layer is difficult to be reproduced accurately *in vitro*, therefore these results do not exclude *CARMN* implication in the context of migration, where instead the two microRNAs have been described as regulator of vSMC migration²⁷⁸.

6.1.2 Characterising *CARMN* axis role in vSMCs.

Functional assays were performed to investigate the potential function of *CARMN* in the pathological context of vascular remodelling. To this end, loss of function approach by GapmeR-mediated targeting of the intronic region of *CARMN* was used to negatively modulate *CARMN* expression. In addition, a GapmeR targeting a common exon to most of the isoforms was included. The modulation of *CARMN* by targeting the intronic region or the common exon revealed wide downregulation of lncRNA transcripts as well as decrease in miR-143/145 expression. This can be considered one of the most critical results of this study, and in this regard, there are different factors that can be considered. The localisation of miR-143/145 within *CARMN* locus, the similar expression pattern of *CARMN* and miR-143/145 in response to stimuli (PDGF-BB, cholesterol) and the existence of a promoter upstream both noncoding RNAs²⁷¹, could suggest that the pri-miRNA transcribing to miR-143/145 overlaps with *CARMN* lncRNA. In this case, the GapmeR-mediated approach might induce the decrease of microRNAs expression by directly targeting the pri-miRNA. At the same time, the existence of a second promoter downstream of *CARMN* and just upstream of miR-143/145²⁷⁶, and the lack of data proving the existence of a pri-miRNA sequence overlapping *CARMN* transcriptional start site which ends where the microRNA localise, indicate the possibility that the two noncoding RNAs might be regulated separately. In addition, the possibility for lncRNA host genes to modulate the processing of their associated microRNAs²⁶⁹, and the possibility of *CARMN* playing a super-enhancer role²⁸⁵, highlight the complexity of this locus whose structure might also suggest a cis-regulation of miR-143/145 by *CARMN* in some cellular contexts. However, the complete characterisation of this highly complex locus and the underlying molecular regulation would require more work towards its structural identification. In this regard, strategies aiming at the study of DNA-DNA interactions (such as chromatin looping experiments) could be useful tools to test possible interaction of *CARMN* with portions of downstream promoter regions. Chromatin immunoprecipitation followed by sequencing (ChIP-Seq) performed in differentiated vSMCs can give information on DNA-binding proteins and histone modifications

enriched in *CARMN* promoter. Moreover, capture-long reads sequencing performed in vSMCs at basal conditions could be a useful tool to uncover the existence of pri-miRNA transcripts overlapping *CARMN* promoter. Moreover, a CRISPR- mediated approach could be used to selectively activate the promoter region upstream of miR143/145 proximal to *CARMN* and test its efficiency in regulating miRNAs expression. In this regard, lncRNA host genes can enhance the transcription of associated miRNAs by facilitating the recruitment of Drosha/DGCR8 complex²⁶⁹. To test whether *CARMN* might favours the miRNAs processing, the deletion of *CARMN* transcript portions (such as using CRISPR/Cas9 approach) might reveal the effects of this deletion on the expression of the microRNA cluster.

To comprehensively characterise vSMCs transcriptomic change in response to the downregulation of *CARMN* axis, RNASeq was performed in treated (PDGF-BB, scratched, water-soluble cholesterol) and untreated vSMCs (basal conditions associated to each phenotype- 0.2%FBS or 0.2% BSA) depleted for *CARMN* or treated with GapmeR control. Here, it is important to consider that RNA sequencing analysis allows to profile gene expression in specific cellular and tissue contexts, but in the *in vitro* context, the cellular heterogeneity, displayed *in vivo* by vSMCs, is not properly illustrated. Principal component analysis showed the close clustering of the repeats belonging to each condition, but also revealed differences in the transcriptional profiles of all the basal conditions used in the study. This can be the result of differences in the three different protocols with respect to the cell culture density and the point of harvesting, following transfection (fully described in the Results paragraph 3.3.10 and Methods paragraphs 2.4 to 2.7). Computational gene ontology analysis revealed that the dysregulated genes were involved in cellular processes related to positive regulation of cell proliferation, and cell migration, cell inflammation and cholesterol metabolism. These categories include multiple interconnected networks involved in the pathophysiology of atherosclerosis. In particular, in the pathological context *in vivo*, de-differentiating vSMCs reduce the expression of contractility markers to be able to migrate and proliferate, increase

macrophage-associated genes to be able to uptake lipids particles, secrete pro-inflammatory cytokines to propagate inflammation and produce metalloproteinases which disrupt the collagen/metalloproteinases ratio in the fibrous cap, therefore contributing to the progression of the plaque to the latest stages³¹⁵. vSMCs depleted for *CARMN* axis at basal conditions displayed reduction of α SMA expression with increased CD68 maker of macrophages versus vSMCs transfected with Gapmer control. In addition, vSMCs knocked down for *CARMN* axis were found to produce less ECM components such as COL3A1, which was found downregulated in ruptured versus stable plaques⁴⁰¹ and ELN whose fragmentation was observed in the progression to vulnerable plaque⁴⁰². While MMP1, previously observed co-localising with cleaved collagen in advanced plaque⁴⁰³, was found overexpressed following downregulation of *CARMN* locus, together with increased expression of IL-1 β , IL-6, whose properties can foster the progression to vulnerable lesions *in vivo*⁴⁰⁴. Dysregulated vSMCs are also able enhance leucocyte recruitment through the secretion of chemoattractants⁸⁵. In this regard, the synthesis of chemotactic proteins was found enhanced in *CARMN* locus-depleted vSMCs versus control, including CCL2⁴⁰⁵ and CXCL1 whose expression is upregulated in the plaque microenvironment^{405,406}. These genes were moreover found displaying a similar expression profile in RNASeq analysis, previously performed in the lab, of stable and unstable plaques²⁴⁴. Functional studies included the validation of the obtained findings through *in vitro* assays. More specifically, vSMCs, silenced for the *CARMN* axis, were induced to proliferate with PDGF-BB treatment, to migrate by scratch assay, and de-differentiate towards foam cell-like phenotype through cholesterol loading. The results indicate that the downregulation of *CARMN* locus enhanced vSMC proliferation rate in response to PDGF-BB stimuli but also under quiescent starvation conditions (0.2% FBS) compared to controls. To place these data in the pathophysiology of atherosclerosis, it was observed that the clonal expansion of vSMCs characterises both, the early “response to injury” process, leading to the intimal formation in which resident vSMCs proliferate⁴⁰⁷, but also the tardive phases of fibroatheroma formation and advancement to vulnerable plaque, where

dysregulated vSMCs continue to proliferate, lose their markers and produce pro-pathological cytokines⁸⁵. Therefore, the reduction in *CARMN* expression as response of vSMCs to pro-pathogenic stimuli during early formation of the lesions, might have a role during the following stages of plaque advancement towards the latest phases of the pathology. In the transition from early pathological intimal thickening to the formation of stable atheroma, vSMCs have been characterised for their pro-migratory phenotype to form the fibroatheroma⁸⁶. In this scenario, the downregulation of *CARMN* locus influences vSMCs' ability to migrate, phenomenon that was previously associated with miR-143/145 modulation^{277,279}. In addition, progressive uptake of cholesterol particles has been shown to downregulate vSMC typical markers while enhancing the expression of macrophage-like genes such as CD68, ABCA1, ABCG1⁹⁸. The expression of these markers was observed dysregulated following *CARMN* axis depletion, where *CARMN* depleted vSMCs in basal conditions show decreased levels of α SMA and MYH11 and increased levels of CD68, ABCA1, ABCG1, whereas the levels of other vSMC markers such as TAGLN and CNN1 did not show similar pattern following *CARMN* modulation. In this regard, previous studies revealed upregulation of these genes upon miR-143/145 overexpression. In particular, the extracellular vesicle-mediated delivery of miR-143, miR-145 and miR-223 in cultured vSMCs was found associated with increased levels of TAGLN and CNN1 proteins⁴⁰⁸. However, no direct association between the increase of miR-143 and/or miR-145 and the levels of these proteins was tested in these studies, indicating a more complex molecular network probably including multiple regulators of vSMC signature. In addition to this, a second study⁴⁰⁹ shown that mimic-mediated overexpression (60nM) of miR-145 increased CNN1 at a protein level in the Dicer knock-out mouse vSMCs. The discrepancy between the observed results and the results from this study can be explained by different factors, such as different dosage and (60nM versus 5nM used in this study), chemistry of miRNA mimic (miRIDIAN versus miRAGEN), species used (human versus mouse) and transfection conditions (hCAsMCs at basal conditions depleted for *CARMN* versus Dicer knock-out mouse vSMCs).

Collectively, functional data obtained *in vitro* suggest that the depletion of *CARMN* locus might prime only a partial de-differentiated vSMC phenotype under basal conditions, characterised by the loss of some key vSMC makers and the upregulation of non-typical vSMC genes.

6.1.3 Study *CARMN* role independently from miR-143/145 modulation.

The role of *CARMN* and miR-143/145 in mediating vSMC function has not been explored yet, and whether *CARMN* has an independent role from miR-143/145 locus is a key question in this project. Considering the complexity of the locus, the modulation of *CARMN* by loss-of-function studies, which has been used herein as well as in previous studies^{285,286}, resulted to not be efficient to disentangle the role of the two noncoding molecules separately. Therefore, co-transfection of miRNAs mimics in combination with GapmeR was applied to efficiently overexpress miR-143/145 in *CARMN*-depleted vSMCs. *CARMN* expression resulted to be activated in response to miR-143/145 upregulation, which might suggest the existence of a feedback loop in which miR-143/145 activate the *CARMN* promoter. However, the increase in *CARMN* expression following mimic stimulation was not enough to counterbalance the effect of GapmeR and restore *CARMN* levels at basal conditions. The evaluation of previously observed phenotypes following co-transfection revealed that the increase in vSMC proliferation observed in *CARMN*-depleted cells at basal conditions was not affected by miR-143/145 overexpression. Previous results have shown association of miR-143/145 with the modulation of rat aortic vSMCs viability and proliferation, in dysregulated vSMCs exposed to treatment with cytokines^{410,411,276}. This study explored the regulation of vSMC proliferation at basal conditions, and it has revealed that *CARMN* can act independently from miR-143/145. However, these results do not exclude the possibility for miRNAs to be part of the regulation of this cell phenotype in response to different stimuli.

CARMN has already been described having super-enhancer signature able to modulate the expression of neighbouring genes in cardiomyocytes progenitor

cells²⁸⁵. RNASeq analysis and qRT-PCR validation revealed the downregulation of CSNK1a1, a protein coding gene adjacent to *CARMN* locus previously associated with cellular proliferation³¹², following *CARMN* depletion.. However, the modulation of CSNK1a1 by siRNAs did not phenocopy the effect observed in *CARMN* depleted cells, therefore suggesting that *CARMN* might modulate vSMCs division independently from the modulation of CSNK1a1. These results do not exclude the possibility for *CARMN* to play a super-enhancer function. To test this hypothesis, chromatin looping experiments combined with ChIP-seq map of chromatin marks in vSMC would be required. Contrarily to the proliferative phenotype, the migration rate and the basal expression of macrophage-like genes were restored following miR-143/145 overexpression, suggesting that miR-143/145 has a primary role in controlling these phenotypes.

6.1.4. Identification of *CARMN* axis role *in vivo*.

To explore the clinical importance of these findings, considering the level of conservation of this locus, the expression and role of the *CARMN* axis was investigated in the pathophysiological context of atherosclerosis in human and mouse. In this regard, animal models have been used to explore the underlying pathological mechanisms of the disease and to evaluate the translational relevance of identified targets. However, specific differences compared to human atherosclerosis still exist, including cholesterol metabolism, inflammatory response, anatomical differences in plaque localisation, and absence of spontaneous rupture with thrombus formation^{412,325}. Moreover, the influence of genetic background and environmental conditions of breeding can influence the plaque composition in mouse models, factor that leads to limited comparative studies in plaque composition in mice versus human⁴¹³. Nevertheless, some analogies have also been identified in the processes characterising the early formation of fatty streak and its progression to advanced lesions in human and mouse³⁷⁶. For this reason, the expression of *CARMN* axis was evaluated in early and late stages of human and mouse atherosclerosis. *CARMN* as well as miR-143/145 were found downregulated in RNA seq analysis and

qRT-PCR validation in human stable versus unstable lesions and in mouse fatty streak versus advanced lesions isolated from Low Density Lipoprotein Receptor knock-out mice (LDLR^{-/-}) by laser capture microdissection. These results confirmed the decrease of the axis observed in response to pathogenic stimuli *in vitro*. The high levels of expression of *CARMN* in human stable plaques (FPKM ~100) suggested the possibility that *CARMN* may also be expressed in different cell types composing the plaque. This can be hypothesised also in mice, where the expression of *CARMN* was observed in early fatty streak, primarily composed by plaque recruited monocytes-macrophages and T cells. In situ hybridisation revealed *CARMN* expression is co-localised with smooth muscle ACTA2 as well as CD68 expressing cells within human plaques. Nevertheless, considering that dysregulated vSMCs can simultaneously co-express vSMC and macrophage makers³⁸⁸, more studies are required to clearly define whether the CD68/*CARMN* double positive cells have SMC origin or not and whether *CARMN* can also be expressed by non-activated macrophages. In this regard, a lineage tracing mouse model, using specific vSMCs markers such as MYH11¹⁰⁴ or SM22α⁹⁷, knocked-out for *CARMN* and induced to atherosclerosis, would be useful to explore the consequence of *CARMN* depletion on vSMC clonal expansion and de-differentiation process towards macrophage-like cells. Moreover, the detection of H3K4dime of *Myh11* locus at single cell resolution in fixed tissue as previously shown⁴¹⁴, in combination with histological examination of Ki-67 proliferation marker or EdU positive cells, could be instrumental to explore the effect of *CARMN*^{-/-} in vSMC-derived dysregulated cells within the plaque. In addition, co-staining with a common leucocyte marker⁴¹⁵, such as CD45, would give the possibility to explore the lncRNA expression also in non-activated inflammatory cells.

The functional consequence of *CARMN* axis genetic depletion *in vivo* was explored in a AAV8-PCSK9 mediated model of atherosclerosis³⁷². This strategy has the advantage of not requiring germline mutation such as of ApoE^{-/-} or LDLR^{-/-} models, and therefore represents a valuable alternative to the use of double knock-out mice. Initial characterisation of *CARMN*^{-/-} mice at baseline showed decrease of microRNAs levels

versus *CARMN*^{+/+} mice, which might indicate the existence of a common promoter for both noncoding RNAs, or the possibility for *CARMN* to cis regulate miR-143/145 expression, which was previously hypothesised²⁸⁵. Histological analysis of *CARMN*^{-/-} and *CARMN*^{+/+} atherosclerotic mice included the evaluation of plaque area, volume and composition in two anatomical sites of the vascular system, the aortic root (AR) and the brachiocephalic artery (BA)³⁸⁵. These vascular beds were selected among the preferred sites where atherosclerosis develops in mice, however it is important to note that differences exist in the mechanisms driving plaque progression at both locations. *CARMN*^{-/-} animals showed increase in the plaque area developed at AR and BA versus wild type controls. In addition, while it was not possible to evaluate plaque volume at AR due to the large size of this vascular bed, plaques developed in the BA displayed increase in volume measured with optical projection tomography. To this end, previous studies have revealed opposite results using miR-143/145 double knock-out animals, showing not only the spontaneous formation of intimal thickening²⁷⁰ and the detrimental effect of miRNAs loss⁴¹⁶, but also the beneficial effect in arterial injury versus control mice^{278,284}. However, while the consequence of miR-143/145 knock-out was not directly explored in atherosclerotic mouse model, their role in cellular function was investigated *in vitro* where the loss of miR-143/145 expression was associated with pro-migratory vSMC phenotype and vSMC marker loss^{416,278} as confirmed in this study. Moreover, the downregulation of miR-145 *in vitro* enhanced the proliferative capability of PDGF-BB treated vSMCs²⁷⁶. In addition, the involvement of miR-143/145 in cholesterol-laden vSMCs was previously hypothesised¹¹³ which was also confirmed by *in vitro* experiments in this study. In contrast to previous studies, herein the expression of the entire *CARMN* locus was affected and demonstrated that the modulation of *CARMN* as well as miR-143/145 can have detrimental effect on the plaque progression *in vivo*. To understand the molecular players involved in response to *CARMN* axis loss during the development of atherosclerosis, plaque composition was evaluated in both vascular beds. Results obtained from the brachiocephalic artery, showed that *CARMN*^{-/-} did not affect the content of ACTA2 and MYH11 positive cells, while the content of LGALS3 protein

expressing cells was increased versus control. In addition, the number of proliferating cells was increased following *CARMN* modulation versus wild type mice. To interpret and contextualise these results, additional important information provided from previous reports need to be taken into account including: i) both, tissue infiltrated macrophages⁴¹⁷ and vSMCs¹⁰⁴ populating the plaque undergo clonal proliferation within the plaque, ii) continuous recruitment of macrophages occurs during the progression of the plaque as reinforcement of macrophage pool at the site of injury²⁵, iii) lineage-tracing studies have identified that a good number of SMC-derived cells within the plaque express macrophage markers including LGALS3⁹⁷ and that not all Lgals3 positive cells have myeloid origin^{97,388}, and iv) more than 60% of smooth muscle-derived cells in the plaque had lost their typical markers³⁸⁸. Taken together these results indicate that *CARMN*^{-/-} alters mouse plaque composition by increasing cell proliferation which was also observed in human vSMCs *in vitro*. However, considering that *CARMN* is also expressed in plaque macrophages, it is not possible to univocally identify the origin of proliferating cells within the plaque of *CARMN*^{-/-} animals by only considering these markers, therefore representing a limitation of this study. regarding order to overcome this, histological staining to detect EdU positive cells in combination with ACTA2 or CD68 or triple staining for EdU/ACTA2/CD68 could give indication on the cell type and the stage of differentiation of proliferating cells in *CARMN*^{-/-} compared to controls. However, since tissue sections used for histological examination following OPT treatment are very fragile, the application of multiple staining enhances the possibility of tissue deterioration, and therefore does not represent an ideal option for the identification of specific cell types positive for EdU incorporation. Whereas the use of lineage-tracing mouse models, as previously discussed, would be a good tool to explore the origin of proliferating vSMCs in *CARMN*^{-/-} versus controls.

Further analysis of plaque composition has shown that plaques of *CARMN*^{-/-} animals displayed less collagen content compared to their *CARMN*^{+/+} controls. In this regard, plaque vulnerability has been associated with decrease in collagen content due to unbalance between collagen synthesis/deposition and degradation, affected also by

increased metalloproteinase-mediated degradation⁴⁴. RNASeq data *in vitro* have also indicated that vSMCs depleted for *CARMN* axis showed reduced Col3a1 and Elastin content, while increased MMP1 production. Taken together these results suggest that *CARMN*^{-/-} increases the content of Lgals3 expressing cells which in turn secrete MMPs during the progression to unstable plaque⁴⁶. In this context, the decrease in collagen content characterising the lesions of *CARMN*^{-/-} animals can be associated to a more advanced phenotype in comparison with *CARMN*^{+/-} lesions. The absence of differences in circulating cholesterol levels or leucocytes content in the blood, bone marrow and spleen indicate that changes in the composition of the plaque are not the results of systemic factors but can be caused by local responses to *CARMN* depletion. Contritely, although plaque area was found increased in the AR of *CARMN*^{-/-} mice, this result was not coupled with differences in plaque composition. These observations can be due to different site-specific responses at the two vascular locations, AR, and BA, influenced by diversity in haemodynamic stimuli and intrinsic cellular factors.

6.1.5 Summary of key findings.

Taken together these findings indicate that the lncRNA *CARMN* has independent role from microRNAs miR-143/145 in the modulation of vSMC phenotypic transition to pro-proliferative phenotype, whose downregulation therefore might constitute a pre-requisite for the cellular transition to pro-pathological phenotype. This is particularly important in the pathophysiology of vascular injury, where a small population of primed vSMCs can readily respond to pro-pathological stress. In this context, the loss of *CARMN* might represent intrinsic factor of selection which favours the activation of vSMC population. In human and mouse atherosclerosis, the expression of *CARMN* axis (lncRNA and microRNAs) was found downregulated in advanced regions of atherosclerotic plaque compared to more stable regions. In this regard, the modulation of *CARMN* axis expression might represent an early target during the pathological vascular remodelling. Moreover, the modulation of *CARMN* *in vitro* and *in vivo* might lead to dysregulation of miR-143/145 expression, which in

turn primes vSMCs to a partial de-differentiation towards macrophage-like phenotype. Therefore, the modulation of *CARMN* levels can be considered a strategic approach to target other aspects of vSMC dysfunction more broadly in vascular injury. In the context of lncRNA/miRNA crosstalk, this study highlights the possibility for *CARMN* host gene to regulate cellular functions independently from the hosted miRNAs. Considering that >70% of miRNAs are included into hosting loci, to functionally dissect the roles of noncoding RNA molecules belonging to the same genomic unit gives the important possibility to develop strategies to globally intervene in the regulation of multiple pathways affecting cell phenotypes. Moreover, in contrast to the vast majority of lncRNA molecules whose expression is limited to one species, the high conservation levels of *CARMN* allowed the study of this lncRNA *in vivo*, providing a novel model of lncRNA knock-out in atherosclerosis.

A graphical representation of the key findings of this project can be found in Figure 6.1.

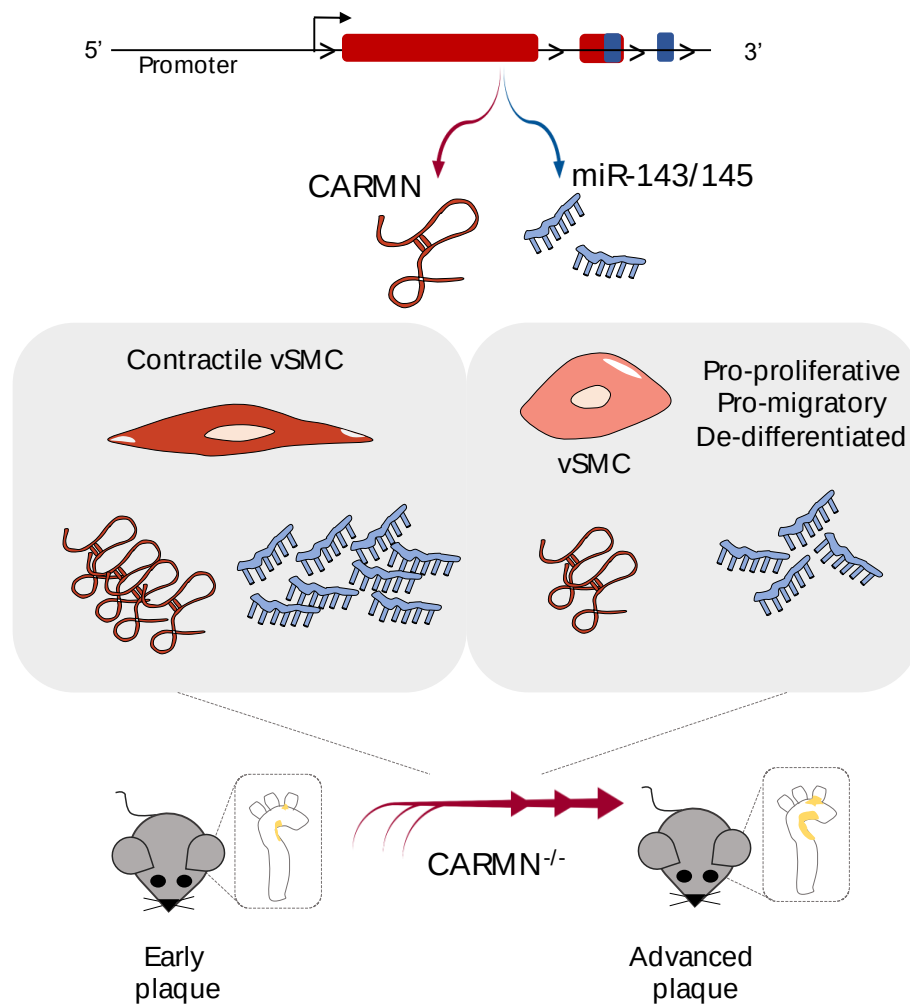


Figure 6.1 Graphical abstract illustrating the main results of this study.

6.2 Limitations of the study

The study of complex loci such as *CARMN* results to be challenging, and limitations within the research enhance the difficulty to comprehensively describe their structure and role experimentally. In this project, limiting factors characterise the *in vitro* experimental design as well as the study of the consequences due to *CARMN* depletion *in vivo*. The use of GapmeR targeting *CARMN* transcripts, and the genetic depletion of *CARMN* promoter revealed to have an effect by negatively modulating miR143/145 expression. Moreover, the co-transfection strategy applied in this study

to downregulate the expression of *CARMN* while maintaining overexpressed the microRNAs, can have target-off effect. To overcome this, CRISPR technology could be used to specifically affect the expression of miR-143/145 *in vivo* with the insertion of a stop codon which allows the expression of *CARMN*, while stopping miR-143/145 transcription. Considering the structural complexity of the locus, the insertion of a stop codon in the region downstream to *CARMN* isoforms and upstream to miR-143/145 could terminate the pri-microRNA translation prematurely inducing transcriptional termination of miR-143/145 pri-microRNA⁴²⁴. This strategy could be used to understand the consequences of miRNAs modulation separately from *CARMN* and to molecularly dissect the functional role of the two noncoding RNAs in vSMCs. In addition, the use of antisense oligonucleotides to selectively suppress the function of miR-143/145 *in vitro* (such as AntimiR) can provide a valuable tool to characterise the molecular consequences of miR-143/145 depletion to vSMC transcriptome independently from the regulation of *CARMN*. Moreover, strategies to restore the expression of *CARMN* could provide useful information to explore whether the increase of *CARMN* can revert or limit the deregulation of vSMC phenotype occurring following *CARMN* depletion. In this regard, the use of viral-mediated expression of single transcript would not be possible due to limited cargo size of available virus vectors, however the endogenous overexpression of *CARMN* and/or miR-143/145 could be achieved by CRISPR-mediated transcriptional activation. This strategy would also allow to test the activity of multiple promoters existing in *CARMN* locus and explore the hypothesis of a separate activation of miR-143/145 from *CARMN*. The study of *CARMN* was limited to vSMCs where the miRNA cluster is highly expressed. In this regard, the use of CSMCs belonging to multiple donor patients as well other sources of SMCs, such as Saphenous Vein SMCs or Pulmonary Aortic SMCs, could be valuable resources to enhance variability in the study and to gain a more comprehensive knowledge of the expression of the axis. Nevertheless, *CARMN* can also be expressed in other cell types composing the vasculature and immune cells. Considering the cellular-specific function of lncRNAs, it would be a valid approach to explore the expression and role of *CARMN* axis in pro-

inflammatory macrophage activation as well as in other vSMC phenotypes characterising the latest stages of plaque development, such as senescent/apoptotic vSMCs and vSMC-derived osteochondrocytes.

6.3 Future perspectives

Targeting the events characterising vascular remodelling in cardiovascular disease has been the main goal of several studies. In particular, the identification of key processes influencing arterial remodelling represented important goal for the possibility to therapeutically treat vascular remodelling. In this context, recent important advances in understanding the vSMC phenotypical transition identified the wide spectrum of associated phenotypes and uncovered beneficial as well as atherosclerosis-prone mechanisms, activated in response to atherogenic stimuli. However, the application of this knowledge in the context of atherosclerosis and its translation into clinical intervention remains still limited. This is the result of multiple factors including i) the lack of pre-clinical murine models resembling human atherosclerosis, ii) the difficulty of dissecting the cellular heterogeneity influencing cell type-specificity and site-specificity of the disease, iii) the limited knowledge of the genetic and epigenetic components contributing to this variability as well as the slow and chronic nature of the pathology. In the pathological context of *CARMN* axis modulation, the re-activation of its expression during the progression of atherosclerosis could be a valuable approach to reduce vSMC phenotypical switch, preventing the early phases of pathological remodelling. However, due to the asymptomatic nature of most of the lesions in their early stage, the restoration of *CARMN* axis expression does not seem a suitable approach in the case of the early vascular remodelling characterising the development of atherosclerosis. While targeting a specific cellular process in the context of atherosclerosis might not be by itself enough to block the progression of the pathology, the identification of specific molecular mechanisms governing vSMC phenotype can offer valuable information for the wider application of the findings of this project to vascular remodelling. In fact, vSMC phenotypical transition has been characterised as key process determining the

outcome of important surgical revascularisation procedures, such as coronary artery bypass grafting that 1 million of patients undergo every year (18,000 in the UK alone <https://www.statista.com/statistics/940785/number-of-coronary-artery-bypass-surgeries-in-united-kingdom-uk/>), with considerable impact on healthcare costs for cardiovascular diseases. This medical procedure is in >90% of cases based on the autologous implantation of saphenous vein into the coronary artery. However, 50% of these interventions develop post-surgery remodelling and long-term occlusion of the vessel and atherosclerosis within 10 years⁴¹⁸. Therefore, monitoring the early remodelling of the graft, by blocking vSMC proliferation, offers an important chance to preserve the long-term patency of the vessel. Together with this, the *ex vivo* access of the target tissue represents an appealing possibility for clinical interventions. During the implantation of the vein into the coronary artery it is possible to expose the tissue *ex vivo* to therapeutics, however also in this case the difficulty to reach long-term therapeutical efficiency still represents a major limitation. This is the main reason leading to failure of pre-clinical studies. In particular, only one study proceeded to clinical trial (PREVENT)⁴¹⁹, based on the delivery of oligonucleotide against E2F transcription factor to reduce intimal hyperplasia, which however failed to proceed to phase III due to lack of long-term efficiency. To overcome this difficulty, the use of gene therapy with viral vectors can be a powerful approach to ensure tissue long-term expression of the desired therapeutic. In this regard, multiple viral vectors have been generated with different means of action, levels of toxicity and packaging size. While AAV vectors are characterised by size limit (~5 kb)⁴²⁰, lentiviral vectors offer high packaging sizes and can transduce efficiently primary cells in culture, displaying however high toxicity levels *in vivo*. Adenoviral vectors instead constitute a safer option for long-term transduction of large genomic portions⁴²¹. Considering the aforementioned points, the next question would be whether gene therapy mediated restoration of *CARMN* axis can prevent the maladaptive remodelling and preserve the long-term patency of the vessel. Overexpressing a multi-transcript host gene lncRNA can be a difficult task if individual transcripts would need to be cloned into viral vectors, and in the case of *CARMN* the size of some

isoforms exceeds the packaging limit of viral particles. In this context, CRISPR strategy can be a powerful approach to endogenously activate the entire locus respecting the physiologic abundances of each isoform. CRISPR technology has rapidly advanced in the last few years and a plethora of new applications based on this strategy have been developed to endogenously repress or activate gene loci⁴²². CRISPR-VPR is one of the most efficient tools for gene activation, which uses deactivated Cas9 endonuclease to selectively bind the promoter of target loci using a single guide RNA (sgRNA)⁴²³. This system would give the advantage to activate the entire panel of *CARMN* transcripts as well as miR-143/145 in the case they are regulated by the same promoter or by *CARMN* mediated cis-activation. Next step for this project would therefore be to generate proof-of-concept data to test the feasibility of *CARMN* axis activation using a single viral vector expressing CRISPR-VPR system. The first application of this system would be *in vitro* in primary cells to assess the efficient activation of the locus. The primary purpose of this strategy would be to transcriptionally induce the expression of the locus in SMCs where *CARMN* is downregulated during disease. However, considering that the data of this study suggest that *CARMN* might be expressed by other cell types, such as macrophages and immunity cells, and that the modulation of *CARMN* might have an impact on these cell populations with determinant function in atherosclerosis and vascular remodelling, it would be important to explore the effect of *CARMN* activation in these cell populations. However, this would imply the previous study of *CARMN* role in these cell types *in vitro*, such as in human or mouse macrophage culture, to better identify the molecular mechanism regulating the axis and the consequence of *CARMN* depletion on inflammation and cell plasticity. In addition to this, strategies based on the cell type specific activation of *CARMN*, such as using an inducible SMC-specific promoter, would give the possibility to explore the effect of this modulation specifically in SMCs. The transability of the CRISPRa-mediated activation of *CARMN* consists of the possibility to intervene *ex vivo* for the pre-treatment of the vein previous implantation to the coronary artery. Nevertheless, this strategy would also provide novel essential information to the field of gene therapy by modulation of

complex loci, and therefore constitutes a powerful tool that can be widely applied for translational research. A schematic representation of the proposed strategy can be found in Figure 6.2.

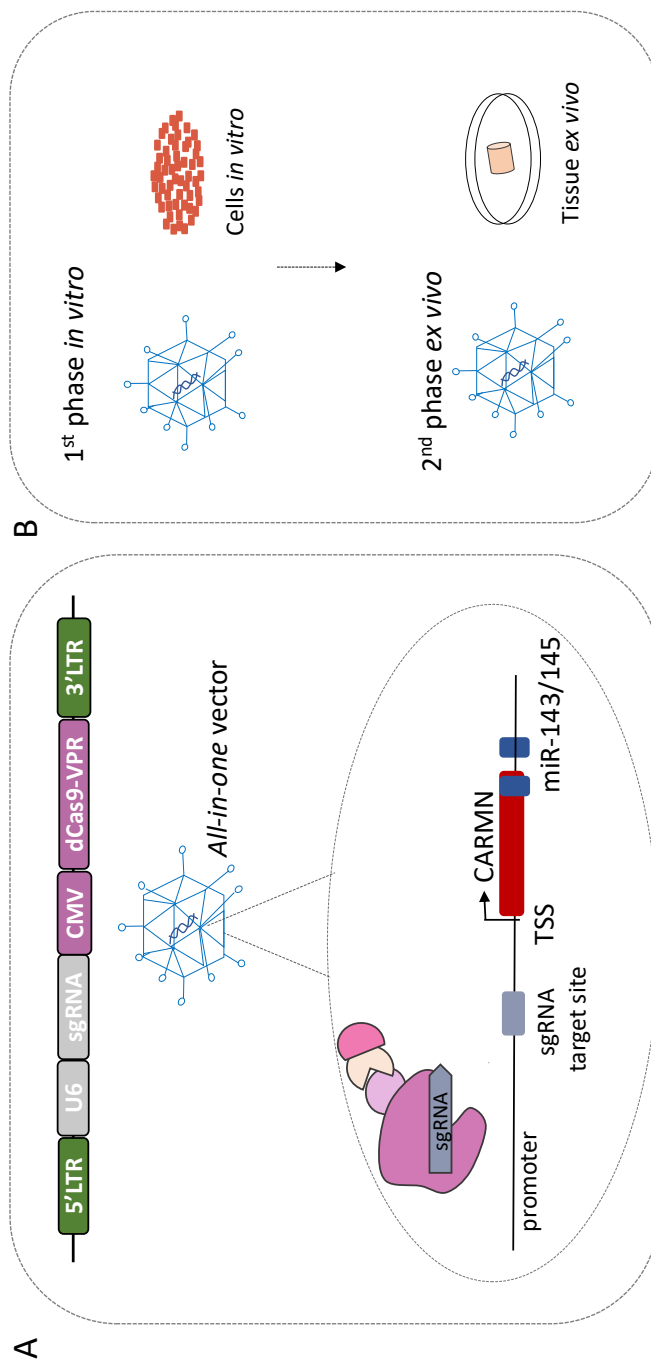


Figure 6.2 Schematic representation of gene therapy-mediated activation of CARMN axis using CRISPR activator.

CARMN axis resulted downregulated during the pathological remodelling characterising atherosclerosis. Considering that CARMN axis has important role in the VSMC pathological switch, the restoration of CARMN axis expression could be a powerful approach to prevent the maladaptive vascular remodelling that characterise the vessel following revascularisation strategies such as vein grafting. A) Diagram displaying the genome structure of the viral vector that can be used to overexpress CARMN axis using CRISPR activator. The molecular mechanism of action mediated by CRISPR-VPR system is represented: the dCas9 machinery linked to 3 transcriptional activators (pink) binds to the single guide RNA (sgRNA, grey arrow) to target the recognised site in the promoter of CARMN axis, therefore driving transcription of the locus. B) Scheme of the proof-of-concept experiments required to assess the feasibility of CRISPR-VPR mediated activation of CARMN axis through infection of primary cells *in vitro* followed by viral infection of the tissue *ex vivo*.

Figure 6.2 Schematic representation of gene therapy-mediated activation of CARMN axis using CRISPR activator.

6.4 Concluding remarks.

This PhD project highlighted the importance of *CARMN* as lncRNA host gene with independent function from the co-located miR-143/145 and uncovered critical mechanisms of lncRNA/microRNA regulation in dysfunctional vSMCs. In this research, the expression of *CARMN* axis (including *CARMN* transcripts and miR-143/145) was comprehensively profiled in the context of human and mouse atherosclerosis, where *CARMN* was identified expressed primarily in the nucleus of vSMCs but also in CD68 expressing cells and found downregulated in advanced plaque phenotype versus earlier stages during lesion development. Mechanistically, the downregulation of *CARMN* axis primes vSMCs at basal condition to phenotypically switch to a pro-proliferative, migratory, partially de-differentiated and pro-inflammatory signature. Downregulation of the lncRNA regulated vSMCs proliferation at basal conditions whereas miR-143/145 primarily regulate vSMCs migration and de-differentiation towards a macrophage-like phenotype. In the pathological context of atherosclerosis *in vitro*, the loss of *CARMN* axis accelerated the process of plaque formation to a more advanced phenotype characterised by increased plaque area, volume, Lgals3 expressing cell content, and decreased collagen content. While the causality of the regulatory molecular interaction between the two noncoding RNAs *CARMN*/miR-143/145 requires more efforts to be clearly identified, these discoveries could be applied for the modulation of the entire *CARMN* axis for therapeutical applications in the management of maladaptive remodelling in vascular injury.

Appendix

Table A1. Genes regulated by *CARMN* involved in lipid metabolism.

Table indicating the list of genes involved in lipid metabolism (GO:0033993) identified by Gene Ontology analysis in vSMC treated with GapmeR targeting *CARMN* (Gap*CARMN*) and control (GapCTR) in the basal condition during treatment (C) with cholesterol (Basal_C).

ENSEMBL name	type	chr	str	GapCARMN_Basa	GapCARMN_Basal.C2	GapCARMN_Basal.C3	GapCTR_Basal.C1	GapCTR_Basal.C2	GapCTR_Basal.C3
ENSG(CFLAR	protein	chr2	+	44.78	40.55	41.11	19.84	18.51	20.59
ENSG(PDK4	protein	chr7	-	0.75	1	1.08	0.83	1.4	1.33
ENSG(DCN	protein	chr12	-	1950.59	2080.88	1945.65	3536.27	3444.27	3560.52
ENSG(LTF	protein	chr3	-	0.09	0	0.02	2.7	2.93	2.96
ENSG(SNAI2	protein	chr8	-	18.44	18.14	18.28	31.7	35.61	35.74
ENSG(CPS1	protein	chr2	+	2.46	3.2	1.98	5.4	3.49	3.37
ENSG(GRAMD1B	protein	chr11	+	2.12	2.41	2.31	0.67	0.99	0.79
ENSG(TNC	protein	chr9	-	26.75	43.51	27.63	6.41	9.71	4.93
ENSG(DSG2	protein	chr18	+	15.42	14.41	15.73	29.35	30.07	31.83
ENSG(DGAT2	protein	chr11	+	7.15	6.31	6.03	2.31	2.02	2.82
ENSG(TIE1	protein	chr1	+	3.18	4.14	3.42	1.39	0.9	0.77
ENSG(TGFBR3	protein	chr1	-	7.05	6.25	6.86	14.2	12.99	15.5
ENSG(SCARB1	protein	chr12	-	26.23	27.7	27.71	11.46	12.39	11.75
ENSG(PTGS2	protein	chr1	-	53.71	58.91	55.69	7.34	6.61	4.8
ENSG(NFKB2	protein	chr10	+	40.59	39.53	38.73	15.36	13.96	14.9
ENSG(EDN1	protein	chr5	+	14.13	12.9	15.31	6.57	5.36	5.91
ENSG(CXCL2	protein	chr4	-	54.04	49.66	48.06	10.63	10.18	10.44
ENSG(MEF2C	protein	chr5	-	1.64	1.24	2.05	1.69	2.27	3.09
ENSG(NCOA1	protein	chr2	+	4.35	3.78	3.91	7.37	7.36	8.51
ENSG(ICAM1	protein	chr19	+	72.46	70.4	72.86	39.03	38.33	39.18
ENSG(TGFBR2	protein	chr1	+	65	63.59	64.27	27.56	20.75	29.76
ENSG(GGT1	protein	chr22	+	11.04	13.26	12.51	3.57	3.35	2.57
ENSG(CSF2RB	protein	chr22	+	4.86	4.29	4.39	4.69	3.17	4.07
ENSG(BDKRB1	protein	chr14	+	70.08	71.1	65.13	27.49	27.49	25.8
ENSG(PCK2	protein	chr14	+	14.66	15.72	13.63	28.02	29.51	28
ENSG(NFKBIA	protein	chr14	-	93.38	91.98	90.6	42.46	36.92	39.36
ENSG(E2F1	protein	chr20	-	2.23	1.97	1.85	0.5	0.36	0.46
ENSG(ACP5	protein	chr19	-	1.2	1.26	1.27	0.57	0.6	0.84
ENSG(RIPK2	protein	chr8	+	31.93	31.62	29.97	9.6	8.64	9.82
ENSG(SFRP1	protein	chr8	-	45.63	47.14	43.36	59.52	62.74	61.22
ENSG(GDAP1	protein	chr8	+	4.21	4.73	4.35	2.13	1.52	2.64
ENSG(CAV1	protein	chr7	+	93.04	94.68	101.65	193.44	186.07	173.96
ENSG(WNT2	protein	chr7	-	0.84	2.35	1.69	2.62	1.71	1.85
ENSG(SERPINE1	protein	chr7	+	998.64	956.52	974.94	185.58	116.79	150.85
ENSG(TGFBR1	protein	chr9	+	20.18	18.35	18.73	13.5	13.62	11.6
ENSG(DKK1	protein	chr10	+	63.39	52.94	53.84	131.56	112.04	147.86
ENSG(CSF3	protein	chr17	+	65.71	62.32	59.87	0.95	0.9	0.91
ENSG(SLC6A4	protein	chr17	+	0.16	0.16	0.32	0.67	1.23	0.57
ENSG(CCL2	protein	chr17	+	1491.38	1426.06	1478.08	266.8	237.93	247.11
ENSG(PF4V1	protein	chr4	+	5.34	4.8	2.91	1.4	1.09	1.18
ENSG(AREG	protein	chr4	+	2.09	4.38	2.25	0.33	1.12	0.15
ENSG(CRYAB	protein	chr11	-	21.09	26.1	25.15	29.27	43.29	32.55
ENSG(CCND1	protein	chr11	+	173.37	192.39	169.33	20.07	16.49	18.91
ENSG(ADTRP	protein	chr6	-	3.77	4.71	4.57	2.65	2.12	1.32
ENSG(LOX	protein	chr5	-	148.72	137.08	141.12	131.29	104.38	126.71
ENSG(PDE4D	protein	chr5	-	2.22	2.2	2.44	0.7	0.85	1.05
ENSG(PDGFRB	protein	chr5	-	14.93	18.52	16.74	47.02	57.57	51.07
ENSG(STC2	protein	chr5	-	94.36	87.32	90.42	70.72	67.69	72.56
ENSG(PLSCR4	protein	chr3	-	87.11	79.62	87.69	63.4	63.56	65.38
ENSG(SDC1	protein	chr2	-	14.61	14.29	15.41	3.67	6.24	3.48
ENSG(ERRF1	protein	chr1	-	15.64	16.99	15.8	32.49	36.04	32.62
ENSG(ID3	protein	chr1	-	123.36	126.83	125.58	13.81	14.61	13.8
ENSG(TNFAIP3	protein	chr6	+	104.39	95.2	101.87	20.28	17.76	15.38
ENSG(CTGF	protein	chr6	-	279.97	276.76	295.02	43.01	44.45	43.87
ENSG(KLF9	protein	chr9	-	13.9	12.32	13.98	11.45	10.35	11.27
ENSG(NR4A3	protein	chr9	+	0.53	1.01	0.65	0.1	0.29	0.17
ENSG(DUSP1	protein	chr5	-	25.28	26.78	24.16	5.76	5.55	4.95
ENSG(SOCS2	protein	chr12	+	0.8	0.62	0.87	3.03	3.42	3.49
ENSG(CLU	protein	chr8	-	266.21	286.26	249	429.49	469.97	452.49
ENSG(PTK2B	protein	chr8	+	4.96	4.46	4.68	22.84	25.73	22.91
ENSG(TRIM6	protein	chr11	+	3.7	5.24	4.24	1.37	2.38	3.05
ENSG(FABP3	protein	chr1	-	1.86	2.8	2.91	5.16	4.78	7.13
ENSG(PTGFR	protein	chr1	+	6.1	7.63	6.43	16.1	13.74	14.82
ENSG(INHBA	protein	chr7	-	61.78	61.22	61.46	24.33	28.75	27.93
ENSG(NR4A1	protein	chr12	+	4.61	9.36	5.21	3.17	3.39	1.82
ENSG(SLPI	protein	chr20	-	1.16	1.19	2.35	5.51	8.12	3.22
ENSG(PMEP1A	protein	chr20	-	18.24	15.99	15.03	8.23	7.53	7.2
ENSG(CXCL6	protein	chr4	+	823.83	772.03	746.05	104.11	66.9	80.6
ENSG(BMP4	protein	chr14	-	0.57	0.41	0.44	2.62	1.5	1.71
ENSG(PTGER2	protein	chr14	+	2.67	3.13	2.53	0.51	0.91	0.46
ENSG(SOX9	protein	chr17	+	18.43	20.91	20.01	6.44	7.85	6.31
ENSG(IL1B	protein	chr2	-	389.29	391.91	383.01	51.45	43.71	52.94
ENSG(ZFP36	protein	chr19	+	8.67	8.39	8.85	17.06	16.47	18.64
ENSG(CD68	protein	chr17	+	142.09	155.72	155.9	78.73	66.93	69.57
ENSG(LDLR	protein	chr19	+	34.59	35.72	35.52	55.4	55.03	66.3
ENSG(ASS1	protein	chr9	+	57.73	62.75	58.98	69.43	75.52	73.08
ENSG(MAP1B	protein	chr5	+	51.96	46.5	50.72	22.27	22.22	22.77
ENSG(GCH1	protein	chr14	-	7.07	6.33	5.62	1.77	1.39	1.67
ENSG(POSTN	protein	chr13	-	40.95	35.83	41.95	68.93	58.77	66.83
ENSG(CCNB1	protein	chr5	+	8.8	8.49	11.08	2.8	2.39	2.93
ENSG(IRAK2	protein	chr3	+	7.54	7.61	7.42	2.19	2.24	2.67
ENSG(PTPN22	protein	chr1	-	6.67	5.95	4.63	0.63	0.66	1.33
ENSG(CD36	protein	chr7	+	0.48	1.05	0.91	1.58	0.73	1.68
ENSG(IL6	protein	chr7	+	389.92	344.2	356.86	18.77	12.58	16.09
ENSG(IL1RN	protein	chr2	+	5.49	6.18	4.75	0.75	0.75	0.28
ENSG(IL36RN	protein	chr2	+	0.63	0.24	0.16	0.12	0.16	0.3
ENSG(IL36B	protein	chr2	+	0.61	0.96	0.36	0.34	0.06	0.14
ENSG(TLR4	protein	chr9	+	25.79	25.18	24.94	10.27	10.64	9.12
ENSG(PIM1	protein	chr6	+	10.73	11.62	8.75	34.32	36.72	36.05
ENSG(MGARP	protein	chr4	-	10.76	10	12.15	12.77	11.75	12.69
ENSG(CASP1	protein	chr11	-	16.05	17.24	20.29	6.65	14.71	11.24
ENSG(THBS1	protein	chr15	+	893.76	838.93	934.91	343	287.97	305.45
ENSG(ANXA3	protein	chr1	+	22.89	21.68	24.95	26.42	35.31	32.1
ENSG(CYP11A	protein	chr15	-	2.61	2.59	3.04	0.14	0.52	0.24
ENSG(ABHD2	protein	chr15	+	45.42	45.69	47.82	18.08	13.24	17.46
ENSG(NTRK3	protein	chr15	-	1.67	1.11	1.3	2.07	1.86	2.64
ENSG(ARRB2	protein	chr17	+	11.98	10.91	10.36	7.25	8.49	7.59
ENSG(TNFRSF11A	protein	chr18	+	5.54	6.02	4.75	1.55	0.99	1.1
ENSG(DUSP10	protein	chr1	-	7.75	9.07	8.3	6.42	6.94	7.7
ENSG(OSR1	protein	chr2	-	1.54	1.65	1.35	7.06	5.35	6.19
ENSG(SLIT2	protein	chr4	+	20.99	19.76	19.67	26.5	24.15	26.06
ENSG(CCNA2	protein	chr4	-	2.18	2.19	2.07	0.62	0.88	1.14
ENSG(CRHRP	protein	chr5	+	1.61	2.33	1.66	0.67	0.18	0.21
ENSG(PAM	protein	chr5	+	89.05	92.47	84.78	319.71	297.52	316.45
ENSG(EGFR	protein	chr7	+	23.96	18.6	23.37	29.54	27.84	28.12
ENSG(MSN	protein	chrX	+	285.53	290.31	289.56	114.35	117.88	117.05
ENSG(PTGES	protein	chr9	-	56.28	49.4	53.66	24.54	24.35	23.6
ENSG(ANKRD1	protein	chr10	-	2.66	2.6	2.54	0.14	0.25	0.1

Table A2. Genes regulated by *CARMN* involved in proliferation.

Table indicating the list of genes involved in proliferation (GO:0008284) identified by Gene Ontology analysis in vSMC treated with GapmeR targeting *CARMN* (Gap*CARMN*) and control (GapCTR) in the basal condition during treatment (P) with cholesterol (Basal_P).

ENSEM name	type	chr	start	end	str	GapCARMN_Basal.P1	GapCARMN_Basal.P2	GapCARMN_Basal.P3	GapCTR_Basal.P1	GapCTR_Basal.P2	GapCTR_Basal.P3
ENSGO CFLAR	protein	chr2	201116104	2.01E+08	+	24.79	21.46	20.15	19.84	19	19.18
ENSGO CCL26	protein	chr7	75769533	75789896	-	7.63	4.5	4.41	1.97	1.44	4.18
ENSGO LTF	protein	chr3	46435645	46485234	-	3.35	2.44	2.82	0.74	0.57	0.7
ENSGO IGF1	protein	chr12	102395867	1.02E+08	-	2.4	3.92	2.78	3.15	3.65	3.3
ENSGO WWTR1	protein	chr3	149517235	1.5E+08	-	59.42	53.63	49.54	47.96	42.02	42.4
ENSGO CD74	protein	chr5	150401637	1.5E+08	-	3.47	3.6	3.52	2.02	2.35	1.63
ENSGO TNC	protein	chr9	115019578	1.15E+08	-	2.13	2.13	1.49	0.55	0.69	0.77
ENSGO PTPRN	protein	chr2	219289623	2.19E+08	-	6.61	6.24	5.37	0.39	1	0.55
ENSGO CDON	protein	chr11	125955796	1.26E+08	-	1.3	1.54	1.57	6.09	6.73	5
ENSGO HIPK2	protein	chr7	139561570	1.4E+08	-	6.93	6.62	6.81	7.96	7.33	6.55
ENSGO ERBB3	protein	chr12	56079857	56103505	+	1.29	1.43	1.19	0.81	0.63	0.8
ENSGO PRKC2	protein	chr1	2050470	2185395	+	1.59	1.77	2.57	0.91	1.17	0.61
ENSGO TGFBR3	protein	chr1	91680343	91906335	-	5.88	4.91	5.96	12.09	10.58	10.58
ENSGO PTGS2	protein	chr1	186671791	1.87E+08	-	2.71	3.66	2.18	1.7	1.03	1.17
ENSGO NOTCH3	protein	chr19	15159038	15200981	-	14.1	11.41	12.54	11.59	11.18	11.53
ENSGO FOSL2	protein	chr2	28392448	28417312	+	30.11	30.09	26.52	15.55	17.25	15.16
ENSGO FGFR1	protein	chr8	38411138	38468834	-	52.61	47.34	53.61	97.05	93.47	99.52
ENSGO EDN1	protein	chr6	12290363	12297194	+	14.76	13.88	11.68	12.23	13.47	12.99
ENSGO MEFC2	protein	chr5	88717117	88904257	-	1.33	1.17	1.56	3.43	6.23	3.69
ENSGO AKR1B1	protein	chr7	134442350	1.34E+08	-	80.21	75.12	70.6	75.09	71.89	69.05
ENSGO PTHLH	protein	chr12	27958084	27972705	-	3.06	2.95	3.23	1.25	2.17	0.95
ENSGO TBX5	protein	chr12	114353931	1.14E+08	-	1.48	1.38	1.69	2.17	1.68	2.08
ENSGO BIRC5	protein	chr17	78214186	78225636	+	0.3	0.14	0.52	0.43	0.77	0.33
ENSGO CHR1	protein	chr3	184380073	1.84E+08	+	1.55	0.89	0.98	0.98	0.51	0.34
ENSGO TGM1	protein	chr14	24249114	24264432	-	1.72	2.45	3.5	0.52	0.92	1.56
ENSGO TGFBR2	protein	chr1	218346235	2.18E+08	+	25.95	24.36	21.2	15.88	9.38	13.2
ENSGO CDC6	protein	chr17	40287633	40304657	+	0.78	0.66	0.26	0.11	0.68	0.21
ENSGO BAMBI	protein	chr10	28677342	28682939	+	19.94	16.85	15.44	20.09	17.22	18.71
ENSGO IL11	protein	chr19	55364389	55370463	-	2.23	2.19	1.66	1.96	1.74	1.41
ENSGO HIF1A	protein	chr14	61695513	61748259	+	327.37	298.18	286.02	174.02	166.56	161.21
ENSGO EZF1	protein	chr20	33675683	33686404	-	0.09	0.38	0.14	0.44	0.37	0.53
ENSGO TIMP1	protein	chrX	47582313	47586789	+	5441.69	4870.54	4942.76	3730.34	3602.66	3647.46
ENSGO HTR2A	protein	chr13	46831550	46897076	-	5.33	3.06	4.5	1.21	0.6	1.39
ENSGO TNFSF13	protein	chr13	108251240	1.08E+08	+	3.42	2.5	2.4	3.49	3.6	2.94
ENSGO FLT1	protein	chr13	28300344	28495145	-	0.88	1.32	0.82	1.68	1.79	1.56
ENSGO HSF4	protein	chr16	67164681	67169945	+	10.02	8.54	9.31	12.72	7.9	10.93
ENSGO RIPK2	protein	chr8	89757747	89791063	+	10.82	10.33	10.6	8.48	6.46	7.53
ENSGO SFRP1	protein	chr8	41261958	41309497	-	29.03	31.97	32.33	56.75	53.13	58.44
ENSGO EBI3	protein	chr19	4229498	4237531	+	1.02	0.97	1.3	0.2	0	0
ENSGO NAMPT	protein	chr7	106248285	1.06E+08	-	240.51	193.53	157.55	72.38	71.6	64.71
ENSGO WNT2	protein	chr7	117276631	1.17E+08	-	0.48	0.62	0.83	1.87	1.23	1.23
ENSGO TGFBR1	protein	chr9	99104038	99154192	+	17.8	18.35	15.8	8.77	8.26	9.45
ENSGO KDM4C	protein	chr9	6720863	7175648	+	5.26	4.58	5.11	6.42	6.53	6.03
ENSGO CXCL12	protein	chr10	44370165	44386493	-	48.96	48.84	52.52	66.31	66.3	71.44
ENSGO CSF3	protein	chr17	40015361	40017813	+	1	1.29	0.66	0.75	0.45	0.26
ENSGO AREG	protein	chr4	74445134	74455009	+	0.58	0.49	0.54	0.36	0.3	0.19
ENSGO BST1	protein	chr4	15702950	15738313	+	18.9	16.93	16.29	21.11	16.62	17.38
ENSGO CCND1	protein	chr11	69641087	69654474	+	35.5	31.54	22.79	34.76	32.95	37.92
ENSGO CD81	protein	chr11	2376177	2397419	+	159.84	161.37	183.49	610.2	595.61	589.4
ENSGO IL23A	protein	chr12	56334174	56340410	+	0	0	0	0.18	0	0.05
ENSGO FOXM1	protein	chr12	2857681	2877155	-	1.02	0.93	0.67	1.47	0.56	1.46
ENSGO HBEGF	protein	chr5	140332843	1.4E+08	-	1.38	1.19	1.2	0.98	0.83	0.8
ENSGO FGF1	protein	chr5	142592178	1.43E+08	-	2.37	1.88	1.82	1.21	0.69	0.73
ENSGO PDGFRB	protein	chr5	150113837	1.5E+08	-	46.54	50.6	51.28	74.21	71.31	74.14
ENSGO HES1	protein	chr3	194136145	1.94E+08	+	1.27	1.24	1.09	0.31	0.33	0.49
ENSGO HYAL1	protein	chr3	50299889	50312381	-	8.57	9.16	8.26	1.15	1.14	0.97
ENSGO VIPR1	protein	chr3	42489299	42537573	+	5.76	5.86	4.2	1.02	0.77	1
ENSGO ITGA4	protein	chr2	181457202	1.82E+08	+	3.73	3.73	3.43	1.24	2.58	1.96
ENSGO IGFBP5	protein	chr2	216672105	2.17E+08	-	2.25	1.58	1.67	0.71	0.58	0.7
ENSGO ODC1	protein	chr2	10439968	10448504	-	35.61	36.62	32.47	28.01	29.36	28.85
ENSGO CDC20	protein	chr1	43358955	43363203	+	0.66	0.7	0.22	0.25	0.24	0.16
ENSGO TNFAIP3	protein	chr6	137867188	1.38E+08	+	24.94	25.69	20.33	10.56	7.71	10.53
ENSGO CTGF	protein	chr6	131948176	1.32E+08	-	198.56	183.53	151.66	102.25	112.03	109.09
ENSGO NR4A3	protein	chr9	99821855	99866891	+	0.14	0.21	0.22	0.24	0.31	0.27

ENSEMBL name	type	chr	str	GapCARMN_Basal.C1	GapCARMN_Basal.C2	GapCARMN_Basal.C3	GapCTR_Basal.C1	GapCTR_Basal.C2	GapCTR_Basal.C3
ENSG00000180133	protein	chr11	+	15.49	19.41	13.46	56.24	54.86	49.64
ENSG00000180134	protein	chr2	+	23.72	26.95	30.9	49.36	56.43	59.57
ENSG00000180135	protein	chr13	-	2.04	2.04	2.02	3.43	4.49	5.17
ENSG00000180136	protein	chr4	+	8.19	6.37	6.93	6.3	5.71	5.28
ENSG00000180137	protein	chr5	+	4.79	4.7	5.85	7.08	7.36	7.04
ENSG00000180138	protein	chr10	-	26.4	27.45	29.34	120.32	166.54	155.59
ENSG00000180139	protein	chr5	-	52.37	49.02	52.19	136.96	130.62	143.14
ENSG00000180140	protein	chr6	+	48.17	46.52	49.59	4.74	7.62	6.6
ENSG00000180141	protein	chr2	-	1.91	5.49	2.23	0.92	0.56	0.54
ENSG00000180142	protein	chr2	-	124.07	114.05	121.92	31.66	27.06	26.62
ENSG00000180143	protein	chr17	+	4.56	6.03	4.28	21.25	20.36	20.96
ENSG00000180144	protein	chr8	-	64.65	62.31	57.94	126.1	113.52	119.63
ENSG00000180145	protein	chr3	-	1.44	1.37	1.58	0.1	0.34	0.08
ENSG00000180146	protein	chr8	-	50.41	50.8	49.19	27.62	18.1	19.89
ENSG00000180147	protein	chr21	-	22.35	21.26	21	24.99	22.77	24.6
ENSG00000180148	protein	chr4	-	4.16	6.68	4.49	36.71	54.76	44.48
ENSG00000180149	protein	chr17	-	17.76	17.53	16.45	32.55	34.67	35.91
ENSG00000180150	protein	chr19	-	9.62	8.94	10.81	4.69	3.23	3.33
ENSG00000180151	protein	chr21	+	5.29	5.22	4.59	0.62	1.11	0.68
ENSG00000180152	protein	chr1	+	3.12	3.62	2.79	6.8	6.02	5.42
ENSG00000180153	protein	chr1	+	20.4	21.5	17.6	0.88	0.83	0.91
ENSG00000180154	protein	chr3	-	3.98	3.48	3.85	5.1	4.4	5.24
ENSG00000180155	protein	chr4	-	58.03	53.49	54.1	4.45	3.55	3.49
ENSG00000180156	protein	chr4	-	12.74	10.96	12.14	1.19	1.04	1.42
ENSG00000180157	protein	chr4	+	1699.9	1627.61	1632.49	102.34	78.01	96.97
ENSG00000180158	protein	chr1	+	42.86	41.02	39.51	10.49	9.64	10.59
ENSG00000180159	protein	chr5	+	63.62	57.52	62.24	7.5	7.05	7.42
ENSG00000180160	protein	chr5	+	3.04	3.26	2.15	0.08	0.36	0.29
ENSG00000180161	protein	chr5	+	5.97	4.67	4.67	10.12	8.77	9.57
ENSG00000180162	protein	chr7	+	1.67	2.4	2.2	0.79	0.53	1.38
ENSG00000180163	protein	chr9	-	19.63	17.42	18.29	10.5	10.36	10.06
ENSG00000180164	protein	chr8	-	4.34	4.68	4.65	0.94	1.59	1.39
ENSG00000180165	protein	chr16	-	0.98	1.02	1.19	0.54	0.64	0.46
ENSG00000180166	protein	chr3	-	2.61	2.94	2.84	0.68	0.39	0.58
ENSG00000180167	protein	chr17	-	38.68	39.99	38.65	77.79	78.69	79.28
ENSG00000180168	protein	chr17	+	4.74	3.69	2.89	2.24	1.99	1.44
ENSG00000180169	protein	chr2	-	7.86	7.56	8.19	3.54	4.02	3.93
ENSG00000180170	protein	chr4	+	669.1	634.53	648.75	22.88	17.51	17.17
ENSG00000180171	protein	chr14	+	1.3	2.43	1.73	2.01	2.8	2.81
ENSG00000180172	protein	chr5	-	5.51	5.45	6.23	3.21	3.99	3.2
ENSG00000180173	protein	chr2	+	3.08	2.18	2.72	3.44	3.78	3.53
ENSG00000180174	protein	chr5	+	2.54	4.11	1.49	0.73	1.88	1.05
ENSG00000180175	protein	chr11	+	2.19	2.35	2.34	0.48	0.45	0.11
ENSG00000180176	protein	chr15	+	4.53	5.9	6.17	7.48	6.72	8.12
ENSG00000180177	protein	chr8	+	4.93	5.72	5.13	3.59	2.67	2.45
ENSG00000180178	protein	chr3	+	84.5	85.67	87.62	52.34	53.5	55.17
ENSG00000180179	protein	chr11	-	34.2	34.81	34.62	5.94	8.77	6.21
ENSG00000180180	protein	chr18	+	25.55	24.96	24.63	11.18	10.46	10.74
ENSG00000180181	protein	chr3	+	3.83	3.15	3.31	1.55	2.02	1.97
ENSG00000180182	protein	chr9	-	2.98	3.23	3.48	1.16	1.18	1.11
ENSG00000180183	protein	chr20	-	5.49	5.25	4.98	1.16	1.5	1.31
ENSG00000180184	protein	chr3	-	2.01	2.91	2.79	1	1.32	1.49
ENSG00000180185	protein	chr5	+	70.94	63.51	68.03	30.95	29.86	30.08
ENSG00000180186	protein	chr9	+	178.44	153.17	171.94	44.73	35.76	39.44
ENSG00000180187	protein	chr8	+	9.37	10.14	8.94	9.97	11.09	10.28
ENSG00000180188	protein	chrX	-	110.35	103.52	108.83	54.5	60.83	54.95
ENSG00000180189	protein	chr5	-	26.15	24.97	25.57	123.26	119.51	118.13
ENSG00000180190	protein	chr1	+	12.42	11.46	12.18	3.45	4.31	3.07
ENSG00000180191	protein	chr1	-	5.52	5.92	5.71	10.42	15.14	12.25
ENSG00000180192	protein	chr14	+	8.1	6.09	6.32	4.5	3.46	3.59
ENSG00000180193	protein	chr1	-	1.53	1.65	1.59	0.23	0.2	0.23
ENSG00000180194	protein	chr10	+	57.63	55.55	62.21	154.14	163.62	171.27
ENSG00000180195	protein	chr2	-	8.27	7.75	8.35	4.62	3.69	3.08
ENSG00000180196	protein	chr9	+	10.27	9.09	10.04	3.56	4.35	3.87
ENSG00000180197	protein	chr9	-	39.23	35.33	38.29	73.07	67.3	72.66
ENSG00000180198	protein	chr20	-	53.4	56.37	57.47	31.12	29.01	29.04
ENSG00000180199	protein	chr11	-	18.53	16.11	17.39	8.89	7.66	8.64
ENSG00000180200	protein	chr2	+	18.89	21.3	19.24	26.27	25.36	25.97
ENSG00000180201	protein	chr17	-	17.95	18.1	19.26	10.82	10.52	9.92
ENSG00000180202	protein	chr17	+	76.3	77.81	74.97	32.26	33.96	36.45
ENSG00000180203	protein	chr7	+	1.95	1.61	1.65	0.48	1.04	0.57
ENSG00000180204	protein	chr8	+	3.36	3.72	3.32	4.21	4.15	4.31
ENSG00000180205	protein	chr17	-	16.01	14.09	13.27	2.92	3.57	2.84

ENSEN_name	type	chr	start	end	str	GapCARMN_Basal.P1	GapCARMN_Basal.P2	GapCARMN_Basal.P3	GapCTR_Basal.P1	GapCTR_Basal.P2	GapCTR_Basal.P3
ENSG0 PGF	protein	chr14	74941834	74955784	-	0.84	1.3	0.77	0.39	0.19	0.11
ENSG0 TEK	protein	chr9	27109141	27230175	+	24.35	22.94	22.01	13.39	13.04	13.68
ENSG0 EGR1	protein	chr5	138465490	1.38E+08	+	6.16	6.58	7.93	14.96	16.41	16.6
ENSG0 PTK2B	protein	chr8	27311482	27459391	+	25.02	24.41	25.24	26.99	27.64	28.34
ENSG0 PTGFR	protein	chr1	78303884	78539749	+	5.52	6.19	5.93	8.88	8.08	8.49
ENSG0 NR4A1	protein	chr12	52022832	52059507	+	2.46	2.57	3.4	6.09	4.87	5.61
ENSG0 RUNX2	protein	chr6	45328157	45664349	+	5.24	5.63	5.27	4.59	5.02	4.8
ENSG0 BMP4	protein	chr14	53949736	53958761	-	2.56	2.5	1.81	2.57	2.78	2.96
ENSG0 SOX9	protein	chr17	72121020	72126420	+	16.77	14.26	13.54	7.11	6.35	6.76
ENSG0 IL1B	protein	chr2	112829751	1.13E+08	-	30.31	28.9	27.15	17.4	19.52	18.59
ENSG0 BMP2	protein	chr20	6767664	6780280	+	2.64	2.4	2.91	3.36	2.72	3.18
ENSG0 ID1	protein	chr20	31605283	31606515	+	49.79	43.57	39.82	18.12	18.75	18.41
ENSG0 STAT5A	protein	chr17	42287547	42311943	+	6.43	6.7	6.76	11.67	9.77	11.16
ENSG0 KDR	protein	chr4	55078477	55125589	-	1.86	1.3	1.56	2.07	1.75	2.43
ENSG0 RAC2	protein	chr22	37225261	37244448	-	33.58	31.32	31.13	34.75	33.54	33.85
ENSG0 LIF	protein	chr22	30240447	30246851	-	11.07	8.74	8.81	8.66	8.04	8.7
ENSG0 ELL3	protein	chr15	43772600	43777543	-	1.2	2.42	1.78	2.26	1.05	1.98
ENSG0 NES	protein	chr1	156668763	1.57E+08	-	7.25	7.21	6.82	5.72	6	5.92
ENSG0 CCNB1	protein	chr5	69167010	69178245	+	2.51	2.33	3	4.15	2.76	2.87
ENSG0 IL6ST	protein	chr5	55935095	55994993	+	381.89	369.35	345.54	497.99	497.88	490.84
ENSG0 TBX3	protein	chr12	114670254	1.15E+08	-	5.8	6.5	5.23	6.1	6.08	5.7

ENSEMBL1	name	type	chr	str	GapCARMN_Basal.P1	GapCARMN_Basal.P2	GapCARMN_Basal.P3	GapCTR_Basal.P1	GapCTR_Basal.P2	GapCTR_Basal.P3
ENSG0000001	MAP3K5	protein	chr6	-	3.87	3.44	3.33	3.58	3.2	3.02
ENSG0000001	DPP4	protein	chr2	-	147.78	151.54	156.22	241.33	224.08	232.72
ENSG0000001	PDCD1LG2	protein	chr9	+	4.17	5.06	4.95	2.53	2.09	2.69
ENSG0000001	NMB	protein	chr15	-	9.57	7.2	9.62	7.19	6.44	6.9
ENSG0000001	IRAK4	protein	chr12	+	3.69	4.05	4.64	6.52	6.45	5.96
ENSG0000001	DDRKG1	protein	chr20	-	39.73	39.49	36.55	24.92	28.6	27.39
ENSG0000001	CARD11	protein	chr7	-	3.46	2.29	2.74	2.89	1.11	1.23
ENSG0000001	NUDT16	protein	chr3	+	7.41	7.65	7.58	11.55	12.53	11.91
ENSG0000001	PNP	protein	chr14	+	22.78	20.03	18.26	13.15	12.58	12.41
ENSG0000001	PRC1	protein	chr15	-	1.64	3.41	2.25	2.67	2.46	2.36
ENSG0000001	TGM2	protein	chr20	-	24.72	24.77	21.89	6.2	5.38	4.9
ENSG0000002	DDR1	protein	chr6	+	38.62	36.68	41.95	52.02	51.08	54.05
ENSG0000002	ADGRG1	protein	chr16	+	2.26	2.12	2.37	0.8	0.57	0.45
ENSG0000002	LBH	protein	chr2	+	27.37	30.53	29.01	32.37	34.74	37.96
ENSG0000002	S1PR3	protein	chr9	+	6.68	5.42	5.44	9.7	7.59	8.73
ENSG0000002	ANG	protein	chr14	+	11.4	13.39	11.66	16.2	15.08	14.22
ENSG0000002	NME1	protein	chr17	+	76.76	83.13	61.83	57.88	54.12	50.75
ENSG0000002	AQP1	protein	chr7	+	0.91	1	1.05	1.21	0.57	0.68
ENSG0000002	ETV5	protein	chr3	-	10.33	9.05	7.56	7.73	7.35	6.98
ENSG0000002	NEAT1	lincRNA	chr11	+	225.15	236.32	275.6	250.54	243.69	241.77
ENSG0000002	MALAT1	lincRNA	chr11	+	39.89	38.62	39.75	43.91	44.77	48.92
ENSG0000002	LYN	protein	chr8	+	3.17	3.09	2.2	3.55	3.23	3.43
ENSG0000002	ITGB3	protein	chr17	+	6.01	6.33	4.92	2.24	2.71	2.95
ENSG0000002	CCL5	protein	chr17	-	3.09	3.12	2.38	1.59	1.6	1.81
ENSG0000002	CD24	protein	chr6	-	35.06	37.18	27.22	8.73	8.65	9.22

ENSEMBL1	name	type	chr	str	GapCARMN_Basal.P1	GapCARMN_Basal.P2	GapCARMN_Basal.P3	GapCTR_Basal.P1	GapCTR_Basal.P2	GapCTR_Basal.P3
ENSG00000011	HILPDA	protein	chr7	+	9.53	13.36	9.08	8.25	7.76	7.37
ENSG00000011	HEY2	protein	chr6	+	1.93	1.79	1.75	2.55	2.95	2.77
ENSG00000011	DYSF	protein	chr2	+	2.67	2.12	1.6	1.73	1.09	1.27
ENSG00000011	RNASEH2B	protein	chr13	+	13.34	8.57	7.87	11.75	13.63	12.52
ENSG00000011	TNS3	protein	chr7	-	31.17	32.74	30.32	52.29	51.4	52.85
ENSG00000011	IL6	protein	chr7	+	55.29	49.72	34.04	15.71	13.15	11.52
ENSG00000011	TLR4	protein	chr9	+	14.3	12.26	10.17	9.17	9.53	8.73
ENSG00000011	IL11RA	protein	chr9	+	6.79	7.44	7.61	13.36	12.85	11.45
ENSG00000011	PIM1	protein	chr6	+	37.11	36.13	36.92	29.54	22.97	25.83
ENSG00000011	SDCBP	protein	chr8	+	97.88	99.41	115.59	218.4	202.91	214.24
ENSG00000011	THBS1	protein	chr15	+	740.57	665.67	612.78	317.39	326.8	323.44
ENSG00000011	ACVRL1	protein	chr12	+	16.29	16.84	16.21	7.45	8.79	7.48
ENSG00000011	NTRK3	protein	chr15	-	3.06	2.25	2.34	7.5	6.85	7.09
ENSG00000011	ITGAX	protein	chr16	+	0.25	0.22	0.22	0.05	0.12	0.09
ENSG00000011	CDH13	protein	chr16	+	22.2	20.09	20.12	62.05	64.72	69.34
ENSG00000011	RPTOR	protein	chr17	+	2.32	2.59	3.16	7.09	6.73	6.29
ENSG00000011	TNFRSF11A	protein	chr18	+	2.02	1.64	1.3	0.64	0.69	0.49
ENSG00000011	VASH2	protein	chr1	+	0.61	0.99	0.44	1.75	0.7	1.76
ENSG00000011	OSR1	protein	chr2	-	5.83	6.48	6.18	10.91	10.84	13.46
ENSG00000011	PLAC8	protein	chr4	-	1.01	0.77	1.02	0.48	0.27	0.55
ENSG00000011	CCNA2	protein	chr4	-	0.57	0.62	0.47	0.22	0.55	0.64
ENSG00000011	TSLP	protein	chr5	+	0.93	0.79	0.72	2.35	2.14	1.65
ENSG00000011	EGFR	protein	chr7	+	16.36	16.47	15.37	28	28.26	27.6
ENSG00000011	TCF7L2	protein	chr10	+	22.56	23.7	27.44	25.94	27.37	24.91
ENSG00000011	ADM	protein	chr11	+	26.75	27.36	30.09	55	59.28	63.92
ENSG00000011	DRD2	protein	chr11	-	0.62	0.74	0.32	0.09	0.12	0.11
ENSG00000011	HMG2A	protein	chr12	+	3.91	4	3.21	3.93	3.22	5.01
ENSG00000011	VEGFC	protein	chr4	-	21.27	16.34	18.5	9.81	9.41	9.71
ENSG00000011	FER	protein	chr5	+	6.04	6.59	4.85	7.21	5.5	5.39
ENSG00000011	AKR1C2	protein	chr10	-	53.98	60.94	60.88	114.85	109.72	110.73
ENSG00000011	BMP6	protein	chr6	+	27.18	26.63	21.64	12.01	12.24	12.42
ENSG00000011	SLC25A27	protein	chr6	+	3.87	3.16	4.46	4.44	5.42	4.78
ENSG00000011	PID1	protein	chr2	-	33.54	30.52	33.24	23.78	22.15	23.45
ENSG00000011	PRKCA	protein	chr17	+	4.67	4.94	5.61	16.41	15.86	16.23
ENSG00000011	TIAM1	protein	chr21	-	3.39	3.34	3.03	3.86	3.7	3.25
ENSG00000011	NRG1	protein	chr8	+	1.8	2.2	2.23	2.11	2.61	2.29
ENSG00000011	LDLRAP1	protein	chr1	+	2.74	2.46	2.1	10.06	8.52	10.63
ENSG00000011	IL6R	protein	chr1	+	2.3	1.62	2.11	2.3	1.67	2.22
ENSG00000011	FGFR4	protein	chr5	+	2.31	2.81	2.08	0.34	0.74	0.43
ENSG00000011	LRP5	protein	chr11	+	5.09	5.6	6.44	6.22	5.59	6.06
ENSG00000011	ATF3	protein	chr1	+	1.75	2.38	1.54	1.06	2.16	1.68
ENSG00000011	IL24	protein	chr1	+	0.76	0.57	0.93	0.21	0.28	1.2
ENSG00000011	DISC1	protein	chr1	+	1.5	1.39	1.43	4	3.88	3.76
ENSG00000011	CXCL5	protein	chr4	-	3.72	2.57	2.09	0.45	0.22	0.15
ENSG00000011	ITGA2	protein	chr5	+	9.97	9.58	8.21	6.6	6.8	6.43
ENSG00000011	ESM1	protein	chr5	-	5.5	4.89	4.04	0.88	0.76	1.02
ENSG00000011	CSF2	protein	chr5	+	0.33	0	0.61	0.54	0.32	0.19
ENSG00000011	OSR2	protein	chr8	+	2.92	3.02	2.22	17.76	15.53	17.09
ENSG00000011	CTHRC1	protein	chr8	+	14.43	15.15	17.37	39.1	35.78	36.46
ENSG00000011	GREM1	protein	chr15	+	227.3	226.56	221.86	327.29	327.77	327.23
ENSG00000011	NKX3-1	protein	chr8	-	1.64	1.4	1.62	1.53	1.08	1.68
ENSG00000011	STAT3	protein	chr17	-	68.47	67.93	65.16	81.36	76.55	81.56
ENSG00000011	LGALS9	protein	chr17	+	1.41	1.02	1.02	2.49	0.8	1.54
ENSG00000011	IRS1	protein	chr2	-	4.76	4.46	4.43	4.86	4.54	4.42
ENSG00000011	ZFPM2	protein	chr8	+	7.72	7.42	7.52	15.18	15.59	16.47
ENSG00000011	CDK1	protein	chr10	+	1.52	0.3	0.69	0.43	0.23	0.35
ENSG00000011	HAS2	protein	chr8	-	0.69	0.64	0.62	0.48	0.41	0.5
ENSG00000011	PDGFD	protein	chr11	-	6.04	6.58	6.09	22.98	22.1	25.35
ENSG00000011	S1PR1	protein	chr1	+	6.38	5.28	6.36	12.32	9.81	12.13
ENSG00000011	P2RY6	protein	chr11	+	1.75	2.95	0.75	0.3	0.11	0.12
ENSG00000011	SCG2	protein	chr2	-	3.65	4.4	4	3.5	3.52	3.89
ENSG00000011	CCL11	protein	chr17	+	91.95	95.64	85.99	18.02	22.72	20.93
ENSG00000011	ARNT2	protein	chr15	+	6.86	8.91	7.4	11.71	9.57	10.99
ENSG00000011	CD248	protein	chr11	-	148.41	155.62	178.83	190.17	188.61	177.92
ENSG00000011	FOSL1	protein	chr11	-	11.34	9.14	10.64	14.32	15.84	13.68
ENSG00000011	SPHK1	protein	chr17	+	16.09	15.83	15.41	25.19	24.78	26.55
ENSG00000011	EGR3	protein	chr8	-	2.32	2.8	2.35	8.22	7	5.95
ENSG00000011	F2R	protein	chr5	+	47.44	47	42.54	45.8	49.25	48.36
ENSG00000011	GAS6	protein	chr13	-	95.79	92.81	87.67	74.66	74.8	77.67
ENSG00000011	TMEM119	protein	chr12	-	34.01	35.74	40.24	45.58	45.03	46.61
ENSG00000011	ZNF703	protein	chr8	+	11.35	10.38	10.93	11.94	12.41	11.76
ENSG00000011	IRAK1	protein	chrX	-	86.55	77.17	74.97	69.78	67.23	71.86
ENSG00000011	PBX1	protein	chr1	+	27.72	28.26	26.46	49.53	46.06	45.36
ENSG00000011	SHC4	protein	chr15	-	2.48	2.52	1.87	1.25	1.69	1.59
ENSG00000011	CCK	protein	chr3	-	0.31	0	0.32	0	0	0
ENSG00000011	ZP3	protein	chr7	+	1.56	1.26	2.04	1.11	1.68	0.84
ENSG00000011	AKR1C3	protein	chr10	+	92.72	103.32	95.97	132.63	123.28	131.58
ENSG00000011	FGF16	protein	chrX	+	0.76	1.46	1.67	1.2	1.33	0.84
ENSG00000011	ADA	protein	chr20	-	9.27	8.88	8.04	11.97	11.4	13.47
ENSG00000011	SERTAD1	protein	chr19	-	10.95	10	9.82	7.62	7.95	8.72
ENSG00000011	PELI1	protein	chr2	-	3.57	5.27	4.48	2.84	3.37	3.84

Table A3. Genes regulated by *CARMN* and involved in migration.

Table indicating the list of genes involved in migration (GO:0030335) identified by Gene Ontology analysis in vSMC treated with GapmeR targeting *CARMN* (Gap*CARMN*) and control (GapCTR) in the basal condition during treatment (P) with cholesterol (Basal_M).

ENSEMBL name	type	chr	str	Gap <i>CARMN</i> _Basal.M1	Gap <i>CARMN</i> _Basal.M2	Gap <i>CARMN</i> _Basal.M3	GapCTR_Basal.M1	GapCTR_Basal.M2	GapCTR_Basal.M3
ENSG00000181363	protein_coding	chr17	+	22.6	22.22	19.86	16.35	14.17	13.04
ENSG00000181363	protein_coding	chr7	-	31.05	37.12	27.59	11.72	14.32	10.98
ENSG00000181363	protein_coding	chr3	+	40.96	39.01	36.51	11.07	13.68	11.37
ENSG00000181363	protein_coding	chr12	-	1.37	0.99	0.69	2.01	2.04	1.27
ENSG00000181363	protein_coding	chr4	+	10.25	10.81	10.42	15.84	16.66	13.05
ENSG00000181363	protein_coding	chr8	-	31.06	30.72	29.72	34.05	38.83	35.67
ENSG00000181363	protein_coding	chr5	-	5.29	7.01	6.75	2.06	2.24	1.79
ENSG00000181363	protein_coding	chr7	-	144.62	143.83	141.57	39.9	33.31	37.33
ENSG00000181363	protein_coding	chr8	+	17.59	17.62	18.83	60.36	58.09	57.37
ENSG00000181363	protein_coding	chr1	-	1.18	1.35	1.45	1	1.72	1.03
ENSG00000181363	protein_coding	chr14	+	3.07	2.64	2.44	2.91	3.31	3.4
ENSG00000181363	protein_coding	chr3	-	5.14	5.43	4.87	6.44	7.9	7.67
ENSG00000181363	protein_coding	chr12	-	14.1	13.55	11.24	13.05	12.18	11.88
ENSG00000181363	protein_coding	chr1	-	26.03	27.68	28.07	12.24	9.07	7.78
ENSG00000181363	protein_coding	chr7	-	6.25	6.64	6.77	1.87	1.83	2.16
ENSG00000181363	protein_coding	chr7	-	76.4	80.14	77.87	107.41	104.48	102.09
ENSG00000181363	protein_coding	chr8	-	38.89	38.77	37.81	79.75	81.35	80.96
ENSG00000181363	protein_coding	chr6	+	14.36	13.32	13.95	10.69	7.97	10.98
ENSG00000181363	protein_coding	chr11	-	1.67	3.8	2.55	1.06	1.48	1.79
ENSG00000181363	protein_coding	chr19	+	80.91	81.04	78.58	50.69	48.36	48.79
ENSG00000181363	protein_coding	chr2	+	37.18	43.17	40.47	5.47	4.68	5.16
ENSG00000181363	protein_coding	chr1	+	101.8	106.45	101.84	42.83	44.63	47.59
ENSG00000181363	protein_coding	chr10	+	5.62	3.67	2.54	2.15	2	1.39
ENSG00000181363	protein_coding	chr14	+	367.63	391.27	360.82	329.21	300.08	311.66
ENSG00000181363	protein_coding	chr14	+	56.56	56.44	67.15	31.63	29.86	28.44
ENSG00000181363	protein_coding	chrX	+	33.4	28.74	30.95	25.2	20.15	19.46
ENSG00000181363	protein_coding	chr13	-	2.37	3.18	3.96	1.37	1.4	1.21
ENSG00000181363	protein_coding	chr15	+	317.19	310.04	344.32	988.29	871.71	940.31
ENSG00000181363	protein_coding	chr7	+	103.36	117.07	103.14	133.63	149.12	142.32
ENSG00000181363	protein_coding	chr7	+	17.72	15.74	17.37	6.28	5.11	6.23
ENSG00000181363	protein_coding	chr7	+	394.64	381.14	414.06	258.59	199.65	212.04
ENSG00000181363	protein_coding	chr7	-	237.18	229.71	235.85	477.82	444.02	466.18
ENSG00000181363	protein_coding	chr9	+	16.95	12.32	13.29	10.77	6.93	9.11
ENSG00000181363	protein_coding	chr10	-	38.7	37.07	42.71	112.16	121.34	119.53
ENSG00000181363	protein_coding	chr10	-	39.15	38.65	31.06	38.11	40.24	44.69
ENSG00000181363	protein_coding	chr17	+	60.54	60.31	66.84	17.77	13.98	11.79
ENSG00000181363	protein_coding	chr17	+	1.68	1.74	2.83	0.7	0.48	0.48
ENSG00000181363	protein_coding	chr17	-	54.58	46.33	46.49	14.51	9.93	10.92
ENSG00000181363	protein_coding	chr4	+	4.38	5.03	5.02	7.27	4.56	3.95
ENSG00000181363	protein_coding	chr12	+	0.27	0.64	0.25	0.1	0.09	0
ENSG00000181363	protein_coding	chr6	-	5.46	5.2	5.3	1.22	1.06	0.89
ENSG00000181363	protein_coding	chr6	-	1691.12	1664.33	1756.04	1854.82	1790.59	1708.47
ENSG00000181363	protein_coding	chr5	-	7.35	7.79	6.39	0.95	1.22	0.69
ENSG00000181363	protein_coding	chr5	-	0.41	0.37	0.45	0.39	0.28	1.29
ENSG00000181363	protein_coding	chr5	-	23.5	23.55	22.05	38.33	42.79	40.94
ENSG00000181363	protein_coding	chr3	-	20.4	18.32	17.51	2.53	2.11	1.98
ENSG00000181363	protein_coding	chr2	+	3.71	3.46	5.08	0.7	0.44	0.89
ENSG00000181363	protein_coding	chr2	+	2.1	2	3.47	2.14	1.95	1.81
ENSG00000181363	protein_coding	chr2	-	3.84	3.69	3.41	0.87	0.89	2.27
ENSG00000181363	protein_coding	chr1	-	3.27	3.59	4.38	1.16	1.26	1.58
ENSG00000181363	protein_coding	chr9	+	0.68	0.5	0.54	0.21	0.19	0.25
ENSG00000181363	protein_coding	chr14	-	4.69	5.09	4.7	1.18	1.43	1.12
ENSG00000181363	protein_coding	chr9	+	19.75	18.26	18.07	17.29	18.33	17.69
ENSG00000181363	protein_coding	chr8	+	9.73	10.05	9.84	22.84	25.3	20.77

ENSEMBL1	name	type	chr	str	GapCARMN_E	GapCARMN_F	GapCARMN_L	GapCTR_Basal	GapCTR_Basal	GapCTR_Basal
ENSG0000001	GLIPR2	protein	chr9	+	18.22	20.12	18.29	21.94	28.27	21.87
ENSG0000001	PLAU	protein	chr10	+	8.79	10.82	14.24	5.64	6.29	6.04
ENSG0000001	TNFAIP6	protein	chr2	+	154.34	148.49	165.01	52.14	46.61	51.37
ENSG0000001	PREX1	protein	chr20	-	3.18	3.31	2.74	2.43	3.6	2.38
ENSG0000001	SNAI1	protein	chr20	+	5.17	4.8	5.28	4.27	3.83	5.39
ENSG0000001	XG	protein	chrX	+	4.87	4.77	2.69	4.79	5.12	7.63
ENSG0000001	BMP4	protein	chr14	-	1.12	0.53	0.7	1.51	1.62	1.09
ENSG0000001	SOX9	protein	chr17	+	19.95	19.83	20.65	8.52	8.17	7.23
ENSG0000001	IL1B	protein	chr2	-	283.56	312.57	297.28	54.27	47.39	45.2
ENSG0000001	TNFSF14	protein	chr19	-	5.35	5.2	5.88	3.64	3.45	2.74
ENSG0000001	BMP2	protein	chr20	+	6.93	7.39	8.84	6.69	4.83	6.06
ENSG0000001	F10	protein	chr13	+	7.4	5.97	5.37	1.22	1.12	1.42
ENSG0000001	STAT5A	protein	chr17	+	5.93	5.65	4.98	13.4	9.87	10.88
ENSG0000001	RHOJ	protein	chr14	+	58.61	61.61	47.45	18.42	18.5	18.22
ENSG0000001	KDR	protein	chr4	-	5.7	6.26	6.15	3.9	4.81	4.21
ENSG0000001	RAC2	protein	chr22	-	101.59	98	94.73	64.58	67.63	62.61
ENSG0000001	ACTN4	protein	chr19	+	135.18	134.82	123.11	312.7	316.85	310.96
ENSG0000001	DIAPH1	protein	chr5	-	23.71	24.71	23	11.49	11.35	13.12
ENSG0000001	LGALS3	protein	chr14	+	300.48	287.44	285	389.59	428.76	403.11
ENSG0000001	POSTN	protein	chr13	-	35.35	35.1	37.41	19.16	21.53	17.14
ENSG0000001	APC	protein	chr5	+	4.04	3.25	4	5.32	4.93	6.01
ENSG0000001	SEMA4F	protein	chr2	+	15.65	15.99	18.15	7.14	8.34	6.94
ENSG0000001	DYSF	protein	chr2	+	3.42	3.89	3.54	3.24	2.73	2.33
ENSG0000001	SPRY2	protein	chr13	-	22.39	22.83	24.62	6.78	5.96	6.09
ENSG0000001	GNPMB	protein	chr7	+	222.25	230.78	234.56	330.86	354.9	339.92
ENSG0000001	IL6	protein	chr7	+	207.26	224.1	214.13	64.12	41.97	48.64
ENSG0000001	SDCBP	protein	chr8	+	125.51	135.33	125.89	177.14	185.99	165.19
ENSG0000001	THBS1	protein	chr15	+	394.89	361.86	330.71	239.35	202.35	215.19
ENSG0000001	SEMA6D	protein	chr15	+	1.78	1.65	3.38	2.61	3.95	2.62
ENSG0000001	SEMA7A	protein	chr15	-	9.14	9.83	9.05	6.16	5.01	4.95
ENSG0000001	ANXA3	protein	chr4	+	43.21	29.66	35.58	22.82	21.58	20.57
ENSG0000001	NTRK3	protein	chr15	-	0.61	1.06	0.84	1.68	1.27	3.46
ENSG0000001	IQGAP1	protein	chr15	+	41.73	40.82	45.87	71.91	70.32	73.32
ENSG0000001	ITGAX	protein	chr16	+	2.2	3.86	2.69	0.31	0.37	0.35
ENSG0000001	CDH13	protein	chr16	+	31.23	25.13	21.53	55.64	51.86	63.59
ENSG0000001	BCAS3	protein	chr17	+	0.44	0.35	1.32	8.1	11.86	9.1
ENSG0000001	SEMA6C	protein	chr1	-	5.03	5.81	5.08	2.01	1.46	1.84
ENSG0000001	RHOB	protein	chr2	+	22.92	23.88	22.4	7.6	7.74	6.62
ENSG0000001	ACKR3	protein	chr2	+	0.54	0.44	0.14	0.11	0.15	0.72
ENSG0000001	PLK2	protein	chr5	-	151.87	140.41	145.39	228	232.39	248.24
ENSG0000001	PLA2G7	protein	chr6	-	2.09	3.28	2.42	0.51	0.29	0.5
ENSG0000001	EGFR	protein	chr7	+	20.72	20.51	18.77	33.65	32.27	36.16
ENSG0000001	VEGFC	protein	chr4	-	22.94	20.32	22.29	9.91	9.95	8.78
ENSG0000001	FER	protein	chr5	+	3.95	3.84	3.66	6.37	6.84	9.53
ENSG0000001	MGAT5	protein	chr2	+	6.34	5.46	5.24	3.75	2.7	3.69
ENSG0000001	DAB2	protein	chr5	-	80.96	77.68	82.23	170.01	166.76	176.93
ENSG0000001	ANGPT1	protein	chr8	-	3.22	4.34	3.46	3.6	2.76	2.44
ENSG0000001	PRKCA	protein	chr17	+	2.2	1.82	1.86	17.08	18.39	18.81
ENSG0000001	TIAM1	protein	chr21	-	3.85	5.88	4.69	1.56	1.25	1.8
ENSG0000001	SH3RF2	protein	chr5	+	12.92	9.58	9.79	10.52	11.47	8.92
ENSG0000001	DMTN	protein	chr8	+	5.5	7.64	7.67	1.87	1.61	2.56
ENSG0000001	CARMIL2	protein	chr16	+	3.47	5.06	3.72	0.6	0.67	0.26
ENSG0000001	IL6R	protein	chr1	+	1.8	1.22	1.09	2.71	2.24	3.5
ENSG0000001	CXCL16	protein	chr17	-	10.87	8.78	7.32	12.18	12.71	13.16
ENSG0000001	ZC3H12A	protein	chr1	+	41.89	46.93	43.67	28.52	27.04	25.08

ENSEMBL	name	type	chr	str	GapCARMN_Basal.M1	GapCARMN_Basal.M2	GapCARMN_Basal.M3	GapCTR_Basal.M1	GapCTR_Basal.M2	GapCTR_Basal.M3
ENSG000	ITGA2	protein_coding	chr5	+	30.56	33.38	36.46	8.19	7.38	6.64
ENSG000	F2RL1	protein_coding	chr5	+	9.28	8.5	10.58	1.13	2.53	0.99
ENSG000	NOS3	protein_coding	chr7	+	2.66	3.13	1.36	0.67	0.56	0.07
ENSG000	AMOT	protein_coding	chr11	+	8.02	6.8	7.44	10.28	11.65	10.37
ENSG000	FAM1	protein_coding	chr3	-	3.73	3.17	2.41	0.94	0.59	0.68
ENSG000	STAT3	protein_coding	chr17	-	51.03	45.02	48.46	82.62	81.45	83.51
ENSG000	ATOX1	protein_coding	chr2	+	69.88	67.76	65.97	56.21	49.49	56.59
ENSG000	LGALS	protein_coding	chr17	+	8.44	8.54	8.43	4.5	3.87	4.15
ENSG000	CXCL8	protein_coding	chr4	+	511.13	537.2	593.29	94.67	74.13	66.3
ENSG000	HAS2	protein_coding	chr8	-	4.4	3.62	4.53	0.75	0.84	1.2
ENSG000	PDGFR	protein_coding	chr11	-	2.13	1.88	2.25	5.89	6.52	6.58
ENSG000	S1PR1	protein_coding	chr1	+	27.25	31.18	26.42	14.14	16.32	12.33
ENSG000	PRKCE	protein_coding	chr2	+	3.11	2.37	2.75	3.78	3.77	4.15
ENSG000	P2RY6	protein_coding	chr11	+	0.99	0.79	0.94	0.89	0.96	0.74
ENSG000	LRRC1	protein_coding	chr3	-	0.62	0.53	0.59	0.73	0.61	0.65
ENSG000	CCL11	protein_coding	chr17	+	23.55	19.84	26.16	29.63	23.93	20.84
ENSG000	RHOD	protein_coding	chr11	+	16	17.79	15.13	12.62	16.06	12.42
ENSG000	SPHK1	protein_coding	chr17	+	36.74	32.66	35.09	28.91	25.31	24.07
ENSG000	FOXC2	protein_coding	chr16	+	2.26	2.12	1.88	1.46	1.67	1.46
ENSG000	ZNF6	protein_coding	chr15	+	5.05	4.68	5.25	15.43	16.26	15.44
ENSG000	FAM8	protein_coding	chr8	-	7.38	7.64	7.62	4.11	4.15	3.62
ENSG000	F2R	protein_coding	chr5	+	51.14	49.9	54.79	35.98	38.71	37.63
ENSG000	GAS6	protein_coding	chr13	-	25.32	26.55	27.24	52.41	50.28	54.38
ENSG000	CCBE1	protein_coding	chr18	-	12.6	13.15	15.06	36.56	34.91	35.97
ENSG000	ZNF7	protein_coding	chr8	+	9.26	9.29	9.86	11.71	13.02	12.03
ENSG000	ZP3	protein_coding	chr7	+	1.42	2.32	1.96	1.49	0.59	0.75
ENSG000	S100A	protein_coding	chr1	-	2.29	2.41	2.07	0.1	0.35	0.31
ENSG000	FGF1	protein_coding	chrX	+	3.12	3.25	2.68	1.09	1.27	1.34
ENSG000	LPAR1	protein_coding	chr9	-	44.34	43.92	48.1	70.74	70.44	69.46
ENSG000	DDRG	protein_coding	chr20	-	66.57	72.13	67.81	31.47	31.3	27.65
ENSG000	LYN	protein_coding	chr8	+	1.95	2.64	3.29	5.22	4.91	5.8
ENSG000	ITGB3	protein_coding	chr17	+	6.59	7.82	7.57	3.06	2.8	3.15
ENSG000	CCL5	protein_coding	chr17	-	3.8	3.74	3.91	1.99	1.9	1.93

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