ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

THE INVESTIGATION OF ANGIOGENIC MECHANISMS ASSOCIATED WITH miRNA-126 SIGNALLING PATHWAY MOLECULES IN ATHEROSCLEROSIS

Ph.D. THESIS

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AĞUSTOS 2016

Çiğdem SEZER ZHMUROV, a Ph.D. student of ITU Graduate School of ScienceEngineering and Technology 521092062 successfully defended the thesis entitled "THE INVESTIGATION OF ANGIOGENIC MECHANISMS ASSOCIATED WITH miRNA-126 SIGNALLING PATHWAY MOLECULES IN ATHEROSCLEROSIS ", which he/she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my beloved parents and my dear husband,

FOREWORD

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Çiğdem SEZER ZHMUROV
(Biologist)

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ABBREVIATIONS

Abca1 : ATP binding cassette transporter 1
AHA : American Heart Association
AKT : Protein kinase B
Apob : Apolipoprotein B
Apoe : Apolipoprotein E
App : Appendix
CD : Cluster of differentiation
CDC : Centers for disease control and prevention
CVD : Cardiovascular disease
cDNA : Complementary DNA
C_T : Threshold cycle
DLK1 : Delta-like ligand 1
DLL1 : Delta-like canonical Notch ligand 1
DLL4 : Delta-like canonical Notch ligand 4
DNA : Deoxyribonucleid acid
dNTP : Deoxyribonucleotide triphosphate
dTTP : Deoxythymidine triphosphate
EC : Endothelial cell
Egfl7 : Epidermal growth factor like domain 7
ECM : Extracellular matrix
EMP : Endothelial microparticles
ERK : Extracellular signal regulated kinase
ETS : E26 transformation specific sequence
EVH1 : Ena/VASP homology 1
FAM^{TM} : 6-carboxy fluorescein
FAK : Focal adhesion kinase
FH : Familial hypercholesterolemia
GWAS : Genome wide association study
HDL : High density lipoprotein
HUVEC : Human umbilical vein endothelial cell
ICAM1 : Intercellular adhesion molecule-1
KLF2 : Krüppel-like factor 2
KO : Knock out
LDL : Low density lipoprotein
Ldlr : Low density lipoprotein receptor
LOX-1 : Lectin-like oxidized low density lipoprotein receptor-1
MAPK : Mitogen activated protein kinase
MCP-1 : Monocyte chemotactic protein-1
mRNA : Messenger RNA
MiRNA : MicroRNA

NCBI : National center for biotechnology information
NIH : National institutes of health
NRP : Neuropilin
NTC : No template control
PBMC : Peripheral blood mononuclear cell
PDGF : Platelet derived growth factor
PDK1 : Phosphoinositide-dependent kinase 1
PI3K : Phosphoinositide 3-kinase
Pik3r2 : Phosphoinositide 3-kinase regulatory subunit 2
PIGF : Placental growth factor
PCR : Polymerase chain reaction
PLC-γ : Phospholipase C–γ
PTB : Phosphotyrosine-binding
qRT-PCR : Quantitative real time polymerase chain reaction
RAS : Rat sarcoma
RAF : Rapidly accelerated fibrosarcoma
RNA : Ribonucleic acid
RTK : Receptor tyrosine kinase
RISC : RNA-induced silencing complex
RT : Reverse transcription
SH2 : Src homolgy region
SNP : Single nucleotide polymorphism
Spred1 : Sprouty related EVH1 domain containing 1
SRA : Scavenger receptor-A
SRB : Scavenger receptor-B
UTR : Untranslated tegion
VCAM1 : Vascular cell adhesion molecule 1
VEGF : Vascular endothelial growth factor
VEGFR : Vascular endothelial growth factor receptor
VLDL : Very low density lipoprotein
WHO : World Health Organization

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THE INVESTIGATION OF ANGIOGENIC MECHANISMS ASSOCIATED WITH miRNA-126 SIGNALLING PATHWAY MOLECULES IN ATHEROSCLEROSIS

SUMMARY

Cardiovascular diseases are mainly the diseases of the blood vessels, the heart, and the vascular diseases of the brain. The main types of cardiovascular diseases that occur due to atherosclerosis include, ischaemic heart disease and coronary artery disease, cerebrovascular diseases, peripheral artery disease and hypertensive heart disease. According to the 2014 Global Status Report on Noncommunicable Diseases published by World Health Organization, 38 million of the 56 million global deaths that took place in 2012 occurred due to noncommunicable diseases, such as cardivascular diseases, cancer, chronic respiratory diseases and diabetes. In 2012, cardiovascular diseases, with a 31% of all global deaths, were the number one cause of death in the world, based on World Health Organization data. 7.4 million of these cardiovascular disease-related deaths took place because of coronary heart disease, while 6.7 million were due to stroke. In Turkey, based on Turkish Statistical Institute Cause of Death Statistics 2014 data, the leading cause of death was cardiovascular diseases, represented with 40.4% of all national deaths that took place in 2014. Cardiovascular diseases remain to be the leading cause of death in the world today. The underlying pathological condition of cardiovascular diseases is atherosclerosis.

Although atherosclerosis is an irreversible process beginning with birth, the diseases it causes can be prevented if major risk factors are taken into consideration. While some of the major cardiovascular disease risk factors cannot be controlled, most of them are treatable, controllable, or modifiable. The major cardiovascular disease risk factors which cannot be changed are; increasing age, male gender and heredity, while the treatable and modifiable ones include smoking, high blood cholesterol levels, high blood pressure, physical inactivity, obesity and diabetes .

In this thesis study, samples from patients with carotid artery disease were studied. Carotid endarterectomy is an operation, in which atherosclerotic plaques are surgically removed from the carotid arteries of symptomatic patients with high grade stenosis ($\geq$ 60%). Carotid artery atherosclerotic plaque samples obtained from 14 patients undergoing carotid endarterectomy for stenosis greater than 70% or stenosis ranging from 50% to 70% associated to clinical symptoms were used in this thesis study.

Atherosclerosis is the chronic, progressive and immunoinflammatory disease of the medium and large-size arteries. It is a complex disease which involves interactions of multiple genetic and environmental factors. Starting with birth, atherosclerotic process progresses slowly, causing diseases, such as coronary artery disease, stroke and peripheral artery disease. In the inflammatory process of atherosclerosis, the inner layer of arteries, the intima layer, gets thicker with lipid rich material (atheroma) and connective tissue (sclerosis). According to the "Response to Injury" hypothesis

of atherosclerosis, potential sources of injury, such as hyperlipidemia, hypertension, metabolites, infections and mechanical factors such as shear stress are suggested to cause the initiation of atherosclerosis. The pathological changes in atherosclerosis occur due to molecular events of endothelial dysfunction, monocyte adherence and entry into the vessel wall, monocyte development into foam cells, smooth muscle cell migration and proliferation, advanced plaque formation with necrotic core and neoangiogenesis, and thrombosis as a result of plaque rupture. Following rupture of a vulnerable plaque, thrombus formation resulting in stroke or a myocardial infarction causes deaths or disabilities. Atherosclerosis would possibly be a more benign disease if thrombus formation could be prevented. The investigation of molecular mechanisms resulting in angiogenesis in advanced plaques, a hallmark event of atherosclerosis, is the main focus of this thesis study.

Angiogenesis is the process of new vessel formation from existing ones. The main signalling pathways that control angiogenesis in cells are the vascular endothelial growth factor (VEGF) pathway and Notch signalling. Intraplaque neoangiogenesis has been linked with progressive atherosclerotic disease. The signalling pathways contributing to angiogenesis represent potential targets for therapies aiming to prevent intraplaque neoangiogenesis. This thesis study focuses on the role of angiogenic mechanisms related to vascular endothelial growth factor pathway and the mechanisms that interact with this pathway, such as microRNAs, the small, non-coding RNAs that post-transcriptionally regulate many cellular processes, including angiogenesis. *Egfl7*, a major player of angiogenesis that is expressed exclusively in embryonic and adult vasculature, encodes two endothelial-specific miRNAs: miR 126-3p and miR 126-5p. This thesis study was conducted with the aim of understanding the role of miR-126 signalling in angiogenesis and its contribution to disease progression. For this purpose, the expression levels of miR 126-3p, the targets of miR 126-3p, miR 126-5p and *Egfl7* were investigated in 14 human carotid artery atherosclerotic plaques and in control samples.

The mRNA targets of miR 126-3p were investigated using the miRNA target prediction databases, TargetScan and miRTarBase. Out of the vast number of miR 126-3p targets, *Egfl7*, *Spred1* and *Pik3r2* genes that were known to be involved in angiogenesis as well as vascular endothelial growth factor signalling pathway were chosen to be studied as targets of the miR 126-3p. Following the preliminary bioinformatic research, total RNA and miRNA isolations were performed following tissue homogenization procedures of 14 carotid artery atherosclerotic plaque and control samples. Complementary DNA synthesis reactions were carried out and then real time quantitative PCR reactions were performed to evaluate the relative expression levels of *Egfl7*, *Spred1*, *Pik3r2*, miR 126-3p and miR 126-5p. It was expected to observe *Egfl7* and miR-126 up-regulation in carotid artery atherosclerotic plaques, due to the vascular injury caused by progressive atherosclerosis, while the expression levels of negative regulators of VEGF pathway, *Spred1* and *Pik3r2*, were expected to be down-regulated, since they are targeted by miR 126-3p.

For real time quantitative PCR analyses, relative quantification strategy employing $2^{\Delta\Delta C_T}$ model was used. All of the target gene and miRNA expression levels of plaque samples were compared to those of control samples and normalized to the levels of the 18S ribosomal RNA and U6 small nuclear RNA endogenous controls. Following data analyses, statistical analyses were performed. The significance of differences

between the results from plaque and control tissues was examined with Student's t test. Demographic information was compared with gene expression data expressed as fold change by using Chi-square and Fisher's Exact tests.

In accordance with the hypothesis, miR 126-3p expression was higher in 11 out of 14 atherosclerotic plaque samples compared to plaque adjacent controls. *Spred* and *Pik3r2* were down-regulated in 8 and 9 samples compared to controls, respectively. Considering the overall gene expression levels, miR 126-3p was up-regulated by 2.14 fold in plaques in comparison to controls (p <0.05). MiR 126-5p was found to be up-regulated in 8 patient plaque samples compared to controls, while down-regulation was observed in 6 of the patient samples. Considering the overall expression, miR 126-5p expression was higher in plaques compared to control samples. *Egfl7* up-regulation was observed in 11 plaque samples out of 14. The overall Egfl7 mRNA expression was higher in plaques (p <0.001) compared to controls.

This thesis study provides the first evidence on miR 126-3p expression in deep intraplaque regions. To the knowledge, the expression levels of miR 126-3p, its targets *Spred1*, *Pik3r2*, miR 126-5p and *Egfl7* in atherosclerotic plaques from human carotid arteries were evaluated for the first time. This work, supported by data on expression levels of miR 126-5p, miR 126-3p and its targets *Spred1*, *Pik3r2* and *Egfl7*, demonstrates the contribution of miR-126 signalling molecules and neoangiogenesis driven by these molecules to atherosclerotic plaque progression in advanced atherosclerosis, despite their known atheroprotective roles in endothelial progenitor cells. The results of this thesis study constitutes preliminary bases for future miRNA biomarker studies to be used in monitoring plaque progression and intraplaque neoangiogenesis, as well as miRNA based therapies for preventing intraplaque neoangiogenesis, therefore thrombosis. In addition, Egfl7 can be considered as a promising prognostic marker of intraplaque neoangiogenesis, to aid in monitoring the unstable plaques, as well as preventing vulnerable plaque rupture. *Egfl7* based anti-angiogenic therapy could be offered as a strategy in eliminating intraplaque neoangiogenesis and stabilization of plaques that are prone to rupture, therefore preventing the devastating consequences of advanced atherosclerosis. The molecular mechanisms of miR 126-modulated dysregulation need further investigation, particularly on the phosphorylation status of the downstream signalling molecules, to be able to fully grasp the affect of miR-126 on vascular endothelial growth factor regulated angiogenesis in atherosclerosis. The interaction of miR-126 with other miRNAs and signalling molecules needs to be elucidated. To be able to fully understand the roles of angiogenesis in different stages of atherosclerosis, the study of patient samples considering the different degrees of stenosis is suggested. Furthermore, larger scale studies must be performed on more than one arterial system, with the aim of determining the specific regulation of angiogenesis by miRNAs in different arterial systems. The potential use of miRNAs as biomarkers to monitor plaque neoangiogenesis and disease progression, as well as the development of miRNA-based therapies are promising areas of research in the battle against the fatal consequences of atherosclerosis.

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ÖZET

Kardiyovasküler hastalıklar, kalp ve kalp damarlarının, beyin damarlarının ve vücuttaki tüm diğer damarların hastalıkları olarak tanımlanırlar. Ateroskleroza bağlı olarak ortaya çıkan kardiyovasküler hastalıklar arasında, koroner arter hastalıkları, serebro-vasküler hastalıklar, periferal arter hastalığı ve hipertansif kalp hastalığı bulunmaktadır. Dünya Sağlık Örgütü tarafından 2014 yılında yayınlanan Kalp ve Damar Hastalıklarını Önleme ve Kontrolü Küresel Atlası verilerine göre, dünyada 2012 yılında gerçekleşmiş olan 56 milyon ölümün 38 milyonu kardiyovasküler hastalıklar, kanser, kronik solunum yolu hastalıkları ve diyabet gibi bulaşıcı olmayan hastalıklar nedeniyle meydana gelmiştir. 2012 yılında kardiyovasküler hastalıklar nedenli ölümler, tüm dünyada birincil ölüm nedeni olup, tüm ölümlerin % 31'ini oluşturmuştur. Tüm kardiyovasküler hastalıklar ile ilişkili ölümlerin 7.4 milyonu koroner arter hastalığına bağlı olarak, 6.7 milyonu ise inme nedenli gerçekleşmiştir. Türkiye İstatistik Kurumu tarafından yayınlanan Ölüm Nedeni İstatistikleri 2014 verilerine göre, kardiyovasküler hastalık kaynaklı ölümler, 2014 yılında gerçekleşmiş olan tüm ölümlerin %40.4'ünü oluşturarak, birincil ölüm sebebini teşkil etmiştir. Günümüzde hala tüm dünyadaki bir numaralı ölüm nedeni olan kalp ve damar hastalıklarının altında yatan temel mekanizma aterosklerozdur.

Ateroskleroz doğumla başlayan geri dönüşsüz bir süreç olsa da, ana risk faktörleri göz önüne alındığında, aterosklerozun yol açtığı kardiyovasküler hastalıklar büyük ölçüde önlenebilmektedir. Kalp ve damar hastalığı risk faktörlerinin bazıları değiştirilememektedir, ancak büyük çoğunluğu kontrol edilebilmekte, tedavi edilebilmekte veya modifiye edilebilmektedir. Değiştirilemeyen veya kontrol edilemeyen risk faktörlerinin başında ileri yaş, erkek cinsiyet ve genetik etmenler gelmektedir. Öte yandan, yüksek kolesterol seviyeleri, hipertansiyon, obezite, diyabet, sigara kullanımı ve fiziksel inaktivite gibi risk faktörleri tedavi edilebilir ve modifiye edilebilir risk faktörleri olarak değerlendirilmektedir.

Bu tez çalışmasında, karotis arter hastalarından elde edilmiş olan aterosklerotik plak örnekleri kullanılmıştır. Ateroskleroza bağlı ortaya çıkan arter hastalığı, beyine kan götüren damarlar olan karotis arterlerdeki kan akışının tıkanıklığa bağlı azalması sonucu meydana gelir. Beyindeki belirli bir bölgeye, karotis arterlerdeki tıkanıklığa bağlı olarak kan akışı azaldığında, o bölgede fonksiyon kaybı ve buna bağlı olarak geçici iskemik atak olarak isimlendirilen semptomlar ortaya çıkar. Görme kaybı (Amaurosis fugax), konuşmada bozukluklar, ekstremitelerde güçsüzlük hissi ve yüzde duyu kaybı bu semptomlardan bazılarını oluşturmaktadır. Beyindeki bir bölgeye kan akışı aniden kesildiğinde ise inme olarak adlandırılan durum meydana gelir. Karotis arter hastalığının teşhisi için, ultrason ve manyetik rezonans anjiyogram gibi görüntüleme tekniklerinden yararlanılmaktadır. Bu testler sayesinde, karotis

arterlerde meydana gelen daralmanın, bir diğer deyişle, stenozun varlığı ve derecesi belirlenebilir. Asemptomatik ve semptomatik karotis arter hastalığının ayrımı önem taşımaktadır. Asemptomatik hastalıkta, geçici iskemik ataklar veya inme olmaksızın, proksimal internal karotis arterde % 50–60 stenoz gözlemlenir. Asemptomatik hastalığın ilerleyişini önlemek amacıyla, hipertansiyon, Diyabet ve yüksek kolesterol gibi risk faktörlerinin tedavisi gibi invazif olmayan terapiler uygulanmaktadır. İleri derecede stenozun ($\geq$ %60) tedavisi için ise, karotis endarterektomisi ve karotis arterlere stent yerleştirilmesi gibi invazif terapiler tercih edilmektedir. Karotis endarterektomi operasyonu semptomatik hastalarda gelecek inme riskinin azaltılması amacıyla gerçekleştirilir. Bu cerrahi operasyon ile karotis arterlerde daralmaya neden olan aterosklerotik plaklar çıkartılır. Bu çalışmada, karotis arterlerinde %70'den daha yüksek derecede veya %50 ile %70 arasında değişen darlık bulunan 14 adet semptomatik hastadan endarterektomi esnasında çıkartılan aterosklerotik plaklar ve plak etrafındaki bölgeden elde edilen kontrol dokuları kullanılmıştır.

Ateroskleroz, orta ve büyük çaplı arterlerin kronik, dejeneratif, immun-enflamatuvar hastalığıdır. Pek çok genetik etmen ve çevresel etmenin etkileşimini içeren kompleks bir hastalık olarak tanımlanır. Doğum ile başlayan aterosklerotik süreç, yavaş ilerleyerek, özellikle 40 ve 50 yaş sonrasında koroner arter hastalığı, inme ve periferal arter hastalığı gibi rahatsızlıklara neden olur. Ateroskleroz enflamasyonun çok etkin olduğu bir süreç olup, arterlerin en iç tabakası olan intima tabakasının kalınlaşması sonucu oluşan lipid bakımından zengin materyal (aterom) ve konnektif doku (skleroz) ile karakterizedir. Aterosklerotik süreci açıklamaya yönelik geliştirilmiş olan hipotezler arasında bulunan ve yaygın kabul gören "Hasara tepki" hipotezine göre, hiperlipidemi, hipertansiyon, çeşitli metabolitler, enfeksiyonlar ve mekanik faktörler gibi potansiyel kaynaklar endotel hasarına sebep olarak aterosklerotik süreci başlatırlar. Ateroskleroz patogenezinde yer alan temel basamaklar, endotel disfonksiyonu, LDL kolesterolün oksidasyonu, monositlerin damar duvarına yapışması ve damar duvarından içeriye girişleri, monositlerin makrofajlara farklılaşması ve makrofajların okside-LDL partiküllerini fagosite etmeleri sonucu köpük hücre oluşumu, düz kas hücrelerinin intima tabakasına göç etmeleri ve proliferasyonları sonucu fibröz başlık oluşumu, ilerlemiş lezyonlardaki makrofajların apoptozuna bağlı olarak plak nekrotik çekirdeğinin oluşumu, plak içerisinde neoanjiyogenez ve plağın yırtılması ile trombüs oluşumu olarak sıralanabilir. Kararsız plakların yırtılması sonucunda oluşan trombüs, inme ve miyokard enfarktüsü gibi durumlara ve bu durumlara bağlı olan sakatlıklara ve ölümlere yol açmaktadır. Trombüs oluşumu engellenebilir olduğu taktirde aterosklerozun çok daha iyi huylu seyreden bir hastalık olacağı pek çok çalışmada önerilmektedir. İlerlemiş aterosklerotik plakların içerisindeki yeni damar oluşumuna yol açan moleküler mekanizmaların araştırılması, bu tez çalışmasının odak noktasını oluşturmaktadır.

Anjiyogenez, var olan damarlardan yeni damar oluşumudur. Hücrelerde anjiyogenezi kontrol eden temel sinyal yolakları vasküler endoteliyal büyüme faktörü yolağı ve Notch sinyal yolağıdır. Aterosklerotik plak içi anjiyogenezin aterosklerozun ilerleyişi ile olan ilişkisi pek çok çalışma ile gösterilmiştir. Bu nedenle, anjiyogenezle ilişkili olan sinyal yolakları, plak içi anjiyogenezi önlemeyi amaçlayan terapiler için hedef teşkil etmektedirler. Bu tez çalışması, vasküler endoteliyal büyüme faktörü yolağı ile ilişkili anjiyogenik mekanizmaların rolünün ve mikroRNAlar gibi, bu yolak ile etkileşimde bulunan moleküllerin araştırılmasını amaçlamaktadır.

MikroRNAlar (miRNAlar) küçük, tek-iplikçikli RNA molekülleridir. Genellikle 18-22 nukleotid uzunluğunda olan ve kodlama yapmayan bu RNAlar, gen anlatımını transkripsiyon sonrası seviyede düzenleyen önemli regülatör moleküller olarak görev yaparlar. MiRNAlar pek çok önemli biyolojik fonksiyon ve hücresel olayda görev alırlar. MiRNAların görev yaptıkları önemli hücresel prosesler arasında hücre proliferasyonu, metabolizma, apoptoz, senesens, tümör oluşumu, damar oluşumu ve gelişim gelmektedir. Anjiyogenezde çok önemli rolü olan ve hem embriyonik hem de yetişkin damar sisteminde anlatımı yapılan *Egfl7*, endotele spesifik iki adet mikroRNA olan miR 126-3p ve miR 126-5p'yi intronundan kodlar. MiR-126 sinyal yolağının anjiyogenezdeki rolünü ve ateroskleroz patogenezine katkısını ortaya çıkarmak amacı ile gerçekleştirilen bu çalışmada, miR 126-3p ve hedef genleri ile miR 126-5p ve *Egfl7*'nin ekspresyon seviyeleri 14 adet karotis arter aterosklerotik plak dokusu ve 14 adet kontrol dokusunda incelenmiştir.

Mir 126-3p hedef genleri miRNA hedef tahmin veritabanları olan TargetScan and miRTarBase kullanılarak elde edilmiş, elde edilen bilgiler ve literatür araştırması sonucunda anjiyogenezde rolü olan ve vasküler endoteliyal büyüme faktörü sinyal yolağının negatif regülatörleri olan *Spred1* and *Pik3r2* genleri ile *Egfl7*'nin çalışılacak miR 126-3p hedef genleri olarak seçilmesine karar verilmiştir. Ön biyoinformatik araştırmayı takiben, 14 adet karotis arter aterosklerotik plak ve kontrol dokusunun homojenizasyonu ve sonrasında elde edilen homojenizatlardan total RNA ve miRNA izolasyonları gerçekleştirilmiştir. RNA izolasyonları sonucunda elde edilen total RNA ve miRNA'ların konsantrasyonları ve saflıkları spektrofotometrik yöntemle belirlenmiş, ve RNA miktarları eşitlenerek komplementer DNA (cDNA) reaksiyonları gerçekleştirilmiştir. Elde edilen cDNA'lar kullanılarak gerçek zamanlı kantitatif PZR reaksiyonları yürütülmüş ve bu sayede plak ve kontrol dokularındaki *Egfl7*, *Spred1*, *Pik3r2*, miR 126-3p ve miR 126-5p rölatif ekspresyon seviyeleri ölçülmüştür.

Gerçek zamanlı kantitatif PZR analizleri için, $2^{\Delta\Delta C_T}$ modelinin kullanıldığı rölatif kantifikasyon stratejisinden yararlanılmıştır. Plak örneklerindeki tüm hedef gen ve miRNA ekspresyon seviyeleri kontrol örneklerindeki hedef gen ve miRNA seviyeleri ile karşılaştırılmış ve 18S ribozomal RNA (hedef genler için) ve U6 küçük nüklear RNA (miRNAlar için) endojen kontrolleri kullanılarak normalizasyon yapılmıştır. Data analizlerini takiben, istatistiksel analizler gerçekleştirilmiştir. Plak ve kontrol dokularından elde edilen sonuçlar arasındaki farkın istatistiksel anlamlılığı Student's t test ile araştırılmıştır. Kat farkı olarak ifade edilen gen ekspresyon verileri, hastaların demografik ve klinik bilgileri ile karşılaştırılmıştır. Bu analizler için Chi-square ve Fisher's Exact testleri kullanılmıştır.

Bu çalışmada, literatürden elde edilen bilgiler derlenerek, ilerlemiş aterosklerotik hastalığa bağlı olarak görülen anjiyogenez nedeni ile, *Egfl7* ve miR 126-3p ile miR 126-5p'nin karotis arter aterosklerotik plaklarında, kontrollere göre up-regüle olacağı, buna karşılık miR 126-3p hedef genleri olan *Spred1* ve *Pik3r2*'nin ekspresyon seviyelerinin plaklarda kontrollere göre azalmış olacağı hipotezi ortaya konulmuştur. Sonuçlara bakıldığında, beklendiği üzere miR 126-3p ekspresyon seviyelerinin 11 adet plak örneğinde, kontrollere göre yüksek olduğu bulunmuş, miR 126-3p hedef genleri olan *Spred* ve *Pik3r2*'nin ekspresyon seviyelerinin kontrollere göre 8 ve 9 adet plak örneğinde daha düşük olduğu gözlemlenmiştir. Genel ekspresyon seviyesi göz önüne alındığında, miR 126-3p'nin plaklardaki ekspresyonunun, kontrollere göre 2.14 kat arttığı bulunmuştur (p <0.05). İncelenmiş olan diğer miRNA miR 126-5p'nin 8 adet

plak örneğinde kontrollere göre up-regüle olduğu gözlemlenmiştir. Genel ekspresyon seviyesine bakıldığında ise plaklarda kontrollere göre yüksek bulunan ekspresyon seviyesi, istatistiksel anlamlılığa ulaşamamıştır. Ek olarak, 11 adet plak örneğinde, kontrollere göre *Egfl7*'nin up-regule olduğu bulunmuş, genel ekspresyon seviyesine bakıldığında ise *Egfl7* ekspresyonunun plaklarda kontrollere göre daha yüksek olduğu gözlemlenmiştir(p <0.001).

Bu tez çalışması, derin plak-içi miR 126-3p ekspresyonu seviyelerininin araştırılması açısından bir ilk niteliği taşımaktadır. Literatürde miR 126-3p, hedef genleri *Spred1*, *Pik3r2*, miR 126-5p ve *Egfl7* ekspresyon seviyelerini insan karotis arter aterosklerotik plaklarında araştıran başka bir çalışmaya rastlanmamıştır. MiR 126-3p, hedef genleri *Spred1*, *Pik3r2*, miR 126-5p ve *Egfl7* ekspresyon seviyesi verileri ile desteklenen bu tez çalışmasında, miR-126 sinyal yolağı moleküllerinin ve bu moleküllerce desteklenen neoanjiyogenezin ileri seviye aterosklerozda gözlemlenen aterosklerotik plakların gelişimine katkısı gösterilmektedir. Bu tez çalışması, aterosklerotik plak gelişiminin ve plak içi neoanjiyogenezin takibi konusunda yapılacak gelecek miRNA biyobelirteç çalışmaları açısından ve plak içi neoanjiyogenezin, dolayısıyla trombüs oluşumunun önlenmesi konusunda yapılacak gelecek miRNA tabanlı terapiler açısından bir ön çalışma çalışması niteliğindedir. Ek olarak, Egfl7 de kararsız plakların takibi ve bu plakların yırtılmasının engellenmesi konularında kullanılabilecek gelecek vaad eden prognostik bir biyobelirteç olarak değerlendirilebilir. *Egfl7* tabanlı anti-anyijogenik terapiler, plak içi neoanjiyogenezin elimine edilmesi ve yırtılmaya meyilli plakların stabil halde getirilmesinde bir strateji olarak önerilmektedir.

MiR-126 tabanlı gen regülasyonunun moleküler mekanizmaları, miR-126'nın vasküler endoteliyal büyüme faktörü ile regüle edilen anjiyogenezdeki etkisinin tam olarak anlaşılabilmesi amacı ile, özellikle vasküler endoteliyal büyüme faktörü yolağında yer alan moleküllerinin fosforilasyon durumlarının da inceleneceği, daha geniş araştırmalar ile ortaya konulmalıdır. Mir-126'nın diğer miRNAlar ve sinyal yolağı molekülleri ile olan etkileşimi açığa çıkarılmalıdır. Anjiyogenezin aterosklerozun değişik evrelerindeki farklı rollerini daha iyi anlayabilmek amacı ile, farklı arteriyel stenoz derecesine sahip hastalardan alınacak örneklerde çalışmalar yapılması önerilmektedir. Bununla beraber, farklı arteriyel sistemlerde görülen hastalıkların benzerlik ve farklılıklarının ortaya konulması ve tüm sistemlerde ortak olan bir regülasyonun belirlenebilmesi için, karotis ve koroner arter hastalarından alınacak örneklerin kullanılacağı, daha geniş çapta yapılacak miRNA profillemesi çalışmalarının faydalı olacağı düşünülmektedir.

1. INTRODUCTION

1.1 Purpose of Thesis

Cardiovascular diseases, mainly described as the diseases of the blood vessels, the heart, and the vascular diseases of the brain, were the number one cause of death among all the deaths attributable to noncommunicable diseases in 2014 [1, 2]. Unfortunately, the devastating consequences of the coronary artery disease and carotid artery disease, such as the myocardial infarction and stroke, remain to be the leading causes of death and disability in the world today. The underlying pathology of cardiovascular diseases is atherosclerosis [3, 4].

In the pathological process of atherosclerosis, the walls of medium and large sized arteries are thickened and hardened. Atherosclerosis is characterized by lipid accumulation and plaque formation in the arteries [5, 6]. Once the disease is clinically manifested as coronary heart disease [7], cerebrovascular disease or peripheral artery disease, many therapies to prevent complications are applied. Since atherosclerosis is a condition that is driven mostly by lipoproteins [7], drug therapies aiming to lower blood cholesterol levels, known as statins have been developed. Besides statins, anti-hypertensive drugs are also used, since hypertension is one of the major risk factors of cardiovascular diseases. Anti-coagulants are widely used as well. Also, drug therapies to increase levels of high density lipoprotein exist [8]. Collectively, these drug therapies are known as primary protection [6, 9]. But, drug therapies have side effects and they are not cost effective. Traditionally, angiography is used to monitor the progression of atherosclerosis. In addition, new imaging techniques such as magnetic resonance imaging, computed tomography, positron emission tomography, ecocardiography and intravascular ultrasound are used as well [10, 11]. But, although atherosclerotic plaques can be detected by means of these imaging technologies, most of the people with atherosclerotic plaques in their arteries do not develop disease [9]. Finally, surgical interventions, such as coronary angioplasty, coronary

artery bypass grafting and carotid endarterectomy are used for the treatment of atherosclerotic cardiovascular diseases. They are considered as invasive, expensive and high risk methods [12]. Detecting atherosclerosis early is very important in preventing cardiovascular diseases. For this purpose, biomarkers that would detect the early lesions of atherosclerosis as well as biomarkers that would enable physicians to monitor the progress of plaques are needed.

Atherosclerosis is a process starting with birth and it progresses throughout life. The earliest lesions of atherosclerosis, known as fatty streaks, are known to be reversible partially [13], but once the disease starts to progress further, more advanced lesions are observed. The rupture of the complex, unstable plaques, which are known as vulnerable plaques, result in thrombus formation [6, 14]. Atherosclerosis itself is not the cause of death. The narrowing of the arteries due to plaque formation cause obstruction of blood flow to the brain, the heart and the limbs [7]. Following rupture of a vulnerable plaque, it is the thrombus formation resulting in stroke or a myocardial infarction that causes death. Therefore, atherosclerosis would possibly be a more benign disease if thrombus formation could be prevented. From this point of view, why atherosclerotic plaques develop is not the question for research purposes anymore. The crucial question is why only few of the many plaques within a given person undergoes the thrombosis-prone phase during lifespan. To be able to prevent the development of vulnerable plaques, as well as finding and treating them is the main focus of atherosclerosis research [14].

The exact molecular mechanisms of how asymptomatic lesions become vulnerable plaques are yet to be elucidated. Intraplaque neoangiogenesis has been linked with progressive atherosclerotic disease and the development of acute lesion instability by many studies [15]. Neoangiogenesis is suggested to cause stenosis, intraplaque hemorrage, thrombosis and consequently a stroke or a heart attack. The signalling pathways contributing to angiogenesis represent potential targets for therapies aiming to prevent intraplaque neoangiogenesis [16,17]. This thesis study focuses on the role of angiogenic mechanisms related to vascular endothelial growth factor (VEGF) pathway, the main driver of angiogenesis in cells, as well as mechanisms that interact with the VEGF pathway, such as microRNAs.

MicroRNAs (MiRNAs), the small, non-coding RNAs that post-transcriptionally regulate many cellular processes are widely studied in cardiovascular diseases and atherosclerosis [18]. *Egfl7*, a major player of angiogenesis that is expressed exclusively in embryonic and adult vasculature, encodes two endothelial-specific miRNAs: miR 126-3p and its passanger strand, miR 126-5p.

The present study aims to understand the role of miR 126 signalling in angiogenesis. This study was designed to answer the following questions:

•What are the expression levels of miR 126-3p, of miR 126-3p targets, miR 126-5p and *Egfl7* in human carotid artery atherosclerotic plaques relative to control samples?

•Are the clinical parameters (cardiovascular disease risk factors) of the patients correlated with relative gene expression levels?

• Through which mechanisms does intraplaque neoangiogenesis affect atherosclerotic disease?

The outcomes of this thesis work are believed to provide useful information on the roles of miR 126-3p and -5p as well as their target partners in angiogenesis and atherosclerosis. A better understanding of the VEGF pathway and its interaction with miR 126 can possibly lead to development of drugs targeting neoangiogenesis. The results of the conducted expression study of these genes and miRNAs may constitute preliminary bases for future miRNA biomarker studies for monitoring plaque progression and intraplaque neoangiogenesis, as well as miRNA based therapies for preventing intraplaque neoangiogenesis, therefore thrombosis.

1.2 Cardiovascular System

There are three basic components in a cardiovascular system: a muscular pump, such as the heart, a circulatory fluid, and a set of interconnecting vessels. In a closed circulatory system, such as the cardiovascular system of humans and other vertebrates, the circulatory fluid is blood [19]. The cardiovascular system transports fluids throughout the body, connecting the aqueous environment of the cells to the organs that function in gas exchange, absorption of nutrients, and disposal of wastes [20]. Before reaching the blood, O_2 inhaled from air diffuses across two layers of cells in the lungs. Then, the oxygen-rich blood is carried to the rest of the body by the

help of the cardiovascular system. As the blood flows throughout the tissues via blood vessels, O_2 in the blood reaches the cells by diffusion. One of the benefits of a closed circulatory system is the relatively high blood pressure, that effectively delivers of O_2 and nutrients to the cells [19–21].

Together, the heart, the blood vessels (or vasculature), the cells and plasma of the blood construct the human cardiovascular system [19]. The heart, located behind the sternum, is an organ about the size of a fist, composed of cardiac muscle called the myocardium mostly [20]. A system of coronary arteries feed the the heart muscle with blood supply. The two atria of the heart function as collection chambers for blood returning to the heart from the systemic circulation or the lungs. The function of the heart is to pump the blood from its atria into large vessels branching into smaller ones which end up infiltrating the organs [19–21].

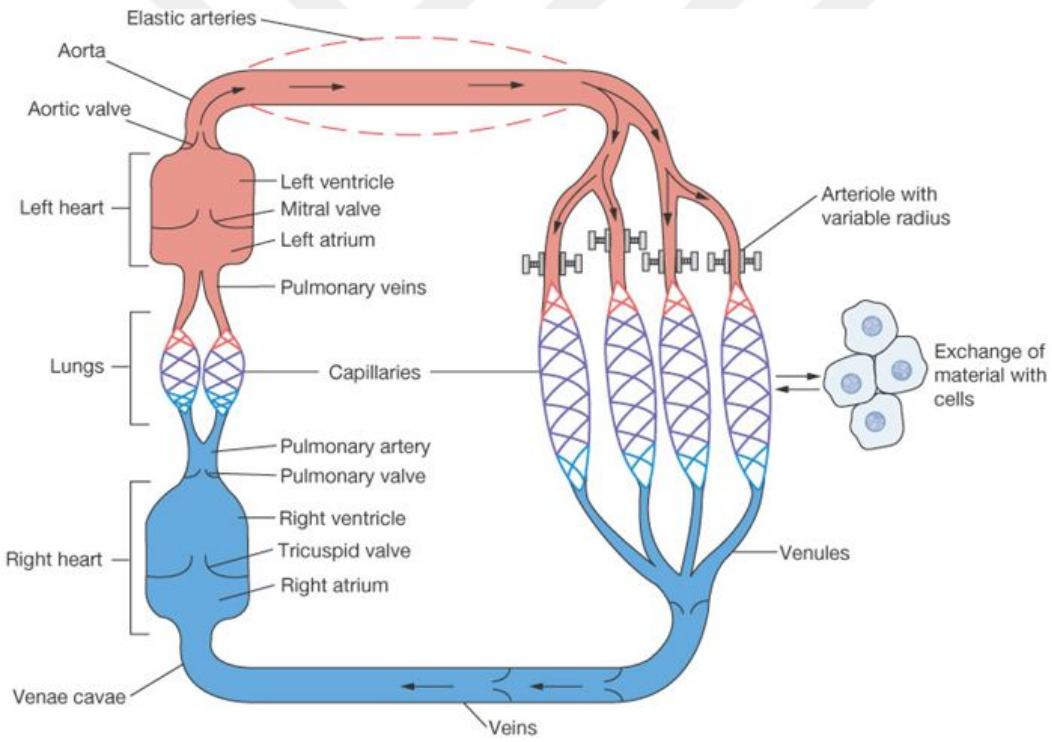


Figure 1.1 : The model of the cardiovascular system [20].

The circulation of blood to and from the heart occurs through an extensive network of vessels. The three main types of blood vessels of the vasculature are the arteries, veins, and capillaries. The blood flows in vessels in one direction. A system of valves in the heart and veins ensures that the blood flow is confined to one direction. Arteries carry the blood to organs where they branch and form smaller vessels called arterioles that

work as outlets with high resistance for arterial blood flow, directing the distribution of blood flow to tissues. Arterioles can adjust their diameter by selectively constricting and dilating. The alterations of the diameter are regulated via autonomous nervous system, hormones and local factors such as tissue oxygen concentration. Blood passes from the arterioles to the capillaries, the microscopic vessels with very thin, leaky walls. Capillaries form a network known as capillary beds and infiltrate every tissue. The porous epithelium of the capillaries allows the exchange of chemicals, including dissolved gases, by diffusion between the interstitial fluid surrounding cells of the tissues and the blood. As demonstrated in Figure 1.1, at their distal ends, capillaries come together to form venules, which further converge into veins, carrying the blood back to the heart [19–23].

The cardiovascular system is composed of two circuits, known as a double circulation. The double circulation arrangement includes the systemic circulation and the pulmonary circulation, which are combined in one single organ, the heart. The blood returning from the organs, deprived of oxygen, enters the right atrium of the heart [22, 23].

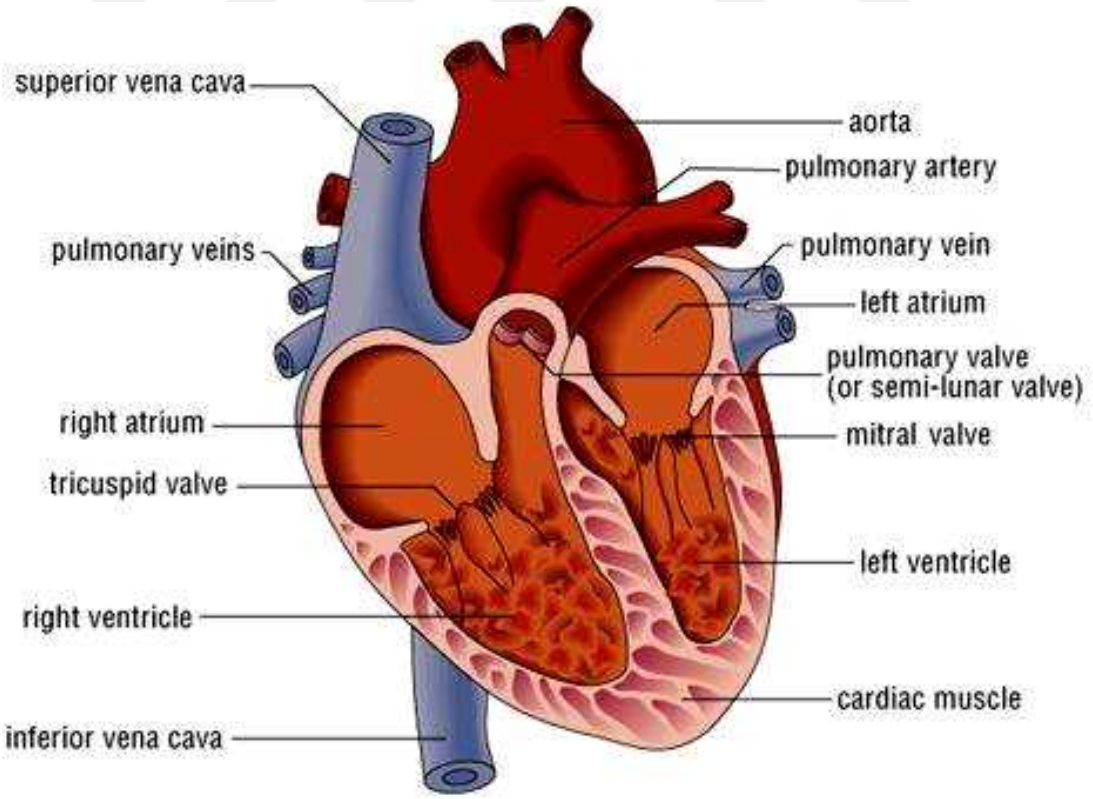


Figure 1.2 : The anatomy of the human heart [24].

From the right atrium, passing through the tricuspid valves, the blood moves to the right ventricle, from where it is pumped into the pulmonary arteries. The pulmonary arteries carry the oxygen deprived blood to the lungs to become oxygenated in the capillaries of the lungs. Then, the blood is carried back to the left atrium of the heart via the pulmonary veins, completing the pulmonary circuit part of the double circulation. As the heart muscle contracts, blood is pumped into the left ventricle through the bicuspid or mitral valves. From the left ventricle, the blood is sent into the aorta, through the aortic valves. Starting in the heart and ending in the abdomen, the aorta is the largest vessel in the body. The aorta branches into systemic arteries and smaller arterioles and finally into capillary beds in organs and tissues to transport the blood from the left ventricle to every organ throughout the body. In the capillary beds, exchange of oxygen with carbon dioxide takes place. The systemic circuit of the double circulation is completed when the CO_2 rich blood is carried back to the right atrium of the heart via veins coming from the lower body and upper body [19–23].

1.3 Diseases of the Cardiovascular System

Cardiovascular diseases (CVDs) are mainly the diseases of the blood vessels, the heart, and the vascular diseases of the brain. The different types of CVDs are summarized below [1, 25].

CVDs caused by atherosclerosis:

• Ischaemic heart disease and coronary artery disease occur due to the disease and blockage in the blood vessels supplying the heart muscle known as coronary arteries. Acute coronary syndromes and heart attacks, also known as myocardial infarction (MI), take place when the vessels supplying blood to the heart muscle are blocked suddenly.

• Cerebrovascular diseases are caused by the disease of the carotid arteries; the blood vessels supplying the brain. Strokes or transient ischemic attacks are the consequences of the cerebrovascular disease.

• Peripheral artery disease, hypertensive heart disease, deep vein thrombosis and pulmonary embolism are the main diseases regarding the circulatory system [1, 25].

Other CVDs:

- Congenital heart disease
- Rheumatic heart disease
- Inflammatory heart disease
- Cardiomyopathies
- Heart failure
- Cardiac arrhythmias [1, 25].

According to the 2014 Global Status Report on Noncommunicable Diseases published by World Health Organization (WHO), of the total 56 million deaths that took place in 2012, 38 million were due to noncommunicable diseases such as CVDs, cancer, chronic respiratory diseases and diabetes [2]. Based on WHO data, CVDs were the number one cause of death in the world in 2012. In 2012, 17.5 million people died from CVDs, making up 31% of all global deaths as demonstrated in Figure 1.3. 7.4 million of these deaths took place because of coronary heart disease, while 6.7 million were due to stroke [25, 26].

In US, based on 2016 update of Heart Disease and Stroke Statistics that is brought together by American Heart Association (AHA), Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH), the overall rate of death due to CVD was reported as 222.9 per 100.000 Americans in 2013. According to this data, more than 2200 Americans die of CVD each day [27].

In Turkey, based on Turkish Statistical Institute Cause of Death Statistics of 2014 data, the leading cause of death was cardiovascular diseases, represented with 40.4% of all national deaths that took place in 2014. 39.6% of these CVD caused deaths occurred due to ischaemic heart disease, followed by the cerebrovascular diseases, represented by 24.7% of all CVD caused deaths [28].

Today, CVDs remain to be the leading cause of death in the world. The main underlying pathological condition causing CVDs is atherosclerosis. While atherosclerosis is an irreversible process beginning with birth, atherosclerotic CVDs can be prevented if major risk factors are taken into consideration. Although some major cardiovascular disease (CVD) risk factors that cannot be controlled exist, most of the CVDs are caused by controllable, treatable or modifiable risk factors.

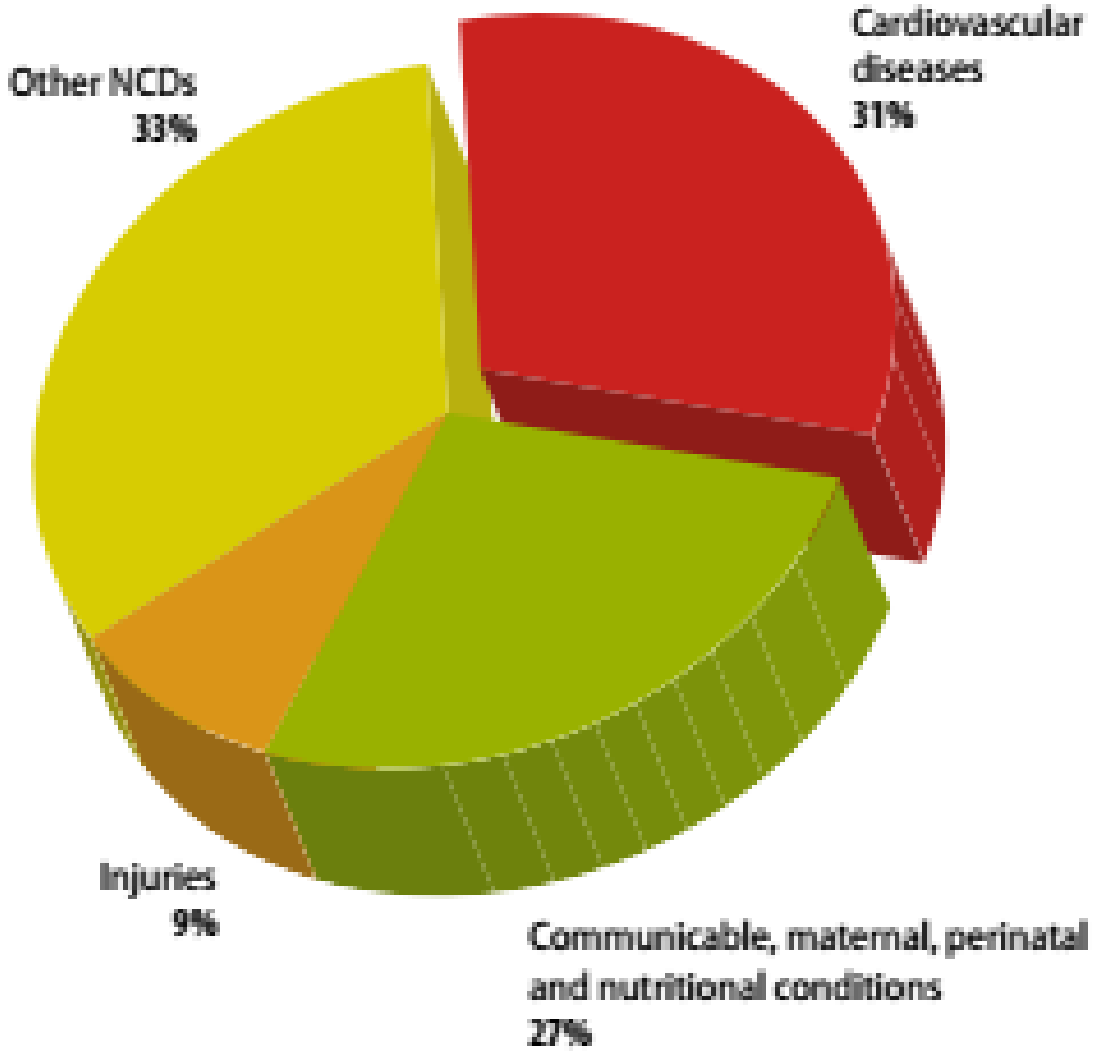


Figure 1.3 : The global distribution of major causes of death in 2012 [25].

The major risk factors are the factors that have been demonstrated to significantly increase the risk of CVDs. On the other hand, the so called "contributing risk factors" have been associated with increased risk of CVD, but their significance in progression of CVD is yet to be determined [29].

In 2011, AHA introduced a new concept of cardiovascular health, characterized by 7 metrics, named as Life's Simple 7. These 7 metrics include health behaviors (having a healthy diet, a sufficient physical activity, not-smoking, normal body weight) and health factors (normal levels of blood cholesterol, blood pressure and blood glucose in the absence of drug treatment). According to the concept, the ideal cardiovascular health was defined by the presence of optimal levels of all 7 metrics together with the absence of clinically manifested CVD [27].

1.3.1 Cardiovascular disease risk factors

The major cardiovascular disease risk factors which cannot be changed are; increasing age, male gender and heredity, while the treatable and modifiable ones include smoking, high blood cholesterol, high blood pressure, physical inactivity, obesity and diabetes mellitus.

The heart changes physiologically with increasing age, even without the presence of any disease. When a CVD affects the heart, the changes in the heart due to old age may further complicate the problem or its treatment. For example, after the age of 65, the deaths attributable to coronary heart disease increase severely.

The risk of a heart attack is greater in men compared to pre-menopausal women. On the other hand, the risk of stroke is similar for men and women.

In the case of heredity, a strong family history of cardiovascular disease poses great risk, since children of parents with cardiovascular disease have more than one major risk factor most of the time. The risk increases if a first-degree blood relative has had a CVD before 55 years of age (for males) or 65 years of age (for females). In addition, compared to Caucasians, African Americans are at greater risk due to their higher blood pressure.

Smoking is one of the major modifiable risk factors for CVDs, especially when it is together with other major risk factors. In coronary heart disease patients, smoking is implicated in sudden cardiac death. AHA considers never having smoked or having quit more than 12 months ago as one of the 7 components of ideal cardiovascular health in adults [29, 30].

High blood cholesterol and abnormal levels of lipids in the blood constitute important treatable and modifiable risk factors for cardiovascular disease. The risk of coronary heart disease increases with high blood cholesterol levels. When present with other risk factors such as high blood pressure and smoking, the risk increases even more. Lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) function in carrying cholesterol in blood. High levels of LDL cholesterol result in rapid progression of atherosclerosis, therefore increase the risk of CVD. HDL carries cholesterol away from the blood stream, thus higher levels of HDL are considered to

reduce the risk of CVD. In addition to HDL and LDL cholesterol, blood triglyceride levels are important. When combined with high levels of LDL cholesterol or low levels of HDL cholesterol, higher levels of triglycerides in blood pose a risk of speeding up the atherosclerotic process. The blood cholesterol levels are affected by age, gender, heredity, diet and physical activity. Tables 1.1, 1.2 and 1.3 demonstrate the normal and abnormal blood lipid levels [30, 31].

Table 1.1 : Total Cholesterol Levels [30, 31].

Total Cholesterol Level	Category
Less than 200 mg/dL	Desirable
200-239 mg/dL	Borderline High
240 mg/dL and above	High

Table 1.2 : LDL Cholesterol Levels [30, 31].

LDL Cholesterol Level	Category
Less than 100 mg/dL	Optimal
100-129 mg/dL	Near Optimal Above Optimal
130-159 mg/dL	Borderline High
160-189 mg/dL	High
190 mg/dL and above	Very High

Table 1.3 : HDL Cholesterol Levels [30, 31].

HDL Cholesterol Level	Category
Less than 40 mg/dL	Poor, major risk factor for CVD
40-59 mg/dL	Better
60 mg/dL and higher	Best, protective against CVD

High blood pressure, otherwise known as hypertension, is the systolic blood pressure (maximum pressure in the arteries as the heart contracts) at or above 140 mmHg and/or the diastolic blood pressure (minimum pressure in the arteries between the contractions of the heart) at or above 90 mmHg. Hypertension leads to stress in blood vessels, causing them to become weaker. Atherosclerotic plaques are mostly observed in vulnerable locations such as arterial branching points where the blood flow is high or disturbed. As the leading cause of CVD, hypertension is a major risk for coronary heart disease and the single most important risk factor for stroke.

Along with physical inactivity, being overweight and obese constitute a major risk factor for CVD progression. Body Mass Index (BMI) is an estimate of the body fat and is used as a tool to screen for obesity and overweight. BMI is calculated by dividing

the weight in kilograms to the square of height in meters. Table 1.4 shows the BMI values and their corresponding explanations [32, 33].

Table 1.4 : Body Mass Index values [32, 33].

BMI	Category
18.5- 24.9	Normal Weight
25- 29.9	Overweight
30- 39.9	Obese
40 and above	Exteme Obesity

Diet and nutrition, alcohol consumption and stress are considered as the other risk factors that contribute to progression of CVDs [30]. In addition to all the mentioned CVD risk factors, some new ones that have been introduced lately include; elevated levels of homocysteine, lipoprotein(a), c-reactive protein and fibrinogen [34].

1.3.2 Carotid artery disease

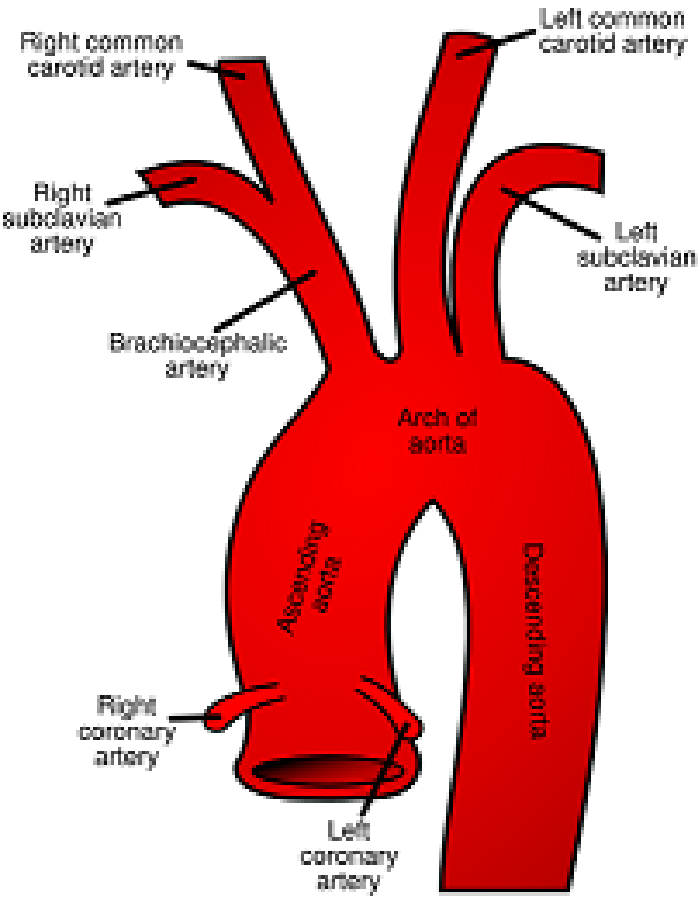


Figure 1.4 : The proximal aorta and carotid arteries [35].

As shown in Figure 1.4, some important branches of the proximal aorta include the coronary arteries, the brachiocephalic artery that further branches into the right

common carotid artery and right subclavian artery, as well as the left common carotid and the left subclavian arteries. The coronary arteries supply blood to the heart muscle, while the left and right subclavian arteries feed the left and the right arms with blood [35].

The two carotid arteries, one located on each side of the neck, deliver blood to the brain. The medical condition known as carotid artery disease is caused by the decreased blood flow to the brain due to a blockage in the carotid artery. The sudden cease of blood flow to a certain area in the brain causes stroke. Atrial fibrillation and blockage in the carotid arteries are the two main causes of stroke. Unfortunately, until the blockage in carotid artery causes the blood flow to brain to stop, there is no way to understand the presence of it. When a brain area is deprived of blood supply due to a blockage, its functions stop, causing symptoms of a stroke or a transient ischaemic attack (TIA), such as painless loss of vision (Amaurosis fugax), slurry speech, difficulty to speak, weakness in limbs and facial numbness. Carotid artery disease is diagnosed with ultrasound of the carotid arteries as well as magnetic resonance angiogram and computed tomography angiogram. These tests provide information about the presence and the severity of a narrowing (stenosis) in the carotid arteries [36]. It is of great importance to differentiate between symptomatic carotid artery disease and asymptomatic disease. Asymptomatic severe carotid stenosis is 50–60% narrowing of the proximal internal carotid artery without the presence of stroke or TIA symptoms [37].

To prevent the progression of disease, non-invasive therapies such as treatment of risk factors such as hypertension, diabetes and high cholesterol are applied. For high grade stenosis ($\geq 60\%$), invasive therapies such as carotid endarterectomy and carotid artery stenting are preferred. Carotid endarterectomy is an operation in which atherosclerotic plaques are surgically removed from the carotid arteries and is performed to prevent future strokes in symptomatic patients, while carotid artery stenting alternative is offered to patients who cannot undergo carotid endarterectomy [36–38].

1.4 Atherosclerosis

Atherosclerosis is the chronic, immunoinflammatory, degenerative disease of the medium- and large-size arteries. It is a complex disease which involves interactions of multiple genetic and environmental factors [39]. Progress of atherosclerosis changes the arterial wall and leads to the build up of atherosclerotic plaques inside arteries. Atherosclerosis is the underlying cause of many CVD diseases, including coronary artery disease, stroke and peripheral artery disease [40]. Starting with birth, atherosclerotic process progresses slowly and shows no symptoms for many decades. When atherosclerosis presents symptoms in later stages of life, its complications, such as myocardial infarction and stroke, are the leading causes of death in the Western world [41].

As shown by the epidemiological studies which have been conducted for more than 50 years, atherosclerosis is a multi-factorial disease, in which environmental factors are interacting with genetic components. The factors that contribute to the development of atherosclerosis, as shown in Table 1.5, can be categorized as environmental factors and as factors with a genetic background [6].

Table 1.5 : Environmental and genetic factors that contribute to development of atherosclerosis [6].

Factors with a genetic background	Environmental factors
High levels of LDL/VLDL	High-fat diet
Low levels of HDL	Smoking
High levels of lipoprotein(a)	Low antioxidant levels
Hypertension	Physical inactivity
High levels of homocysteine	Infectious agents
Family history of CVDs	
Diabetes and obesity	
Depression	
Male gender	
Metabolic syndrome	
Systemic inflammation	
LDL: Low density lipoprotein	
VLDL: Very low density lipoprotein	
HDL: High density lipoprotein	
CVD: Cardiovascular disease	

1.4.1 Pathogenesis of atherosclerosis

The wall of a normal blood vessel has three layers, the tunica intima, tunica media and tunica adventitia. The intima layer is made up of a monolayer of endothelial cells, which reside on a basement membrane containing type IV collagen, laminin and heparan sulphate proteoglycans. The intima and the media layers are separated by internal elastic lamina made up of elastin. The focal intimal thickenings rich in sulphated polysaccharide and hyaluronic acid are exclusive to humans and intimal smooth muscle cells (SMCs) can be found in these areas. Below, vascular SMCs embedded in an interstitial matrix form the tunica media layer. The adventitia layer contains capillaries, loose connective tissue, fibroblasts and fat cells, as shown in Figure 1.5 [42].

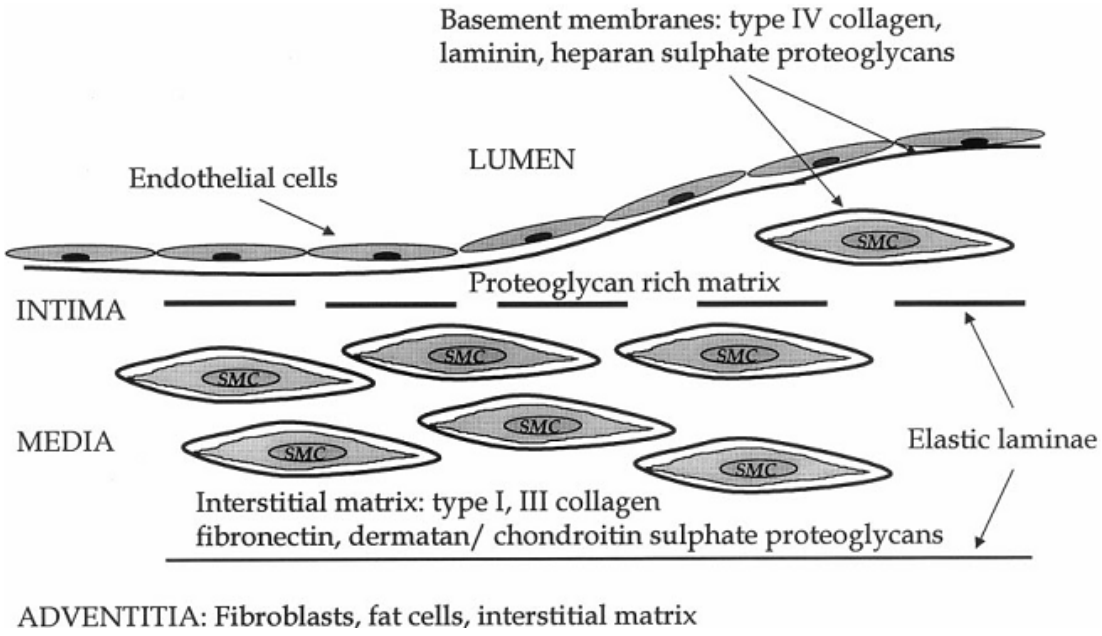


Figure 1.5 : The three compartments of an artery [42].

In the inflammatory process of atherosclerosis, the intima layer gets thicker with lipid rich material (atheroma) and connective tissue (sclerosis) [43]. Today, it is generally believed that a response to injury initiated by circulating factors causes atherosclerosis, owing to the "Response to Injury" hypothesis developed and published by Russell Ross and John A. Glomset in 1973. In this hypothesis, it is stated that, it is the injury to the endothelium that initiates the pathogenesis of atherosclerosis [44]. In this model of atherogenesis, potential sources of injury, such as hyperlipidemia, hypertension,

metabolites, infections and mechanical factors such as shear stress were suggested to cause the initiation of atherosclerosis. The aggregation of platelets, retention of oxidized lipids and migration of smooth muscle cells to the intimal layer are the events that were suggested to follow the initial injury to eventually cause plaque formation. This hypothesis was supported by various results from studies with animal models [45].

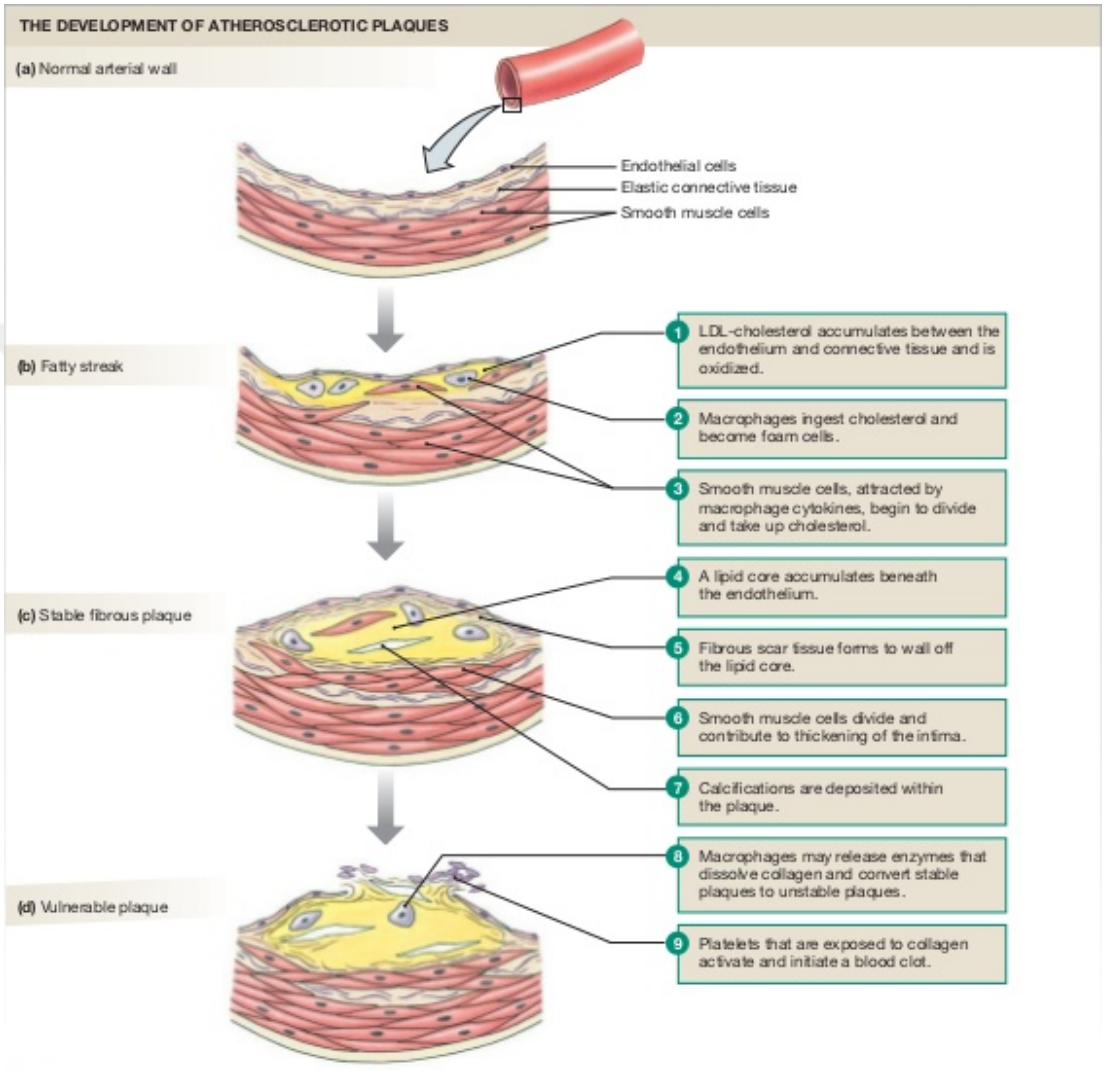


Figure 1.6 : Stages of atherosclerosis [20].

As demonstrated in Figure 1.6, the development of atheromatous plaques inside the arteries is referred to as atherogenesis, which takes place in distinct stages:

•The normal vessel wall,

•The first lesions; fatty streaks,

•Atherosclerotic plaques,

•Ruptured plaques with thrombosis [40].

Atherosclerosis is characterized by vascular inflammation and the accumulation of lipids, cholesterol, calcium, and cellular debris inside the intima of the vessel wall. The pathological changes in atherosclerosis occur due to molecular events of endothelial dysfunction, monocyte adherence and entry into the vessel wall, monocyte development into foam cells, smooth muscle cell migration and proliferation, and platelet adhesion and aggregation [39, 40].

1.4.1.1 Initiation of atherosclerotic lesions

The vascular wall homeostasis is regulated by the endothelium. The mediators such as nitric oxide, prostacyclin and endothelin that are released by endothelial cells function in controlling vascular tone and maintaining low levels of oxidative stress. The endothelium regulates processes including vascular permeability, platelet and leukocyte adhesion and aggregation, as well as thrombosis [46]. The healthy endothelium changes to a non-adaptive state, in which the homeostatic mechanisms of endothelial cells are dysregulated, in response to various stimuli such as diabetes, dyslipidaemia, hypertension, shear stress related cell injury, smoking and most importantly, oxidized low density lipoprotein (ox-LDL) [47]. This situation, which the endothelium can no longer protect the vessel wall is named as endotelial dysfunction [40, 46].

Fluid shear stress is one of the many physical forces that affect endothelial cells. For a healthy vessel function, the physiologic, laminar shear stress needs to be maintained. Shear stress affects the morphology of endothelial cells; where blood flow is uniform and laminar, the cells, aligned in the direction of flow, are ellipsoid shaped. On the other hand, in regions where there is disturbed blood flow, such as arterial branching sites, endothelial cells are polygonal shaped and without a particular orientation. The areas of disturbed flow are prone to lesion formation, because nonlaminar flow alters the endothelial gene expression, cytoskeletal arrangement and leukocyte adhesion [48]. The cells in the these areas are more permeable to molecules such as LDL [6].

The first step in the formation of early lesions is endothelial dysfunction taking place as a response to various triggers, such as ox-LDL [49]. The most important events that take part in fatty streak formation are, the recruitment of monocytes, differentiation of monocytes into macrophages and the uptake of LDL-derived cholesterol by these

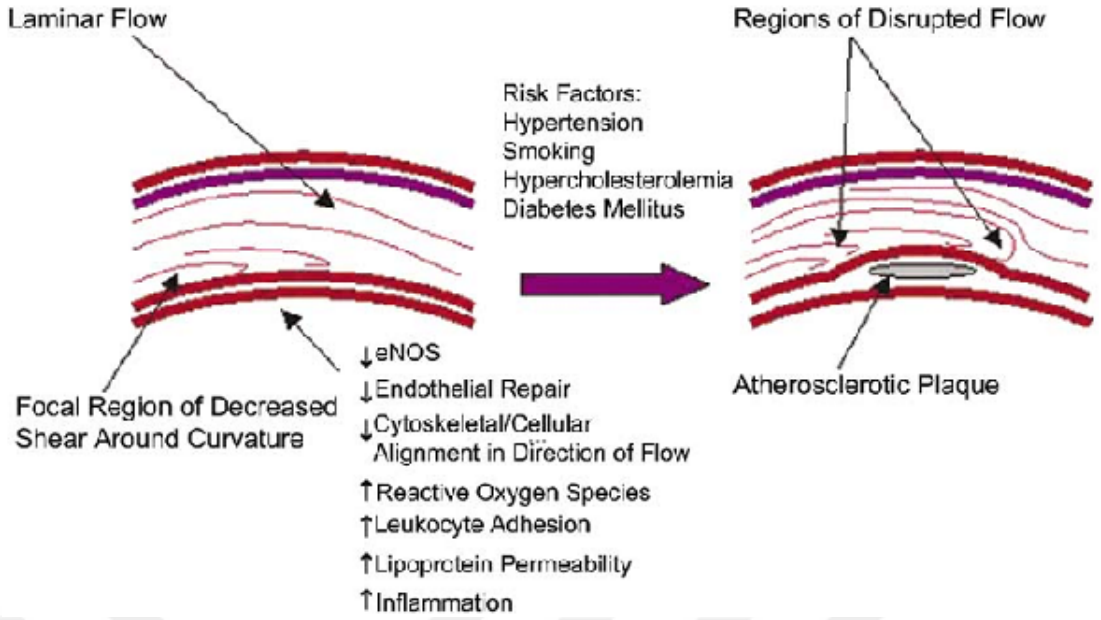


Figure 1.7 : Role of shear stress in atherosclerosis progression [48].

macrophages. The oxidative modifications in LDL, especially in the lipid and the apolipoprotein B components, cause endothelial dysfunction and initiation of inflammatory response, thus contribute to fatty streak formation [50].

1.4.1.2 Foam cell formation

Inflammation runs in almost all stages of atherogenesis, starting with endothelial dysfunction. Cell adhesion molecules expressed by endothelial cells, including Vascular Cell Adhesion Molecule-1 (VCAM-1), E selectin and P selectin, and Intercellular Adhesion Molecule-1 (ICAM-1), are upregulated in response to these stimuli that cause endothelial dysfunction. As a result, monocytes and T cells in circulation are recruited to the areas which are prone to atherosclerosis [14, 51]. Together with cell adhesion molecules, ox-LDL serves as a chemoattractant molecule too for monocytes. In addition, the expression of chemotactic molecules, such as monocyte chemotactic protein-1 (MCP-1), is triggered by ox-LDL [52].

After monocytes are attracted to the arterial wall in response to chemotactic signals, they move to the subendothelial region and differentiate into macrophages within the intimal layer and internalize ox-LDL via their scavenger receptors. This protective function of monocytes to remove the ox-LDL particles or apoptotic cells ends up with the uptake of ox-LDL molecules by macrophages, causing foam cell formation. The

presence of foam cells in the intima is a hallmark of the earliest atherosclerotic lesions, fatty streaks [14, 49, 51].

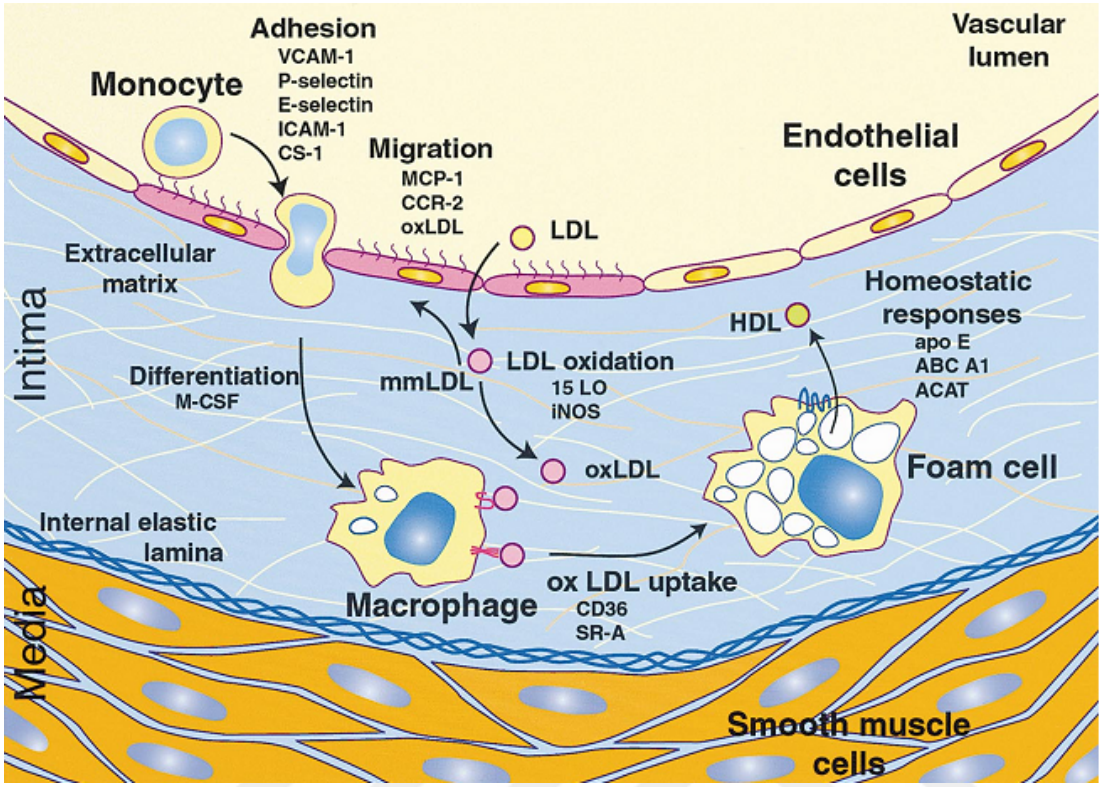


Figure 1.8 : Foam cell formation [51].

According to the "Oxidative modification hypothesis of atherosclerosis", for the rapid uptake of LDL by macrophages, it needs to be highly oxidized. The uptake results in foam-cell formation. This process is mediated by scavenger receptors (SRs). Certain scavenger receptors, such as SRA, SRB and Lectin-like ox-LDL receptor-1 (LOX-1) have been implicated in atherogenesis due to their role in receptor mediated uptake of modified LDL [49, 53].

1.4.1.3 Fibrous plaques

The initial lesions of atherosclerosis, the fatty streaks can be observed in the aorta in the first decade of life, while they emerge in the coronary arteries in the second decade, and the cerebral arteries in the third or fourth decades in humans. Though fatty streaks do not have clinical significance, their importance as precursors of more advanced lesions have been recognized. The characteristics of these more advanced lesions, named as fibrous plaques, are the accumulation of smooth muscle cells and necrotic debris rich in lipids [6].

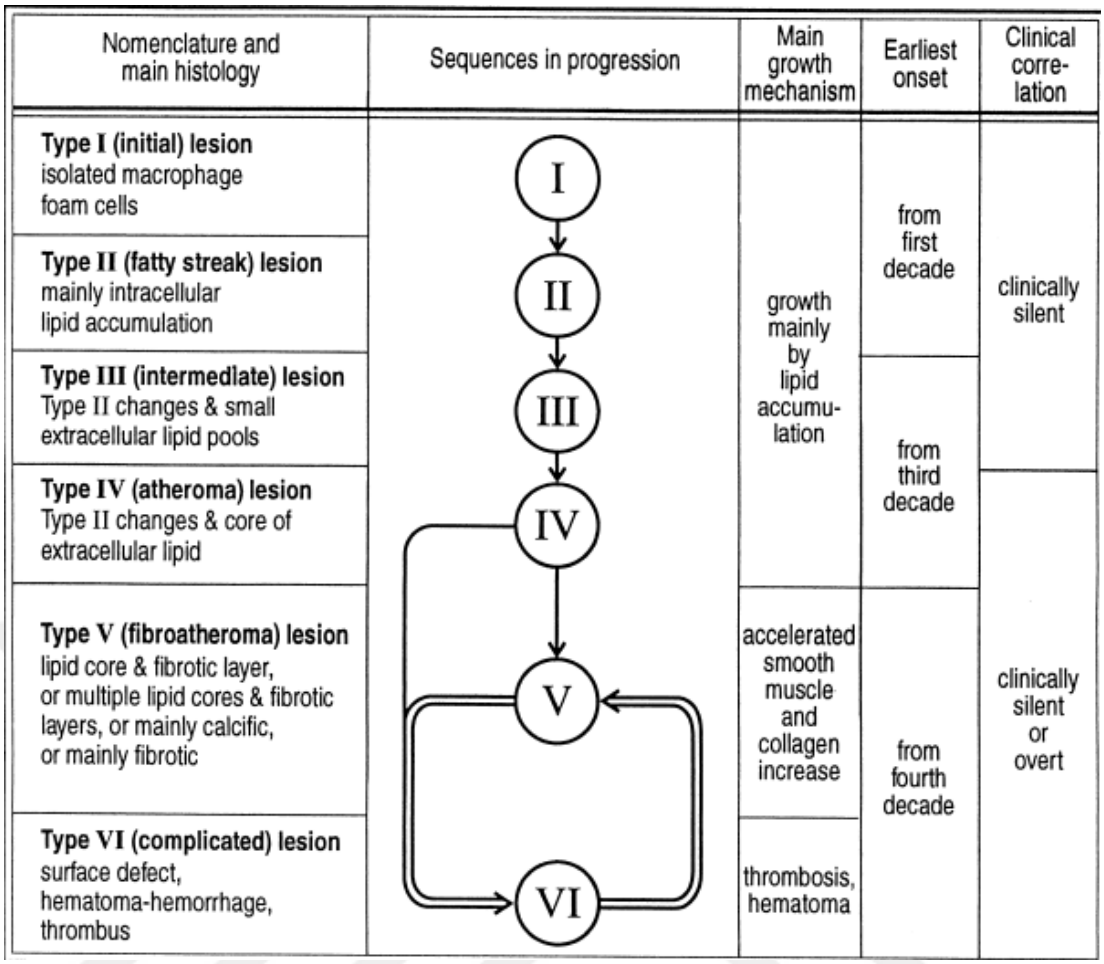


Figure 1.9 : Progression of atherosclerotic lesions [47].

As the disease progresses, a transition from simple fatty streak to a complex lesion takes place. The smooth muscle cells migrate from the tunica media layer, passing the internal elastic lamina, into the tunica intima. They proliferate in intima in response to mediators such as platelet derived growth factor. The intimal smooth muscle cells further contribute to the formation of foam cells by taking up modified lipoproteins [43, 54]. As the SMCs in intima start producing extracellular matrix molecules, such as interstitial collagen and elastin, the emergence of the fibrous cap is observed. Apoptosis of the foam cells result in extracellular lipid accumulation, creating the necrotic core within the plaque [14, 55].

1.4.1.4 Advanced plaques, neoangiogenesis and thrombosis

The arterial lumen narrows with the progressing lesion, resulting with a decrease in the blood flow and eventually tissue ischemia. Finally, the ischemia is precipitated as the plaque is physical disrupted. The characteristics of plaques that are prone

to rupture (vulnerable plaques) include a large lipid-rich core, a thin fibrous cap with fewer smooth muscle cells and many macrophages, angiogenesis and adventitial inflammation. Devastating consequences of the plaque rupture are myocardial infarction and stroke [14, 49].

In carotid artery disease, strokes mostly take place due to thrombosis resulting from the rupture of atherosclerotic plaques [56]. Thrombus formation is known to be associated with plaque instability, instead of plaque size [57]. Angiogenesis inside the plaques has been associated with qrowth of the plaques, its rupture, intraplaque hemorrhaging and thrombosis [58,59]. It is not clear at which stages of plaque formation neoangiogenesis occurs, although new vessel formation has been reported in fatty streaks and was found to increase with the progressing lesions [60].

In studies performed with apolipoprotein E knock-out mice, lesion growth was suppressed when neoangiogenesis was inhibited, suggesting the contribution of neoangiogenesis to plaque vulnerability in atherosclerosis [61].

1.4.2 Genetic susceptibility to atherosclerosis

The molecular mechanisms leading to atherosclerosis are partially understood. Multiple risk factors have been identified by large-scale epidemiological studies performed in families with premature cardiovascular disease and in monozygotic and dizygotic twins. There is a significant genetic component behind each of the traditional risk factors, such as dyslipidemia, obesity, diabetes mellitus, and hypertension. On the other hand, population migration studies emphasize the impacts of environmental factors. Thus, the overall risk of atherosclerosis is determined by the interaction of genetic and environmental factors [41, 62, 63].

In most premature forms of atherosclerosis, there is involvement of increased plasma levels of pro-atherogenic lipoproteins. Almost 50% of the total variance in plasma total cholesterol, HDL cholesterol and triglycerides levels have a genetic background. The plasma levels of lipoproteins are determined by multiple genes, except for a few rare Mendelian forms. For this reason, single nucleotide polymorphisms (SNPs) in genes involved in lipid and lipoprotein metabolism are considered as primary candidates for susceptibility genes to atherosclerosis [64].

While atherosclerosis is a complex (polygenic) disease, single-gene forms of atherosclerosis exist as well. These Mendelian forms of the disease have provided valuable bases for elucidating the factors contributing to atherosclerosis. Of the many monogenic diseases that elevate plasma levels of LDL, familial hypercholesterolemia (FH) was the first to be discovered. In FH, LDL plasma levels are elevated because the hepatic LDL receptors, which normally function in clearing LDL from the plasma, are impaired. More than 600 mutations have been identified in the *Ldlr* gene of patients with FH. Severe coronary atherosclerosis is observed in patients with homozygous FH. These patients suffer from myocardial infarction and die in childhood [39, 65].

Similar to *Ldlr*, *Apolipoprotein B* (*Apob*) and *Apolipoprotein E* (*Apoe*) genes are higly polymorphic. Mutations in the *Apob* gene result in reduced binding of apolipoprotein B-100 to LDL receptors, elevating plasma LDL cholesterol levels and causing familial ligand-defective apolipoprotein B-100 disorder (FDB). Familial hypobetalipoproteinemia (FHBL) is the other genetic disorder caused by mutations in *Apob* gene [66, 67]. However, the most crucial protein in the lipid transport system is Apolipoprotein E (APOE), which takes part in formation of HDL particles as well as modulating serum cholesterol levels. *Apoe* gene has three major alleles (*Apoe2-4*). The wild-type protein is encoded by *E3* allele, while *E2* is implicated with type III hyperlipoproteinemia and allele *E4* has been demonstrated to contribute to athorosclerosis pathogenesis [68–71]. In addition, ATP binding cassette transporter 1 (*Abca1*) gene mutations cause Tangier disease, which is characterized by severe HDL deficiency and premature atherosclerosis. Similar to genes and loci implicated in dyslipidemia, many candidate genes have been identified for the other risk factors, especially for Mendelian forms of high and low blood pressure [41, 72, 73].

However, in the case of atherosclerosis, multiple genes take part in disease pathogenesis. Up to date, candidate gene studies and genome-wide linkage studies to find atherogenesis-regulating quantitative trait loci have been useful tools in elucidating the genetic basis of atherosclerosis. Via whole genome sequencing, genome-wide association studies (GWAS) to identify responsible genes could be conducted. The largest GWAS for coronary artery disease was the CARDIoGRAM study that included more than 100,000 subjects of European descent. Together with smaller studies of Asian populations, nearly 30 loci were identified [74, 75]. Some of

the genes that have been found to be atherosclerosis-related by candidate gene studies, linkage analysis and association studies are given in Table 1.6 [3].

Table 1.6 : Genetic studies related to Cardiovascular Diseases [3, 76–83].

Defective gene	Related Syndrome
[(1),(2)]5-lipoxygenase	Atherosclerosis
[(2)]Phosphodiesterase 4D	Stroke, atherosclerosis
[(3)]Lymphotoxinα	Myocardial infarction
[(3)]Connexin 37	Myocardial infarction
[(3)]Plasminogen activator inhibitor 1	Myocardial infarction
[(3)]Stromelysin	Myocardial infarction
[(2)]Myocyte enhancer factor 2	Myocardial infarction
[(2)]Linkage chromosome 14	Myocardial infarction
[(1)]Toll-like receptor 4	Atherosclerosis
[(1)]Candidate gene studies	
[(2)]Linkage analysis	
[(3)]Association studies	

For identifying the polymorphisms in genes for risk factors of atherosclerosis, such as hypertension and diabetes, large-scale sequencing studies are being performed. The fact that whole genome sequencing studies for diabetes, dyslipidemia, and hypertension associated loci have yielded only limited number of loci points out to the complex nature of all these traits [6].

For investigating the genes that take role in atherosclerosis, animal models, especially mice and rats, are used widely [51]. Most of the animal models are used in studies concerning lipid metabolism. Wild-type mice are resistant to atherosclerosis, mainly because cholesterol circulates in high density lipoprotein particles, as opposed to humans, which have circulating cholesterol in low density lipoprotein. To overcome this barrier, many genetically modified mouse models have been generated [84]. One well known example is the apolipoprotein E-knockout (apoE-KO) mice, in which the apolipoprotein (apo) E gene has been deleted. These mice develop atherosclerosis spontaneously due to hypercholesterolemia, therefore represent a powerful tool for modeling and prevention of the disease [85].

Some other mouse models used for lipid metabolism studies include, LDL receptor deficient KO mice [86], Apo E3-Leiden transgenic mice [87] and Hepatic lipase-KO mice [88]. Similar to LDL receptor deficient KO mice, Watanabe Heritable

Hyperlipidemic (WHHL) rabbits naturally lack LDL receptors and thus are used in these studies [89].

1.5 Atherosclerosis and Angiogenesis

All of the cells of a human body need a well-controlled supply of oxygen. Since oxygen diffusion in tissues is limited with 100 to 200 μm, our highly developed vascular system has evolved to meet the need of this close proximity to an oxygen supply. Every metabolically active tissue in the body is only a few hundred micrometers from a blood capillary that is in charge of exchanging nutrients and metabolites with its surroundings. This well-developed system is maintained by angiogenesis; the process of new vessel formation from existing ones [90, 91]. In the embryonic stage, the first organ system to develop is the cardiovascular system [92]. In the circulatory system, the single layer of endothelial cells coating the luminal surface of vessels originate from mesoderm. Mesodermal stem cells give rise to hemangioblasts which differentiate into hematopoietic stem cells and angioblasts. In the embryonic stage, blood vessels are formed by the process of vasculogenesis via differentiation of angioblasts that come together to form primitive vessels. Vasculogenesis is directed by growth factors and morphogenes. Cell-cell and cell-extracellular matrix interactions are needed for this dynamic process [93, 94]. When new vessels start to grow during vasculogenesis, a switch is triggered in endothelial cells that enable them to change from their quiescent state into a proliferating, migrating state. This is accomplished by hypoxic conditions (insufficient oxygen concentration) that usually occur during tissue expansion. In such conditions, angiogenesis is engaged by a genetic regulatory master switch that is activated via the oxygen sensing transcriptional system. Angiogenesis continues throughout the adult life, occurring in both health and disease [95].

Any blood vessel of the body is made up of two different cell types: endothelial cells (ECs) and mural cells. In the capillaries, only the ECs make up the vascular wall, whereas in larger vessels such as the arteries, the vascular wall consists of many layers as demonstrated in Figure 1.10. The inner layer, termed as tunica intima, is formed exclusively by the ECs. The elastic connective tissue of the middle layer, named as the tunica media, consists of mural cells, particularly the smooth muscle cells. Tunica

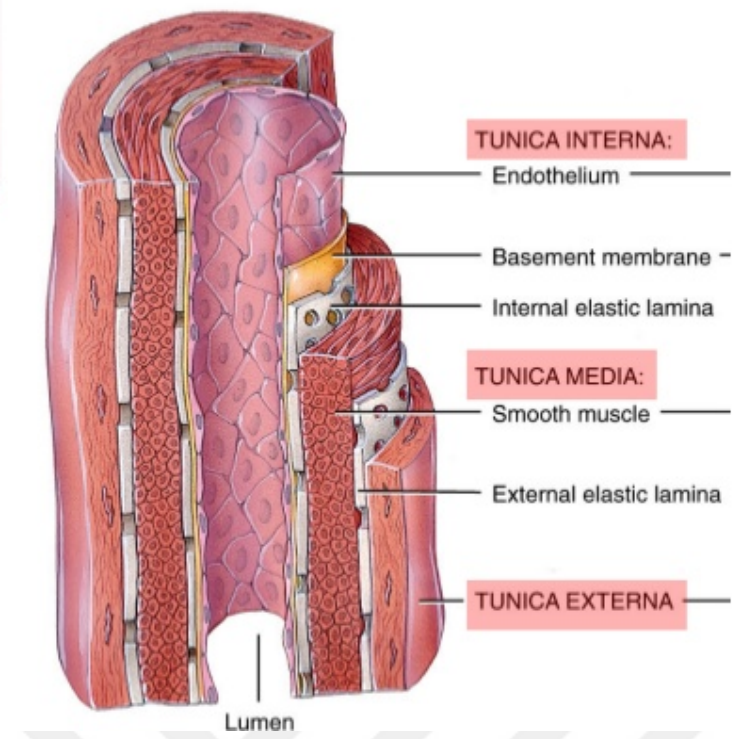


Figure 1.10 : Structure of an artery [96].

adventitia, the outer layer, is made of connective tissue mainly. While endothelial cells form the inner vessel wall, the mural cells, such as vascular smooth muscle cells and pericytes, take part in coating the endothelial cell tube. Vascular smooth muscle cells form multiple layers around arteries and veins, while pericytes interact with the smaller diameter blood vessels such as arterioles and capillaries, sharing their basal membrane with the endothelium. Endothelial cells are the most important type of vascular cells that take part in angiogenesis. The two main types of angiogenesis taking place in tissues include sprouting angiogenesis and intussusceptive angiogenesis as demonstrated in Figure 1.11 [95, 97].

As the name implies, the characteristic structures of sprouting angiogenesis are sprouts made up of endothelial cells growing toward angiogenic stimuli. Sprouting angiogenesis is capable of adding blood vessels to parts of tissues where there were no blood vessels before. However, in the case of intussusceptive angiogenesis, elements of interstitial tissues invade existing vessels and form transvascular tissue pillars that expand [91].

Sprouting angiogenesis usually begins in poorly perfused tissues. Parenchymal cells such as myocytes, hepatocytes, neurons and astrocytes secrete a proangiogenic growth

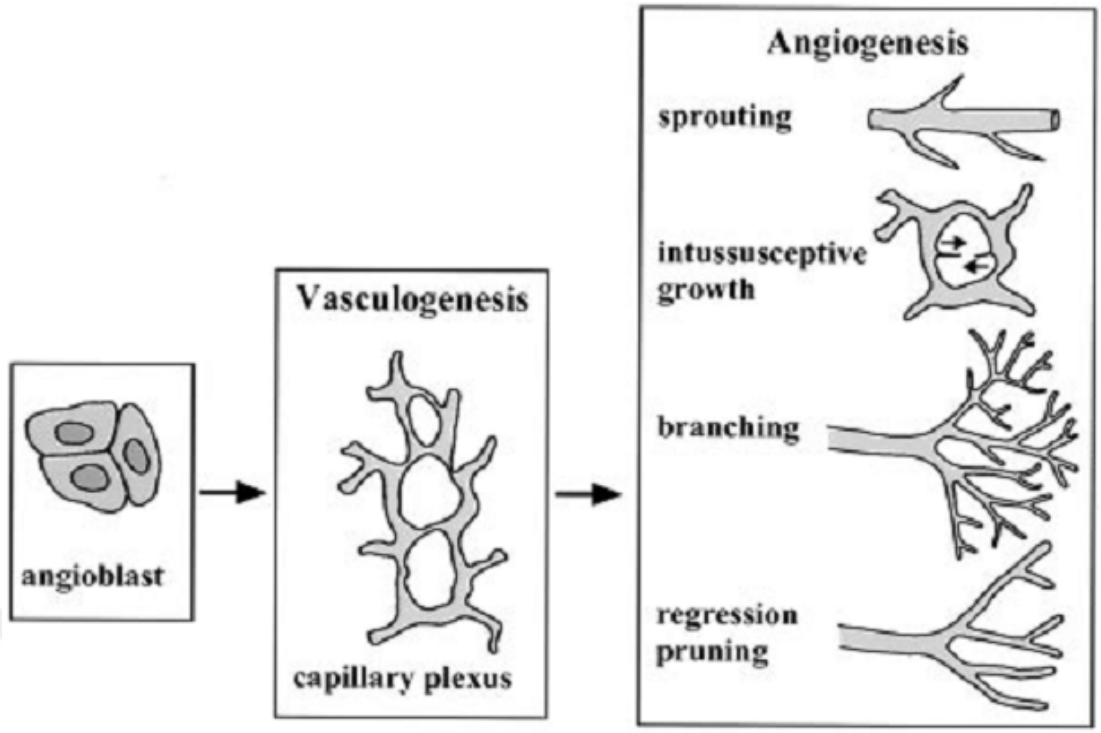


Figure 1.11 : Formation of blood vessels [98].

factor called vascular endothelial growth factor (VEGF-A) to respond to the hypoxic environment. As a result, a vessel sprout is directed through the extracellular matrix (ECM) towards the VEGF-A by an endothelial tip cell [99–101]. The capillary sprout is elongated by the proliferation of endothelial stalk cells that follow the tip cell. A lumen is formed within a series of stalk cells as vacuoles develop and fuse inside them. When more than two tip cells of capillary sprouts converge at the VEGF-A source, they fuse together to form a continuous lumen through which oxygenated blood flows. VEGF-A levels return to near normal as the local tissues are oxygenated [91, 102, 103].

1.6 Signalling Pathways In Angiogenesis

Angiogenesis is tightly controlled by a system that employs both the pro-angiogenic and anti-angiogenic factors. The angiogenic process is physiologically balanced between the stimulatory and inhibitory signals. In adults, angiogenesis takes place during wound healing, organ regeneration, ovulation, menstruation, and during the formation of the placenta in females. The dysregulation of angiogenesis has been linked to several pathological processes such as tumor growth, rheumatoid arthritis, diabetic retinopathy, and psoriasis. For example, aberrant angiogenesis has been

shown to promote cancer and neovascular age-related macular degeneration, while ischemic diseases of the heart, brain and limbs are known to be caused by insufficient angiogenesis [90, 104].

The role of angiogenesis in atherosclerosis has been the subject of debate and remains as an unresolved issue, therefore research on elucidating the role of angiogenesis and signalling pathways taking part in angiogenesis is required.

1.6.1 Vascular endothelial growth factor (VEGF) pathway

Vascular endothelial growth factor (VEGF) signaling pathway is very important for regulating sprouting angiogenesis in physiological and pathological conditions. Endothelial differentiation, migration, proliferation, survival, and permeability are controlled via this signalling pathway. The superfamily of vascular endothelial growth factor (VEGF) includes VEGF ligands, receptors and the molecules that interact with them. The VEGF ligands and receptors are attractive targets for therapeutic approaches since excessive or insufficient angiogenesis is the crucial element in many diseases. Therefore, understanding the mechanism of action of this family of ligands that act through three receptor tyrosine kinases (RTKs) is important.

VEGFs are members of Vascular endothelial growth factor (VEGF) / Platelet-derived growth factor (PDGF) group of the cystine-knot superfamily of hormones and extracellular signaling molecules. All of the members of this superfamily contain a VEGH homology domain that is made up of eight conserved cysteine residues forming the typical cystine-knot structure. All VEGF family members modulate their abilities of binding to VEGF receptors as well as co-receptors such as neuropilins by increasing their complexity via alternative splicing and processing [90, 105, 106]. The vascular endothelial growth factor family has six secreted dimeric glycoprotein members in mammals; VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). All of these members of the VEGF family bind to the three VEGF tyrosine kinase receptors (VEGFR1, VEGFR2, VEGFR3) with different affinities [107].

1.6.1.1 VEGFR2 signalling

Just like many other signalling proteins, VEGF family members act through by binding to receptor tyrosine kinases for achieving their biological functions. Upon ligand binding, the receptor chains of RTKs dimerize, causing the kinase domains of the two receptor chains to be brought together. When they are in close proximity, they become activated and phosphorylate each other on multiple tyrosine residues in a process known as trans-autophosphorylation. The phosphorylation of tyrosine residues in the kinase domain inreases the kinase activity, while the phosphorylation of tyrosines outside the kinase domain result in docking sites for intracellular signalling proteins to bind. Some of the important intracellular signalling proteins that function in relaying the signal forward include phospholipase C-γ (PLC-γ) and phosphoinositide 3-kinase (PI3K). These intracellular signalling proteins are able to bind to phosphotyrosines due to their highly conserved phosphotyrosine-binding domains such as Src homology region (SH2) or Phosphotyrosine-binding (PTB) [108].

VEGF family members bind to three VEGF receptors; (VEGFR)-1, VEGFR-2, and VEGFR-3. VEGFR1 that binds to VEGF, VEGF-B, and PlGF plays a role in vasculogenesis, and is suggested to facilitate hematopoiesis and recruit endothelial cell progenitors to tumor blood vessels from bone marrow. It has not been completely elucidated through which mechanisms VEGFR1 functions. In addition, the role of VEGFR1 in adult angiogenesis is not well-known [109].

VEGF-C and -D bind to VEGFR3, which is associated to formation of the lymphatic vessels and tumor development. The importance of VEGFR3 mediated activation of lymphatic endothelial cells in tumor metastasis has been demonstrated. Along with the three VEGF receptors, the co-receptors Neuropilin-1 (Nrp-1) and Neuropilin-2 (Nrp-2) bind to the heparin binding isoforms of VEGF and enhance the binding of VEGF to VEGFR1, and VEGFR2 [111].

VEGFR2 is the main receptor that plays a role in VEGF-driven angiogenesis. VEGF binding to VEGFR2 results in activation of downstream effector molecules such as PLC-γ, protein kinase C, Rat Sarcoma (RAS), Rapidly Accelerated Fibrosarcoma (RAF), the Mitogen Activated Protein Kinase (MAPK)/ Extracellular

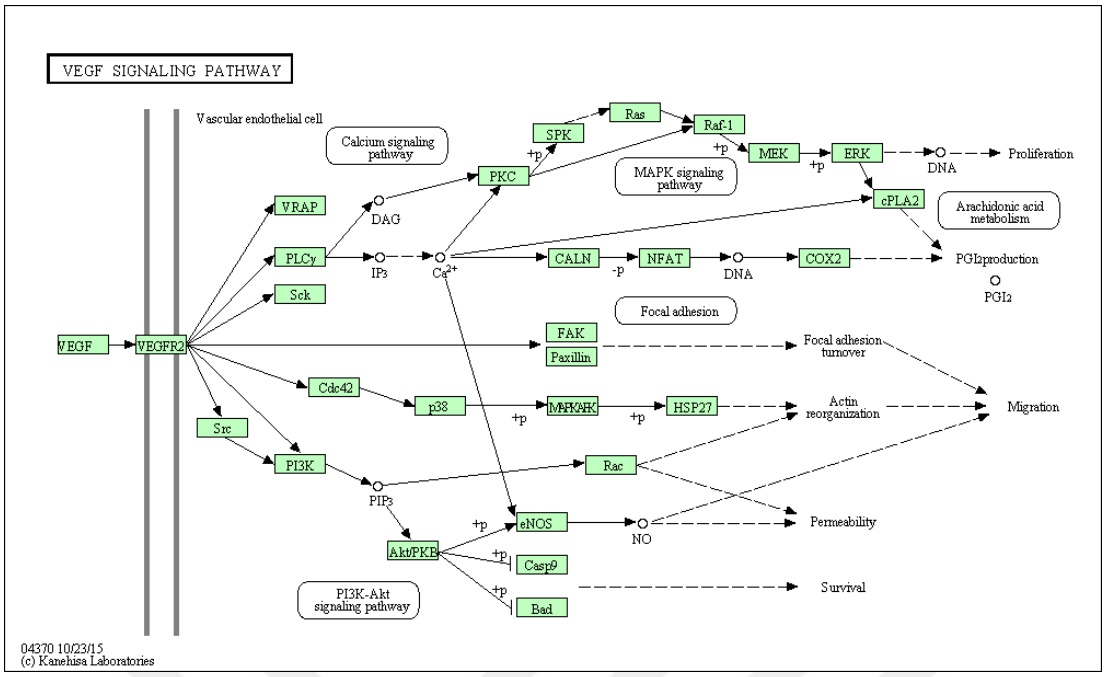


Figure 1.12 : VEGFR2 signalling [110].

Signal-Regulated Kinase (ERK) signaling cascades, the PI3K and Focal Adhesion Kinase (FAK) pathways. As is demonstrated in Figure 1.12, activation of MAPK, PI3K/Protein Kinase B (AKT) signalling pathways lead to angiogenesis, endothelial cell proliferation, migration, and survival [112–115].

1.6.1.2 VEGF pathway's negative regulators

Angiogenesis depends on the ability of endothelial cells to correctly process the signals from growth factors that bind to their RTKs on the cell surface, activating many intracellular signaling pathways whose outputs are cell growth, migration, and morphogenesis. Endothelial cells can modulate their response to these growth factors by also inhibiting the downstream signalling cascades. Elucidating the control mechanisms and regulators of these signalling cascades is crucial to understanding physiological and pathological angiogenesis.

Sprouty family of proteins include Sprouty proteins and Sprouty-related proteins, both of which take part in organogenesis, especially during the branching of endothelial tubes. They are highly conserved modulators of MAPK/ERK pathway and negatively regulate growth factor signaling [116, 117]. A member of the family, Sprouty-related Ena/VASP homology 1 (EVH1) domain-containing protein 1 (SPRED1), encoded by *Spred1* gene, works as homodimer or as a heterodimer together with SPRED2 and

regulates the activation of the MAP kinase signalling [118]. It was discovered that in response to growth factors, SPRED1 was phosphorylated and that through the formation of RAS-SPRED1 complex, it suppressed the phosphorylation, therefore activation of RAF1, an upstream kinase of the MAPK/ERK pathway. Via this mechanism, SPRED1 inhibits MAP kinase activation and represses the VEGF signalling [119].

Phosphoinositide 3-kinase (PI3K) family members are heterodimeric lipid kinase enzymes, with their distinct regulatory (p85) and catalytic (p110) subunits encoded by different genes. They take part in phosphorylating the D3 hydroxyl group of membrane phosphatidylinositols when stimulated by receptor tyrosine kinase and G-protein-coupled receptor or upon RAS activation. PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which binds to the pleckstrin-homology (PH) domains of AKT and phosphoinositide-dependent kinase 1 (PDK1). When in close proximity, PDK1 phosphorylates AKT, causing phosphorylation of many proteins affecting cell growth and survival. Just like MAPK/ERK, PI3K/AKT is a crucial signalling cascade for cell fate [120–122]. PI3K regulatory subunit p85 is made up of the α and β proteins [123]. Similar to SPRED1, phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2 or p85 β) functions as a negative regulator of VEGF signalling by inhibiting PI3K cascade [124].

1.6.2 Notch signalling and angiogenesis

Along with VEGFR2 signaling, there are many other factors that take part in regulation of angiogenesis. Notch signalling, an evolutionarily conserved pathway with crucial roles in the determination of cell fate in all metazoans, is needed for normal embryonic development, the regulation of tissue homeostasis, and adult stem cell maintenance. There are four Notch receptors (Notch 1–4) and five ligands (Delta-like-1, -3, -4 and Jagged-1 -2) in mammals. Both the receptors and the ligands are located on the cell surface. Notch receptors are synthesized as single-chain precursors and cleaved into extracellular and transmembrane subunits in the Golgi apparatus. Held together on cell membrane by non-covalent bonds, these subunits are activated when they interact with ligands present on the cell membrane of neighbouring cells. A series

of proteolytic cleavages of Notch take place upon ligand binding, starting with the removal of extracellular subunit by a disintegrin and metalloprotease (ADAM) and followed by an intramembranous cleavage by γ-secretase. Via cleavage by γ-secretase, active form of Notch (NIC) is released, which then translocates into the nucleus to modulate transcription [125, 126].

The role of Notch signaling in sprouting angiogenesis is to determine which cells should become tip and which should become stalk cells. Upon VEGFA stimulation, Notch ligand Delta-like 4 (Dlt4) is upregulated, causing binding of DLL4 to the Notch1 receptors on the neighboring endothelial cells. Notch1 receptor activation causes downregulation of VEGFR2 and as a consequence, VEGFR2 signaling is inhibited in these cells. Activation of Notch during vascular injury suggests that this pathway plays important role in limiting damages to the vascular structure. Via regulating the number of sprouts, DLT4/NOTCH1-mediated signaling modulates VEGF-A-driven angiogenesis [125, 127].

1.7 The contribution of angiogenic signalling pathways to pathogenesis of atherosclerosis

The argument that intraplaque neoangiogenesis contributes to atherosclerotic lesion development is supported by the fact that once the thickness of the vessel wall exceeds a critical depth due to neointimal growth, the supply of oxygen and other nutrients to the media and neointima will be resticted. When the interior of the artery becomes hypoxic, hypoxia-inducible transcription factors (HIFs) that induce expression of VEGF and other angiogenic regulators are stimulated. The stimulation of VEGF results in angiogenesis, promoting plaque growth. Evidence demonstrating the presence of VEGF-A in human atherosclerotic lesions showed a rise in VEGF-A during disease progression. VEGFR2 signalling pathway is implicated in triggering neoangiogenesis in the intima, thereby in promoting lesion progression [16, 127].

Notch is crucial for cardiovascular system development and maintenance, under normal and pathological conditions such as ischaemia, neoangiogenesis. How Notch contributes to molecular mechanisms underlying atherosclerosis is not yet elucidated. Atherosclerotic lesions occur in arterial branch points, where there is disturbed blood flow and low shear stress which cause endothelial dysfunction, a hallmark

of atherosclerosis progression. Studies focused on modulation of Notch signalling by shear stress have demonstrated a decrease in expression of Notch signalling components in atheroprone regions of mouse aortic arch. These results suggest that in areas with disturbed blood flow, Notch signalling is affected, therefore there areas are predisposed to atherosclerosis. The extent of angiogenesis and the number of sprouting is determined by a balance between the Notch signalling and growth factors [128].

New blood vessel formation in atherosclerotic plaques is puzzling and no consensus has been reached on whether angiogenesis either contributes to the pathogenesis of atherosclerosis or is a treatment approach in CVDs [15]. Despite the fact that an association between intraplaque angiogenesis and atherosclerosis progression exists, the use of VEGF to stimulate blood vessel formation in an approach called therapeutic angiogenesis has been a valuable tool for the treatment of ischemic heart and limb. On the other hand, to stabilize the plaques and to avoid intraplaque hemorrage, eliminating neoangiogenesis within atherosclerotic lesions via molecular therapies is proposed [16, 17].

1.8 MicroRNAs

When the nematode *Caenorhabditis elegans* (*C.elegans*) *lin-4* gene that controls the timing of larval development was demonstrated to be a non-coding gene, microRNA genes were discovered for the first time as genes that produce a pair of small RNAs [129]. Following the discovery of *lin-4* RNA, the 22 nucleotide long let-7 RNA encoded by *let-7* gene was demonstrated as a small RNA that plays a role in larval development of *C.elegans* [130]. Since then, a vast number of genes encoding these small temporal RNAs were revealed in worm, fish, fly and mammalian genomes. The small RNA *lin-4* is accepted as the founding member of these small RNAs now called microRNAs [131, 132].

MicroRNAs, miRNAs in short, are small, single-stranded RNA molecules. They are usually 18 to 22 nucleotides in length and they are non-coding RNAs with regulatory functions. They take roles in many important biological functions and cellular events. Some of the crucial cellular processes that miRNAs take part in regulation include,

cell proliferation, metabolism, apoptosis, senescence, tumorigenesis, angiogenesis, differentiation and development [40].

1.8.1 MicroRNA biogenesis

Almost a quarter of the genes encoding miRNAs are located in the introns of pre-mRNAs and are co-expressed with their host genes, while some of them are clustered in the genome. MicroRNA expression is known to be specific to developmental stages and it is also tissue-specific. In *Homo sapiens*, nematodes and *Drosophila*, miRNAs are thought to account for nearly 1% of all genes and conservation of miRNA genes is observed among closely related species.

In Eukaryotes, miRNAs biogenesis begins when RNA polymerase II transcribes a primary product that is several thousand nucleotides long which contains stem–loop structures (pri-miRNA). Following transcription, cleavage of this pri-miRNA occurs in nucleus and pre-miRNA with a small hairpin structure is liberated. This step is performed by the nuclear RNAse III-endonuclease Drosha.

After its cleavage in nucleus, pre-miRNA is transported to the cytoplasm via a nucleo/cytoplasmic cargo transporter, Exportin-5, as demonstrated in Figure 1.13. Another RNAse III-endonuclease located in cytosol, Dicer, cleaves the pre-miRNA hairpin structure into a small double-stranded RNA duplex that is made of a mature RNA stand and its complementary strand. These two strands are also termed as miR-5p and miR-3p [133, 134].

Upon cleavage, the double stranded duplex generated by Dicer is loaded into an AGO protein and RNA-induced silencing complex (RISC) is formed. AGO proteins are part of the Argaunate family proteins. As the integral parts of the RISC complex, they bind to small non-coding RNAs and play role in RNA silencing as effector molecules. When the AGO protein associates with RNA duplex, the pre-RISC complex is formed. In pre-RISC, the complementary strand of the duplex is degraded and mature RISC is generated. [135].

The RISC complex chooses the RNA strand with the lowest thermodynamic stability at its 5' end as the guide strand, while the complementary strand is often degraded. The guide strand leads RISC to its target [40, 136].

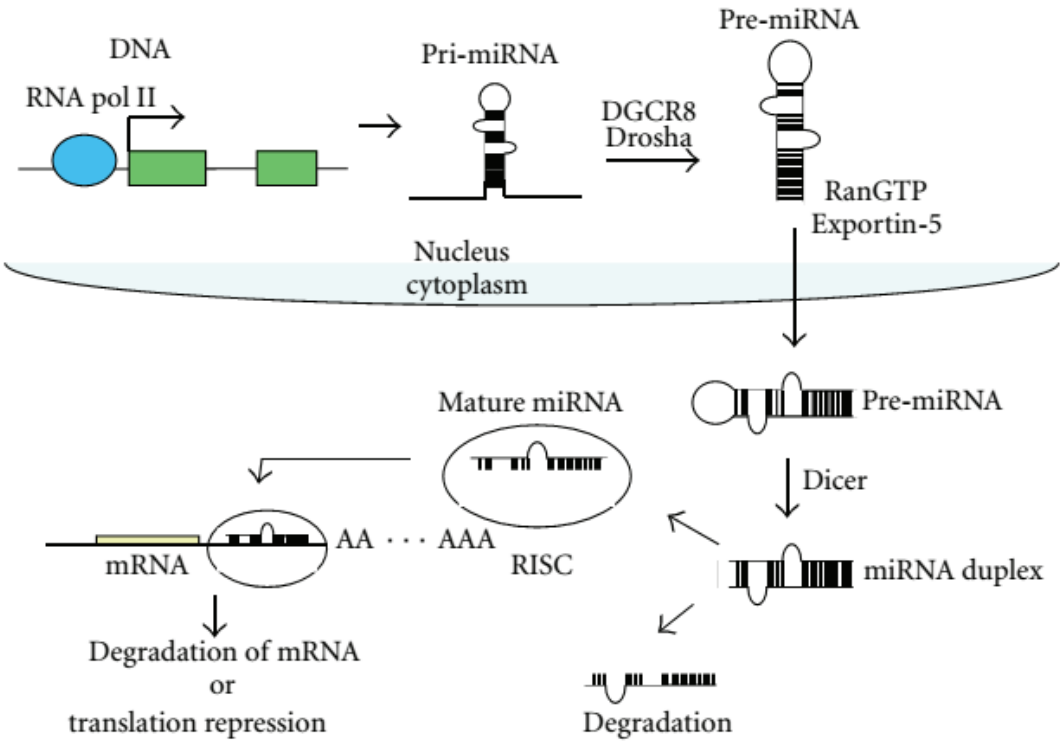


Figure 1.13 : MicroRNA biogenesis [40].

1.8.2 Regulation of gene expression by microRNAs

RNA interference is gene-silencing at the post-transcriptional level by small RNAs, such as microRNAs, small interfering RNAs (siRNA) and short hairpin RNAs (shRNA). When a miRNA is incorporated in RISC complex, it binds to the 3' untranslated (UTR) region of the targeted messenger RNA (mRNA) to accomplish gene silencing via different mechanisms. The mRNA target recognition is mediated via a complementation between the 2nd–8th nucleotides of the miRNA, known as the seed sequence, and the target mRNA [137, 138]. Gene silencing by microRNAs through RISC is accomplished in two main ways: mRNA decay and translational repression as demonstrated in Figure 1.14 [139–141].

Nearly %50 of human transcriptome is known to be under regulation of miRNAs. Many crucial cellular processes in which miRNAs exert their regulatory function have been elucidated in the last ten years. MiRNAs have been linked to several pathological conditions, some of which include, cancer, cardiovascular disorders, neurological pathologies, autoimmune diseases, metabolic diseases, obesity, hepatological disorders

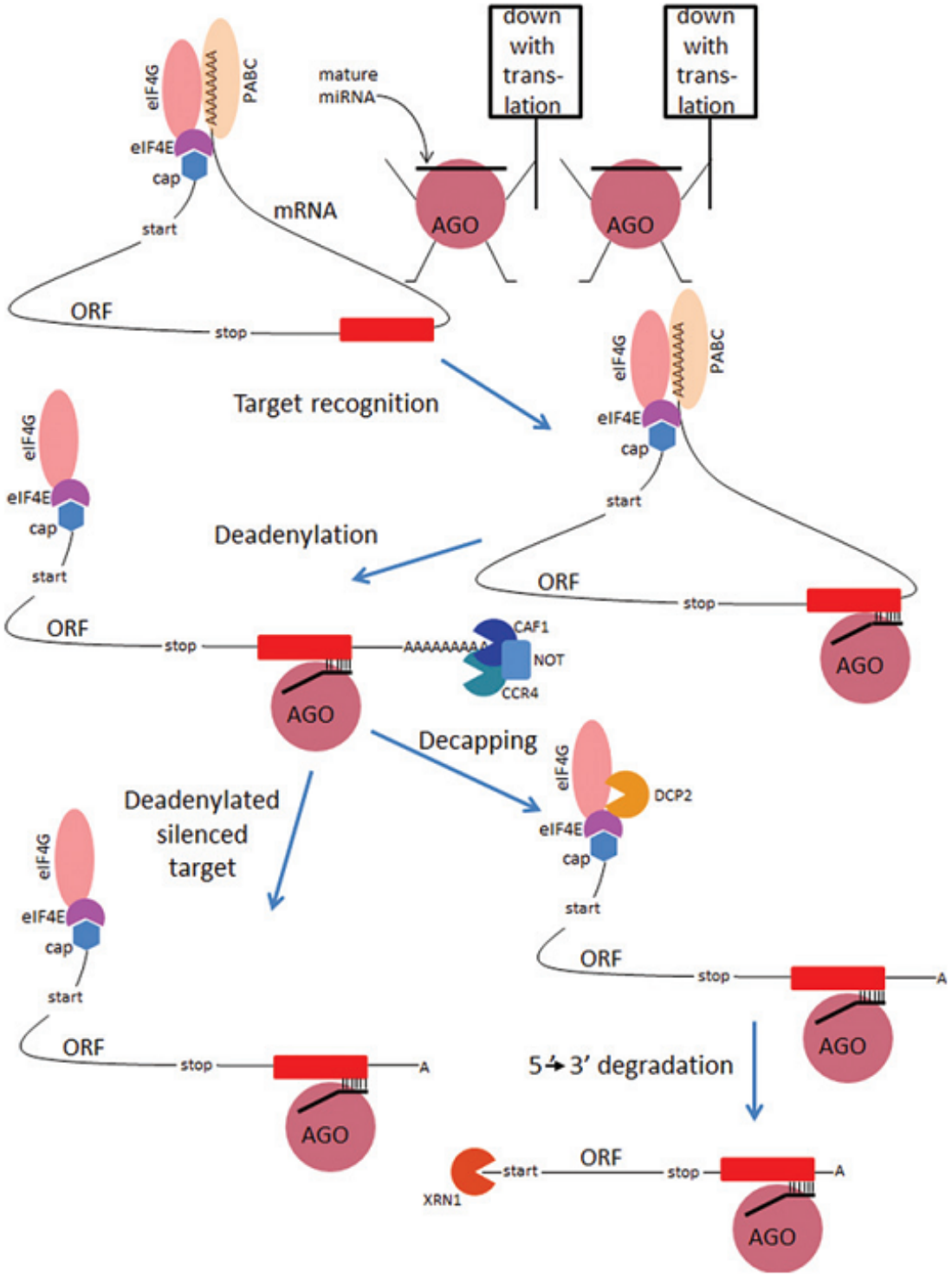


Figure 1.14 : Mechanism of gene silencing by miRNAs [139].

and viral diseases. For these reasons, investigating and elucidating the roles of miRNAs in the regulation of such conditions is one of the rising topics of research.

Researchers working with miRNAs make use of online resources and algorithms identifying miRNAs, predicting their targets and investigating their functions. Of these

databases, servers and algorithms, MiRBase, TargetScan, PicTar and Miranda are the most widely used ones [142–146].

1.8.3 MicroRNAs in cardiovascular diseases and angiogenesis

MicroRNAs are important regulators of biological processes. They are linked to atherosclerosis, angiogenesis and many cardiovascular pathological conditions, such as ischaemic heart disease, hypertension and arrhythmias [18, 147]. They have been suggested to regulate atherosclerotic process by taking part in its stages, including endothelium dysfunction, cellular adhesion, plaque development, and plaque rupture [147, 148].

Dicer and Drosha deletion studies have provided insights to the role of miRNAs in angiogenesis and endothelial function. The microRNAs that take part in regulation of angiogenesis are named as angiomiRs. Defects in vascular remodeling during development have been observed in Dicer hypomorphic mouse lines. Similary, the result of knockdown of Dicer or Drosha in human endothelial cells was found to decrease angiogenesis [149].

Extracellular signals, such as growth factors or hypoxia, turn the related signal transduction pathways on and in return, specific effectors such as, mitogen-activated protein kinase, or Akt are activated or gene expression is induced, resulting in an angiogenic response. MiRNA biogenesis, expression or degradation can be modulated through the the activation of the signal transduction pathways. Figure 1.15 shows the effect of stimuli such as VEGF, hypoxia, or mitogens on promoting angiogenesis and participation of miRNAs in these processes [150].

MiRNA expression is known to be specific to tissues and different cell types. Studies aiming to profile miRNA expression patterns in endothelial cells have demonstrated that miRNAs, including let-7b, miR-16, miR-21, miR-23a, miR-29, miR-100, miR-221, miR-222 and miR 126 are expressed in endothelial cells [147]. Some of the miRNAs that are known to play roles in angiogenesis include, let-7f, miR-17$\sim$92 cluster, miR-126, miR-21, miR-27b, miR-31, miR-210, miR-221/222, miR-296 and miR-378. However, the only miRNA that was found to be expressed exclusively both in endothelial cells and endothelial progenitor cells is miRNA-126 [149].

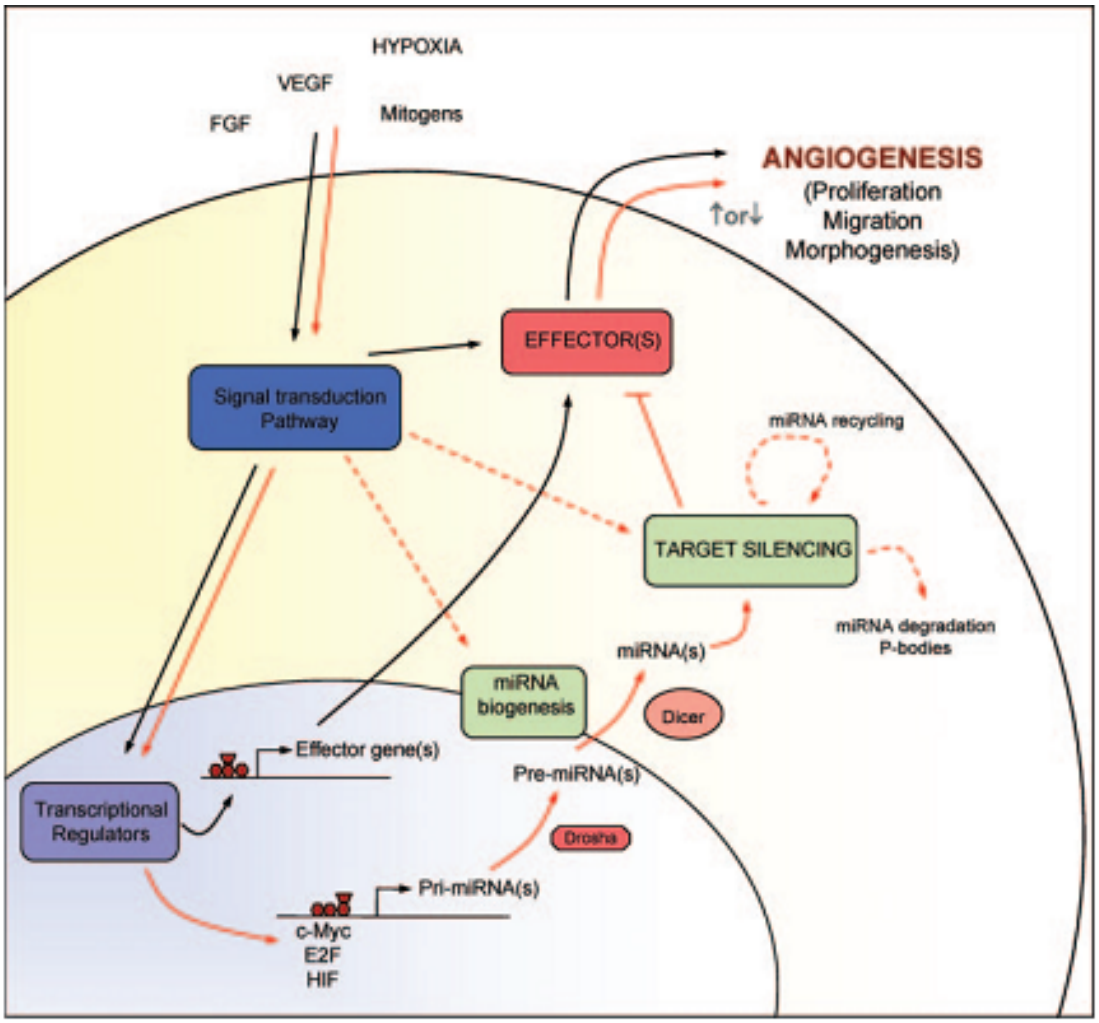


Figure 1.15 : Regulation of angiogenesis via miRNAs [150].

Several miRNAs have been implicated in the regulation of cellular processes in atherosclerosis. To date, the miR 126, miR 145 and miR 155 triad have been studied. MiR-145 was found to be down-regulated in smooth muscle cells. This down-regulation was suggested to contribute to atherosclerotic lesion formation. On the other hand, in macrophages, the high expression levels of miR-155 had a relieving effect on advanced atherosclerosis [151].

1.8.4 MicroRNAs 126-3p, -5p and their host gene *Egfl7*

MiR 126-3p and miR 126-5p are a set of crucial miRNAs for endothelial cells. Both of them are conserved among species and they originate from the precursor molecule pre-miR-126. Unlike many miRNAs, passenger strand of miR 126-3p, miR 126-5p (also shown as miR-126*) is not degraded [152]. Both of the MiRNAs are

encoded by the miR-126 gene, which resides within the intron of *Egfl7* gene located at chromosome 9q34.3 [153].

MiR-126 is up-regulated in embryonic stem cell- derived endothelial progenitor cells during embriyonic developmental stages. In the embriyogenic stage, for generation of initial vascular plexus, VEGF is needed, and VEGF-driven angiogenesis is modulated by miR-126. The VEGF signaling suppressor molecules, Sprouty-related protein SPRED1 and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) are targeted by miR-126 [154]. The inhibition of *Pik3r2* results in an up-regulation in angiopoietin-1, which functions as a proangiogenic signalling factor with a role in stabilizing and maturation processes of the vessels, which is normally down-regulated by PI3K/Akt signaling [155].

MiR 126-3p is highly expressed in vascularized tissues such as heart, liver and lungs. Expression studies demonstrated its specific expression in the endothelial cell lineage, hematopoietic progenitor cells,and endothelial cell lines [152]. Deletion of MiR-126 in mice (without altering Egfl7 expression) results in major defects in blood vessel development, such as delays in angiogenesis, haemorrhage and early embryonic lethality [156, 157]. The observation of similar defects when studies were performed with loss of VEGF signalling in mice and zebrafish suggests that miR-126 modulates VEGF signalling. In endothelial cells, decreased phosphorylation of AKT and ERK1/2 in response to VEGF treatment was observed in knockdown of miR-126. MiR-126 targets PIK3R2 and SPRED1, the negative regulators of VEGF pathway [154, 156–158].

In adults, the roles of miR-126 are quite different [153]. MiR-126 induces angiogenesis, as well as vascular tissue remodeling in response to vascular injury. It has been demonstrated by Voellenkle *et al.*(2012), under hypoxic conditions, the levels of miR-126 increase [159].

Several studies have been conducted to assign the specific roles to the endothelial specific miR 126-3p. Although the results of such studies yielded to categorize miR 126-3p an an angiomiR, specific information on the expression of its passanger strand, miR 126-5p is scarce, therefore requires investigation.

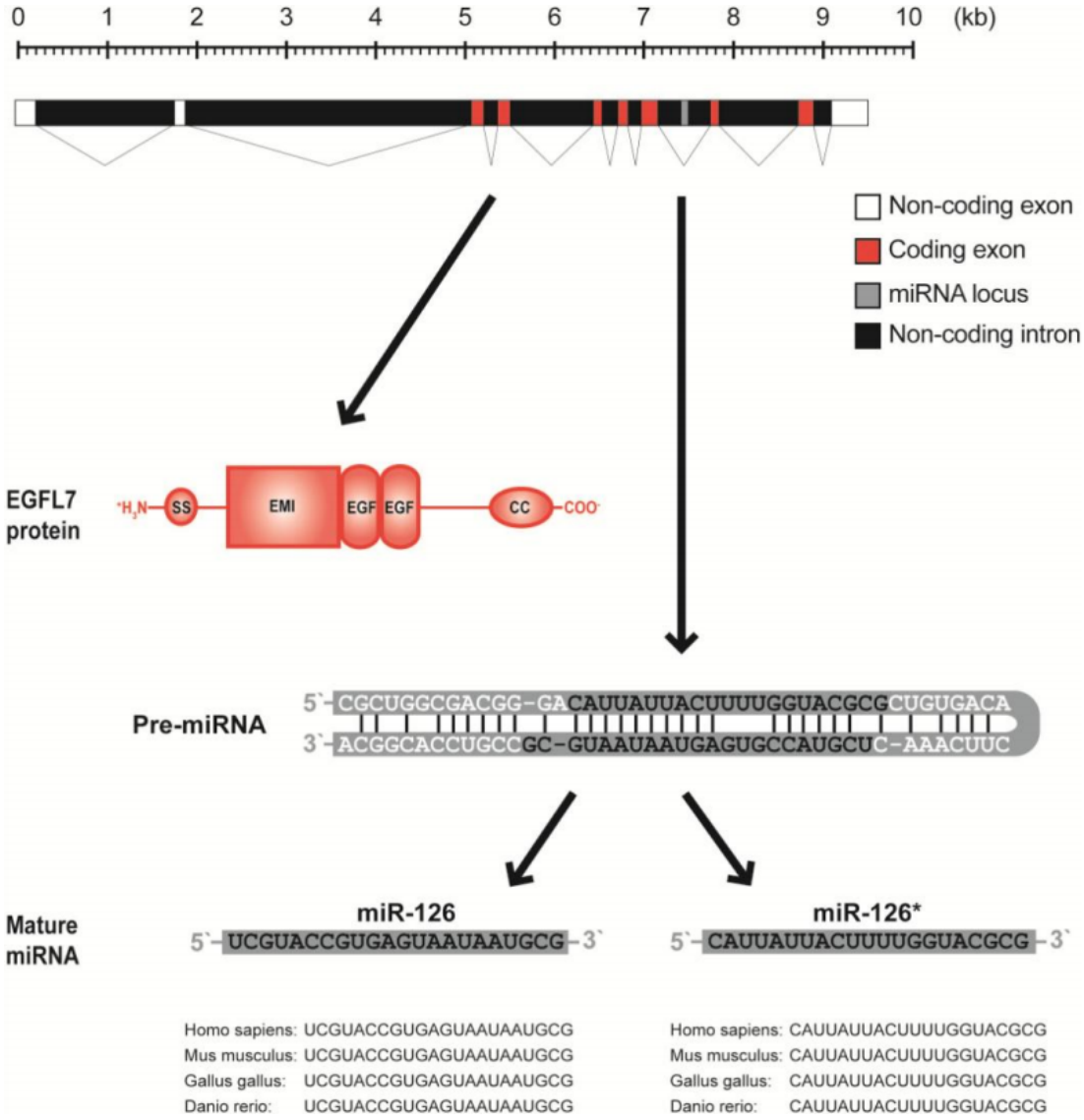


Figure 1.16 : MiR-126, miR 126* and putative protein sequence of EGFL7 [152].

The host gene of miR 126, epidermal growth factor (EGF)-like domain 7 (*Egfl7*) is located on chromosome 9 in humans. Along with the two miRNAs mentioned before, it encodes EGFL7 that is highly conserved in vertebrates. The 41 kDA angiogenic factor EGFL7 is specific to endothelial cells. It acts solely on endothelial cells, binds to components of the extracellular matrix and regulates blood vessel development via Notch signalling. The protein sequence of EGFL7 is composed of a putative amino-terminal signal peptide domain, an Emilin-like domain (EMI) and 2 centrally located EGF-like domains; the centrally located one for Notch receptor-ligand interactions and the distal one for Ca^{2+}-binding as can be seen in Figure 1.16. These protein motifs are often found in secreted and extracellular matrix–bound proteins. EGFL7 expression is highest during embryogenic development while after

birth expression is down-regulated in the vascular system. In adults, significant protein levels are maintained only in vascularized tissues such as the lung, heart, kidney, spleen and uterus. In cases of physiological and pathological angiogenesis, increase in expression levels is observed [160–162].

The role of *Egfl7* in sprouting angionesis has been demonstrated by studies conducted on *Egfl7* knock-out mice, in which endothelial cells without EGFL7 had impaired migration and created clumps and over-sized sprouts instead of proper vessel sprouts. These findings suggested that EGFL7 created an environment where cells can be properly positioned to construct new vessels. A study conducted by Schmidt *et al.* (2014) has provided a link between Notch signaling and EGFL7 by showing the binding of EGFL7 to Notch receptors. EGFL7 is suggested to compete with NOTCH ligands and inhibit NOTCH receptor activation, therefore affect angiogenesis [160].

1.9 Hypothesis

Atherosclerosis related cardiovascular diseases (CVDs) are considered as the number one cause of death in developed countries. Atherosclerosis begins due to endothelial dysfunction. Subsequently, leukocyte infiltration and foam cell formation as a consequence of a deposition of oxidized low-density lipoprotein particles is observed. As the disease progresses, apoptosis, necrotic core formation, migration and proliferation of vascular smooth muscle cells together with intraplaque angiogenesis cause lumenal narrowing. When the fibrous cap structure in the core of the plaques ruptures, thrombosis causing stroke or myocardial infarction, takes place [163, 164]. Several studies have linked intraplaque angiogenesis with the formation of vulnerable plaques, which are prone to rupture [14, 15].

Egfl7 and miR-126 are considered as important regulator molecules of angiogenesis. During embryonic development, miR-126 induces angiogenic signaling, promotes the differentiation of embryonic stem cells to endothelial cells and endothelial progenitors. On the other hand, as reviewed by Chistiakov *et al.*(2016), miR-126 maintains the vascular homeostasis via inhibition of angiogenesis, just as its host gene *Egfl7* is down-regulated in adults to help in maintenance of the quiescent endothelial phenotype in terms of angiogenesis. It is suggested that in response to vascular injury or under

hypoxic conditions, miR-126 is up-regulated for new vessel formation, as well as to rectruit endothelial progenitor cells that function in vascular repair [153].

Furthermore, the host gene of miR 126, *Egfl7* too was found to be up-regulated in pathological angiogenesis [160–162]. In addition, in response to vascular injury in rats, *Egfl7* was up-regulated as demonstrated by Campagnolo *et al.*(2005) [165]

In light of the literature reviewed, it is expected to observe *Egfl7* and miR-126 up-regulation in carotid artery atherosclerotic plaques, due to the vascular injury caused by progressive atherosclerosis. In contrast, the expression levels of negative regulators of VEGF pathways, *Spred1* and *Pik3r2*, are expected to be down-regulated, since they are targets of miR 126-3p, as suggested in Figure 1.17.

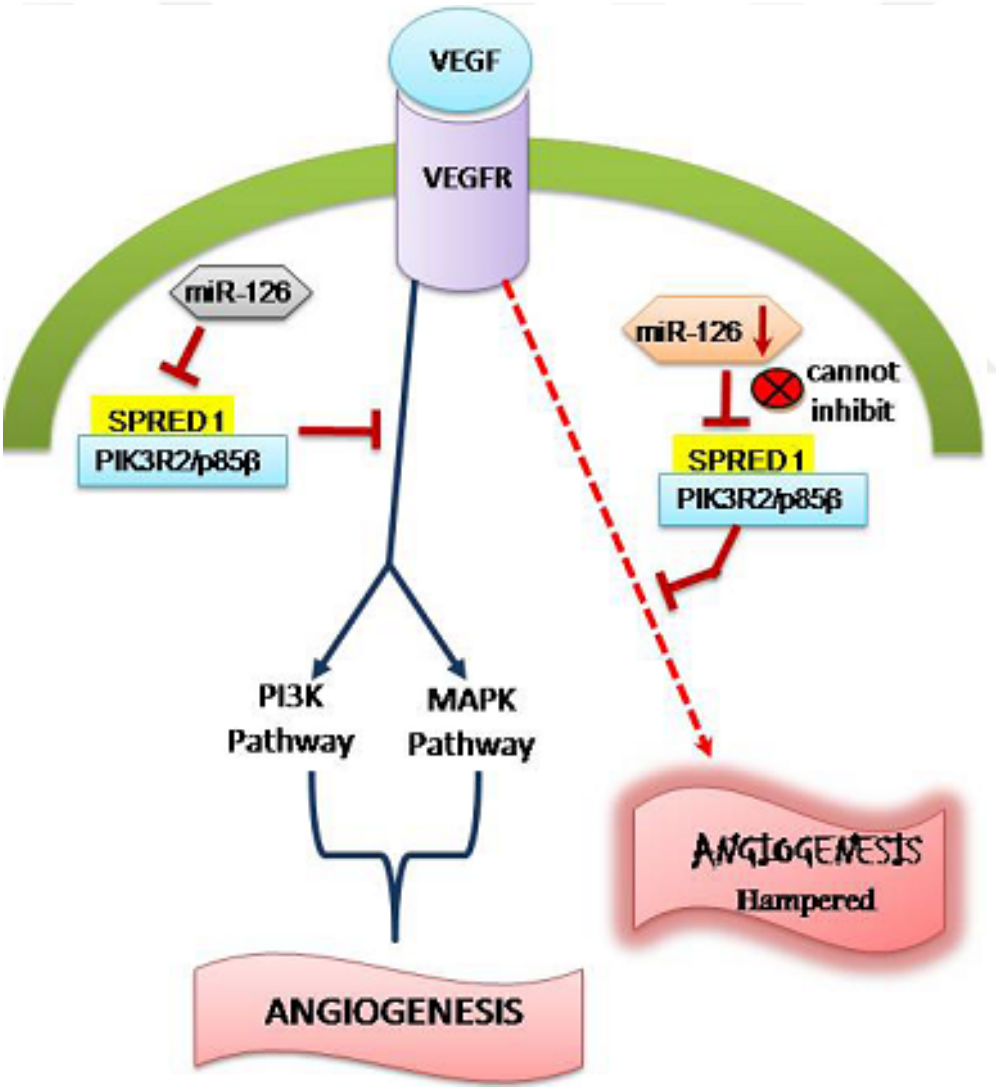


Figure 1.17 : Role of miR-126-3p, SPRED1 and PIK3R2 in angiogenesis [166].

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Subjects and specimens

Carotid artery atherosclerotic plaque samples were obtained from patients (n=14) undergoing carotid endarterectomy for stenosis greater than 70% or stenosis ranging from 50% to 70% associated to clinical symptoms according to AHA guidelines. The patients were enrolled at Istanbul Medicana International Hospital. Symptomatic patients were defined subjects with a history of transient attacks, stroke or amourosis fugax. The 2-3 cm of plaque area and the tissue from the region immediately adjacent to plaque were collected from patients. The region immediately adjacent to plaque was used as control tissue in the experiments. All samples were transferred to cryogenic tubes immediately after they were obtained and placed in liquid nitrogen. Samples were transferred to Istanbul University Experimental Medicine Research Institute (DETAE) Molecular Medicine Department and stored in -80°C for the subsequent experiments. This thesis study was performed according to the current version of Helsinki Declaration. Informed consent was obtained from each patient. The experiments were reviewed and approved by the Committee of Experimental Medicine Research Institute at Istanbul University.

2.1.2 Chemicals and kits

All of the purchased chemicals and commercially available kits used in this thesis study are given in Appendix A with their suppliers. App B lists the ID numbers of the commercially available TaqMan®miRNA and gene expression assays.

2.1.3 Laboratory equipment

The equipment that is used to perform the experimental procedures of this thesis study is listed in App C.

2.2 Methods

2.2.1 MicroRNA target prediction

The result of thorough literature review pointed out the role of microRNA 126 in angiogenesis. The miRBase database was searched for microRNA 126 [167]. Together with data obtained from the literature review, microRNA 126-3p and its passanger strand, microRNA 126-5p were chosen as the microRNAs to be studied.

Next, the mRNA targets of miR 126-3p were investigated using the TargetScan [123] and miRTarBase [168] miRNA target prediction databases.

Finally, the mRNA targets were searched in National Center for Biotechnology Information (NCBI) Gene Database [169]. Supported by the information obtained from literature review, the genes that were involved in angiogenesis as well as VEGF signalling pathway were chosen to be studied as mRNA targets of the miR 126-3p.

2.2.2 RNA isolation from samples

For isolation of total RNA and microRNAs from plaque and control samples obtained from patients, mirVana^{TM} miRNA Isolation Kit (Ambion, Austin, TX, USA) was used. For purification of total RNA or small RNAs, this kit combines methods of organic extraction with acid:phenol:chloroform and solid-phase extraction by immobilization of RNA on glass-fiber filters. For all RNA isolations, the following protocols were used:

-Samples were removed from -80°C and placed on ice immediately to prevent the release of RNases as a result of partial thawing. The weight of each sample was measured and for standardization, 100 mg of frozen tissue was used in every isolation. Using a sterile scalpel, each plaque and control material was divided into two 100

mg samples; one to be used for isolation of total RNA and the other for isolation of miRNAs.

-1000 μl of lysis buffer was added to microtubes and tubes were placed on ice.

-For homogenization, tissues were ground to powder with liquid nitrogen in a pre-chilled pestle and mortar.

-The ground tissues were scraped into lysis buffer containing microtubes on ice using a prechilled metal spatula and mixed rapidly until all visual clumps were dispersed. The lysate volume was measured.

-For organic extraction of total RNA and miRNAs, 100 μl of miRNA Homogenate Additive was mixed with cell lysates, mixed well by vortexing and incubated for 10 minutes on ice.

-Acid:phenol:chloroform with a volume equal to the cell lysate volume before the addition of miRNA homogenate additive was added to samples and vortexed for 30-60 seconds to mix.

-Samples were centrifuged at room temperature for 5 minutes at 10.000 x g to separate the aqueous and organic phases.

-The aqueous upper phase was transferred into a new 2 ml microtube without disturbing the lower phase. The removed volume was noted.

-At this point, two different procedures were used to isolate total RNA and miRNAs.

Total RNA Isolation Procedure

- 1.25 volumes of room temperature 100% ethanol was added to the aqueous phase which was recovered in the previous step.

- A filter cartridge was placed into a 2 ml collection tube supplied with the kit, the lysate/ethanol mixture was pipetted onto the filter cartridge and centrifuged for 15 seconds at 10.000 x g to pass the mixture through the filter.

- The flow-through was discarded and the above step was repeated until all of the lysate/ethanol mixture was passed through the filter.

- 700 μl of wash solution 1 was applied to the filter cartridge and centrifuged for 5-10 seconds. The flow-through was discarded.

- 500 μl of wash solution 2 was added onto the filter cartridge and centrifuged for 5-10 seconds. The flow-through was discarded. This step was repeated with a second 500 μl aliquot of wash solution 2. After discarding the flow-through, the assembly was centrifuged for 1 minute to remove the residual fluid from the filter.

- The filter cartridge was transferred into a fresh collection tube. 100 μl of pre-heated (95°C) elution solution was applied to the center of the filter and centrifuged for 20-30 seconds at maximum speed to recover the total RNA.

Enrichment Procedure for MicroRNAs

- This enrichment was accomplished by first immobilizing large RNAs on the filter with a relatively low ethanol concentration and collecting the flow-through containing mostly small RNA species. To begin with, 1.25 volumes of room temperature 100% ethanol was added to the aqueous phase which was recovered in the organic extraction step.

- A filter cartridge was placed into a 2 ml collection tube supplied with the kit, the lysate/ethanol mixture was pipetted onto the filter cartridge and centrifuged for 15 seconds at 10.000 x g to pass the mixture through the filter.

- The filtrate was collected.

- 2/3 volumes of room temperature 100% ethanol was added to the filtrate, the filtrate/ethanol mixture was pipetted onto a filter cartridge and centrifuged for 15 seconds at 10.000 x g.

- The flow-through was discarded and the above step was repeated until all the filtrate/ethanol mixture was passed through the filter.

- 700 μl of wash solution 1 was applied to the filter cartridge and centrifuged for 5-10 seconds. The flow-through was discarded.

- 500 μl of wash solution 2 was added onto the filter cartridge and centrifuged for 5-10 seconds. The flow-through was discarded. This step was repeated with a second 500 μl aliquot of wash solution 2. After discarding the flow-through, the assembly was centrifuged for 1 minute to remove the residual fluid from the filter.

- The filter cartridge was transferred into a fresh collection tube. 100 μl of pre-heated (95°C) elution solution was applied to the center of the filter and centrifuged for 20-30 seconds at maximum speed to recover the miRNAs.

The purity and quantity of all RNA samples were assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The purity of RNA samples were assessed from the ratio of A_{260}/A_{280}. Highly pure RNA samples with a ratio of 1.8 - 2.1 were used. All RNA samples were stored at -20°C until further use.

2.2.3 Complementary DNA synthesis

To synthesize complementary DNA (cDNA) from plaque and control total RNA samples, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA) was used. The protocol for reverse transcription (RT) reactions is as follows:

-All the kit components were thawed on ice. For each reaction, a 2X RT master mix was prepared with the components shown in Table 2.1.

Table 2.1 : Components to Prepare 2X RT Master Mix.

Component	Volume per Reaction (μl)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
Multiscribe^{TM}Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	10

-To be able to perform real time quantitative polymerase chain reaction (qRT-PCR) analyses with the equal concentrations of cDNA, 100 ng of total RNA per reaction was used for each reaction.

-10 μl of 2X RT mix for each reaction was mixed with 10 μl of total RNA sample.

-The PCR tubes were briefly centrifuged to spin down the contents.

-The samples were loaded into the thermal cycler and the reaction volume was set to 20 μl.

All reverse transcription reactions including the reactions for miRNAs described in the following paragraphs were performed with BIO-RAD Thermal Cycler

T100^{TM}(Bio-Rad, Hercules, CA, USA). The conditions used for RT-PCR are given in Table 2.2.

Table 2.2 : RT-PCR Reaction Conditions for Total RNA Samples.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA) was used for syntheses of cDNA from miRNAs isolated from plaque and control samples. For reverse transcription of miRNA samples, the given protocol was used:

-All kit component were thawed on ice.

-A RT master mix of 7 μl was prepared using the components shown in Table 2.3.

Table 2.3 : Components to Prepare miRNA RT Master Mix.

Component	Volume per Reaction (μl)
10 mM dNTPs (with dTTP)	0.15
Multiscribe^{TM}Reverse Transcriptase)	1.0
10X Reverse Transcription Buffer	1.5
RNase Inhibitor, 20U/	0.19
Nuclease-free H_2O	4.16
Total volume	7.0

-The components were mixed gently and centrifuged to bring the solutions to the bottom of PCR tubes. The tubes were placed on ice.

-MiRNA samples and 5X RT primers were thawed on ice.

-5X RT primers were vortexed to mix and centrifuged.

-7 μl master mix was combined with 5 μl miRNA (10 ng per reaction) for each reaction.

-3 μl of 5X RT primer was mixed with 12 μl reaction mix to a final volume of 15 μl.

For miRNA reverse transcription reactions, the primers were specific for each of the TaqMan®miRNA assays (miRNA-126-3p, miRNA-126-5p and miRNA endogenous control U6 assays) to be used for the following qRT-PCR reactions. Working with the same miRNA template, RT-PCR was performed to synthesize cDNA samples for

miRNA-3p, miRNA-5p and U6, using the different primers specific to each of these TaqMan® miRNA assays.

The reaction conditions to perform reverse transcription of microRNA samples are given in Table 2.4.

Table 2.4 : RT-PCR Reaction Conditions for MicroRNA Samples.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	16	42	85	4
Time	30 min	30 min	5 min	∞

Following RT-PCR reactions, obtained cDNA samples were kept in -20 °C until qRT-PCR analyses.

2.2.4 Real time quantitative PCR

Following cDNA syntheses from total RNA and miRNA samples, real time quantitative PCR (qRT-PCR) reactions were performed to quantify the relative expression levels of miR-126-3p, miR-126-5p as well as *Egfl7*, *Spred1* and *Pik3r2* genes in plaque and control samples.

TaqMan®miRNA-126-3p and miRNA-126-5p assays (Applied Biosystems, Foster City CA, USA) were used to detect miRNA-126-3p and miRNA-126-5p expression levels in plaque and control samples. The levels of small nuclear RNA U6 were also quantified using Taqman® endogenous control small nuclear RNA U6 assay (Applied Biosystems, Foster City CA, USA) for normalizing the levels of miR-126-3p and miR-126-5p.

The assays were composed of small RNA-specific forward PCR primer, small RNA-specific reverse PCR primer and small RNA-specific 6-carboxy fluorescein (FAM™) dye-labeled Taqman® probes.

All PCR amplification reactions were carried out in 96-well plates. In one 96-well plate, 5 plaque and 5 control samples were analyzed in each run. MiR-126-3p and miR-126-5p assays were run separately. The reactions were performed in a total volume of 20μl.

To prepare the qPCR reaction;

1. The 96-well plate was placed on ice.

2. TaqMan® Assays (20X) and cDNA samples were thawed on ice.

3. The number of reactions needed for each assay were calculated. Each plate included a TaqMan® assay (20X) for each cDNA sample, a Taqman® Endogenous Control Assay for each cDNA sample and a no template control(NTC). For a plate containing 20 cDNA samples (5 plaque and 5 control samples run in duplicates) and 1 NTC, a reaction mixture for 22 reactions was prepared for each assay.

4. The components shown in Table 2.5 were used to prepare qRT-PCR reaction mix without the addition of cDNA.

Table 2.5 : Components to Prepare qRT-PCR Reaction Mix for TaqMan® miRNA Assays.

Component	Volume per 20μl Reaction
TaqMan® Small RNA Assay (20X)	1.00 μl
TaqMan®Universal PCR Master Mix 2	10.00 μl
Nuclease-free water	7.67 μl

5. The tube was inverted several times to mix and centrifuged briefly.

6. 1.33 μl of cDNA was pipetted into each of two wells of 96-well plate for each sample.

7. 18.67 μl of the qRT-PCR reaction mix prepared for each assay was pipetted onto each cDNA sample that was previously transferred to the 96-well plate wells.

8. The plate was covered with optic seal and centrifuged briefly.

9. The plate was loaded into the instrument.

10. The experiment was set up and the plate was run with the conditions shown in Table 2.6.

Table 2.6 : qRT-PCR Reaction Conditions for TaqMan® miRNA Assays.

Stage	**Temp (°C)**	**Time (mm:ss)**
Hold	50	2:00
Hold	95	10:00
Cycle (40 Cycles)	95	0:15
	60	1:00

To detect *Egfl7*, *Spred1* and *Pik3r2* gene expression levels in plaque and control samples, TaqMan® Gene Expression Assays (Applied Biosystems, Foster City CA,

USA) were used. Levels of housekeeping gene 18S ribosomal RNA were also quantified in plaque and control samples for normalizing expression levels of *Egfl7*, *Spred1* and *Pik3r2*. For normalization, TaqMan® Endogenous Control Assay 18S (Applied Biosystems, Foster City CA, USA) was used. All of the assays were composed of specific forward and reverse PCR primers and 6-FAM™ dye-labeled TaqMan® probes. All the TaqMan® Gene Expression Assay ID numbers are listed in App B.

Quantitative Real Time PCR reactions were carried out in 96-well plates. In one 96-well plate, 4 plaque and 4 control cDNA samples were amplified using *Egfl7*, *Spred1* and *Pik3r2* TaqMan® Gene Expression Assays along with the Endogenous Control Assay 18S in each run. The reactions were performed in a total volume of 20μl.

To prepare the qPCR reaction;

1. The 96-well plate was placed on ice.

2. TaqMan® Gene Expression Assays (20X) and cDNA samples were thawed on ice.

3. The number of reactions needed for each assay were calculated. Each plate included a TaqMan® Assay (20X) for each cDNA sample, a TaqMan® Endogenous Control Assay for each cDNA sample and a no template control(NTC). For a plate containing 16 cDNA samples (4 plaque and 4 control samples run in duplicates) and 1 NTC, a reaction mixture for 18 reactions was prepared for each of the assays.

4. The components shown in Table 2.7 were used to prepare qRT-PCR reaction mix without the addition of cDNA.

Table 2.7 : Components to Prepare qRT- Reaction Mix for TaqMan® Gene Expression Assays.

PCR Reaction Mix Component	Volume per 20μl Reaction
TaqMan® Gene Expression Assay (20X)	1.00 μl
TaqMan®Universal PCR Master Mix 2	10.00 μl
RNase-free water	5.00 μl

5. The tube was inverted several times to mix and centrifuged briefly.

6. 4.00 μl of cDNA was pipetted into each of two wells of 96-well plate for each sample.

7. 16.00 μl of the qRT-PCR reaction mix prepared for each assay was pipetted onto each cDNA sample that was previously transferred to the 96-well plate wells.

8. The plate was covered with optic seal and centrifuged briefly.

9. The plate was loaded into the instrument.

10. The experiment was set up and the plate was run with the conditions shown in Table 2.8.

Table 2.8 : qRT-PCR Reaction Conditions for TaqMan® Gene Expression Assays.

Stage	**Temp (°C)**	**Time (mm:ss)**
Hold	50	2:00
Hold	95	10:00
Cycle (40 Cycles)	95	0:15
	60	1:00

All qRT-PCR reactions were performed with the Mx3005P Real-Time PCR (Agilent, Santa Clara, USA). Quantitative real-time PCR analysis depends on detection and quantification of PCR products via fluorescent signals emitted by the dyes used in the reaction. The principle of this system relies on TaqMan® probes. As integral parts of the TaqMan® assays used in the qRT-PCR reactions, TaqMan® probes are linear oligonucleotides labeled with a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. The signal from the reporter molecule is quenched when both of the molecules are in close proximity in the solution. In contrast, when the probe is hybridized to its target sequence, it is cleaved by the 5' nuclease activity of the Taq polymerase during the extension phase of the PCR. A fluorescence signal based on the fluorescence resonance energy transfer (FRET) principle is emitted when the reporter and quencher molecules are separated. In each PCR cycle, reporter molecules are separated from their probes and as a result, fluorescence intensity increases proportionally with the amount of amplified template. The increase in fluorescence signal is detected in real time by the instrument. During amplification, data on about the fluorescence emission is collected by the software of the instrument and output is shown as amplification plots [170, 171].

QRT-PCR amplification is comprised of four successive phases: The baseline phase; the exponential phase; the linear phase and the plateau. The exponential amplification of the template begins during the PCR cycles of baseline phase.This phase is named

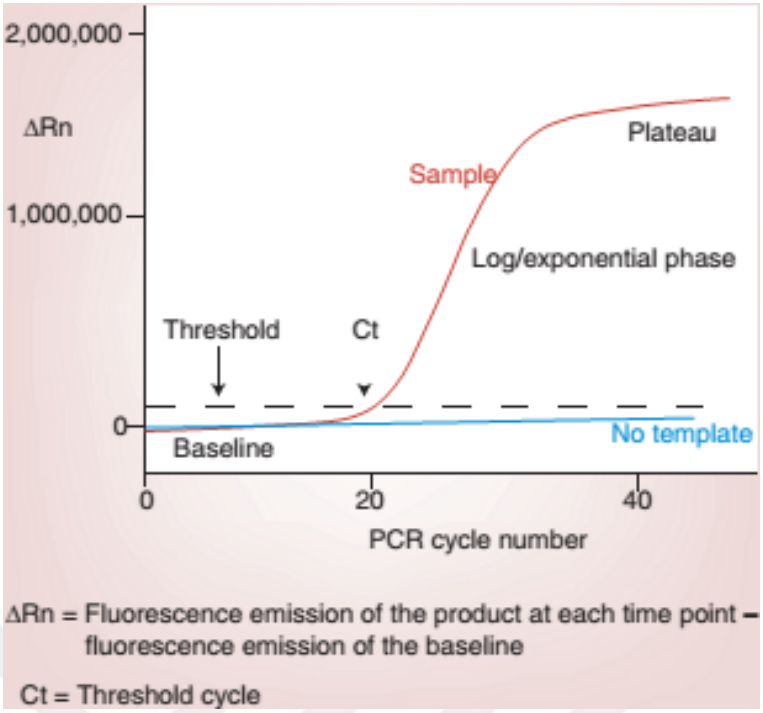


Figure 2.1 : Single amplification plot [171].

so because the accumulating fluorescent signals are below the detection limits of the instrument. During the run, the instrument software calculates a ΔRn value and plots it versus the cycle number as shown in Figure 2.1. The ΔRn value is calculated by the equation shown below (2.1) in which Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission of the baseline:

$$Rn = Rnf - Rnb \tag{2.1}$$

Based on the baseline, a threshold is chosen by the software and a fluorescent signal detected above the threshold can be used to define the threshold cycle (Ct) for a sample.C_T points to the amplification of template molecule and represents a cycle at which the sample fluorescence signal from the sample exceeds a chosen threshold above background fluorescence signal. PCR amplification continues at maximal rate during the exponential phase in which the earliest detectable signals are received. In a reaction that is 100% efficient, 2 complete molecules are synthesized from every one template molecule during the exponential phase. In the linear phase, the amplification efficiency starts to diminish. In the latest plateau phase, no further increase in signals take place [172].

Following qRT-PCR runs, the raw C_T values were used for the analysis of gene expression levels.

2.2.5 Analysis of gene expression

Relative quantification strategy, also known as comparative quantification, was employed for qRT-PCR analyses. This method quantifies the changes in mRNA expression levels of a gene or genes in many samples. The change is expressed relative to the expression levels of a reference gene or genes, such as housekeeping genes. For this reason, standards with known concentrations are not needed in relative quantification strategy, as is needed for absolute quantification [173]. In addition, the studied gene expression levels can be quantified relative to those of healthy individuals or non-treated controls [174]. The $2^{\Delta\Delta C_T}$ method [175], the mathematical model of normalized gene expression, was used for relative quantification. The alternative mathematical model is the model with kinetic PCR efficiency correction as can be observed in Figure 2.2 [173].

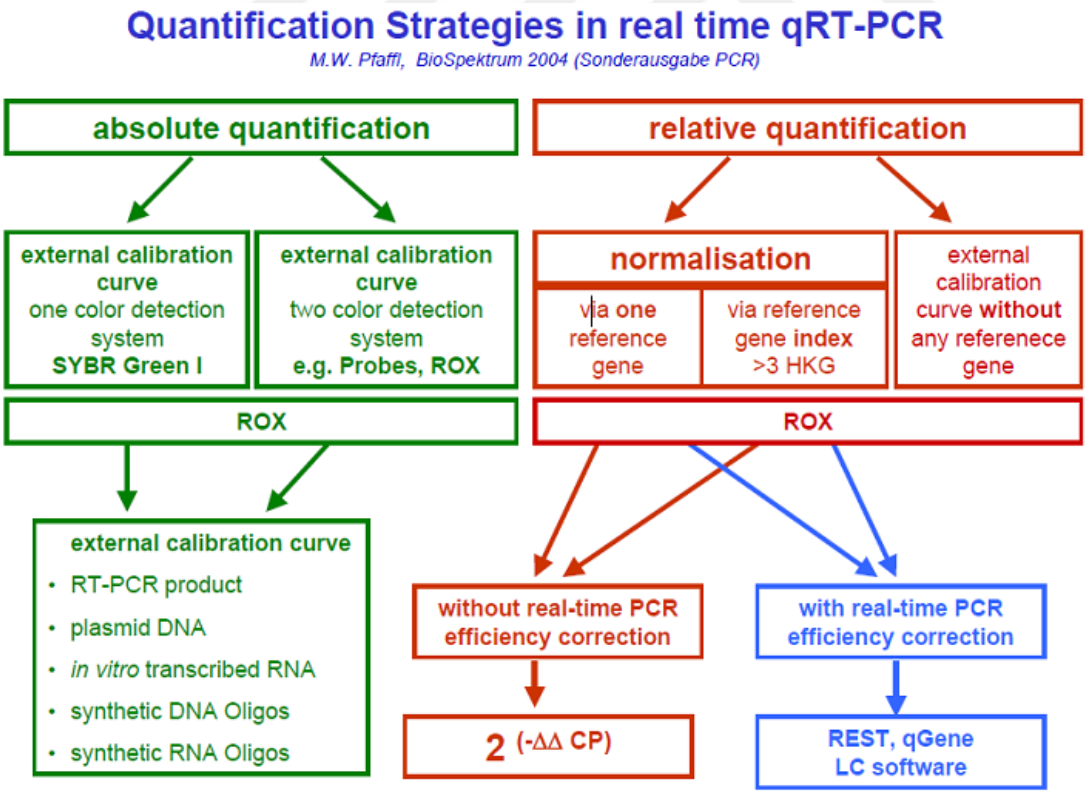


Figure 2.2 : Quantitative real time PCR strategies for quantification of gene expression [173].

Quantitative real time PCR quantifies the mRNA levels differentially. Many factors, such as biological conditions (pathologies, diseased state...etc.) and non-reproducible factors that are non-specific to the biological conditions contribute to this differential expression. Although all the technical variables are carefully controlled, variations from sample to sample and from run to run still cause the aforementioned factors. Therefore, data normalization with the help of internal reference genes is very important in qRT-PCR, since it minimizes the effects of these non-specific factors. Housekeeping genes, genes that are needed for survival and that are expressed stably and abundantly, are chosen as internal reference genes [176]. Most commonly used internal reference genes include, Beta actin (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18S), Phosphoglycerate kinase 1 (PGK1), Peptidylprolyl isomerase A (PPIA) and TATA box binding protein (TBP) [176, 177]. In addition to internal reference genes mentioned before, housekeeping genes used for normalization of miRNA expression exist. Let-7a, miR-16, small nuclear RNA (snRNA) U6 are among the housekeeping genes used for normalization of miRNA expression levels [178].

$2^{\Delta\Delta C_T}$ model was chosen to be used in the relative quantification strategy employed. For normalization of gene expression and miRNA expression, 18S rRNA and U6 snRNA were used. All of the target gene and miRNA expression levels of plaque samples were compared to those of control samples and normalized to the levels of the 18S rRNA and U6 snRNA (U6) using the following procedure and equations:

- Mean C_T values for *Egfl7*, *Spred1*, *Pik3r2* genes as well as housekeeping gene *18S* measured in each of the control and plaque samples were exported to Microsoft Excel following qRT-PCR experiments.

- For each plaque and control sample, ΔC_T values were calculated for *Egfl7*, *Spred1*, *Pik3r2* target genes using their respective C_T values with the equations (2.2) and (2.3).

$$\Delta C_{tPlaque} = C_{tTargetgene} - C_{tReference} \tag{2.2}$$

$$\Delta C_{tControl} = C_{tTargetgene} - C_{tReference} \tag{2.3}$$

-For each sample, $\Delta\Delta C_T$ values were calculated for *Egfl7*, *Spred1*, *Pik3r2* target genes using the equation (2.4)below.

$$\Delta\Delta C_t = \Delta C_{tPlaque} - \Delta C_{tControl} \tag{2.4}$$

-The relative quantification ratio R was calculated for each target gene by the equation (2.5).

$$R = 2^{\Delta\Delta C_t} \tag{2.5}$$

-For each target gene, differences in expression levels were denoted as log-transformed ratios to show fold change between the carotid artery plaque tissues and control tissue.

Following qRT-PCR analyses, mean C_T values for miR 126-3p, miR 126-5p and the housekeeping gene *snU6* measured in each of the control and plaque samples were exported to Microsoft Excel following qRT-PCR experiments. The procedures explained above were replicated for the analyses of miR 126-5p and miR 126-3p expression levels in plaques and controls. The expression levels in plaque samples relative to controls were expressed as fold change values.

2.2.6 Statistical analysis

To perform statistical analyses, IBM SPSS Statistics for Windows, version 20.0 software (IBM Corp., Armonk, NY) was used. The significance of differences between the results from plaque and control tissues was examined with Student's t test. Demographic information was compared with gene expression data expressed as fold change by using Chi-square and Fisher's Exact tests. Values were expressed as mean $\pm$ standard deviation (SD). P values less than 0.05 denoted statistical significance.

3. RESULTS

3.1 Analysis of the Clinical Parameters of the Study Population

The demographic and biochemical features of the study population (n=14) were obtained from the surgical team who have performed the carotid endarterectomy operations on patients in Istanbul Medicana International Hospital. The data, including gender, age, body mass index (BMI) of patients, as well as their smoking habits, the presence of major cardiovascular risk factors, the presence of other cardiovascular diseases and their statin use is reported in Table 3.1 below.

Table 3.1 : Demographical and Clinical Characteristics of the Studied Patients.

Parameters	Total (n=14)
Gender (F/M)	9/5
Age (year $\pm$ SD)	66.50 $\pm$ 7.41
BMI (kg/m $\pm$ SD)	30.62 $\pm$ 2.60
Smokers n(%)	50
Family History of CVD n (%)	57.1
Diabetes n (%)	42.9
Hypertension n (%)	78.6
Cerebrovascular Event n (%)	64.3
Coronary Artery Disease n (%)	35.7
Peripheral Artery Disease n (%)	28.6
Cholesterol (mmol/L)$\pm$ SD (mg/dl)	223.57 $\pm$ 79.57
LDL Cholesterol $\pm$ SD (mg/dl)	141.86 $\pm$ 70.97
HDL Cholesterol $\pm$ SD (mg/dl)	39.64 $\pm$ 10.47
Triglycerides $\pm$ SD (mg/dl)	30.62 $\pm$ 2.60
Use of Statin n (%)	35.7

n: number of individuals
Values are reported as mean $\pm$ standart deviation (SD) or
number of patients (percentage of the total group)
BMI: Body mass index
CVD: Cardiovascular disease
LDL: Low density lipoprotein
HDL: High density lipoprotein

Age and male sex are among the major risk factors for atherosclerotic cardiovascular diseases, although the risk for females after the menopause is almost the same as men with the same age. The population pyramid that shows the age and sex information about the study group is given is Figure 3.1 below. As can be seen in the graphic, 64.3% of the study population is female within the age groups 55-60, 60-64,65-69 and 75-80.

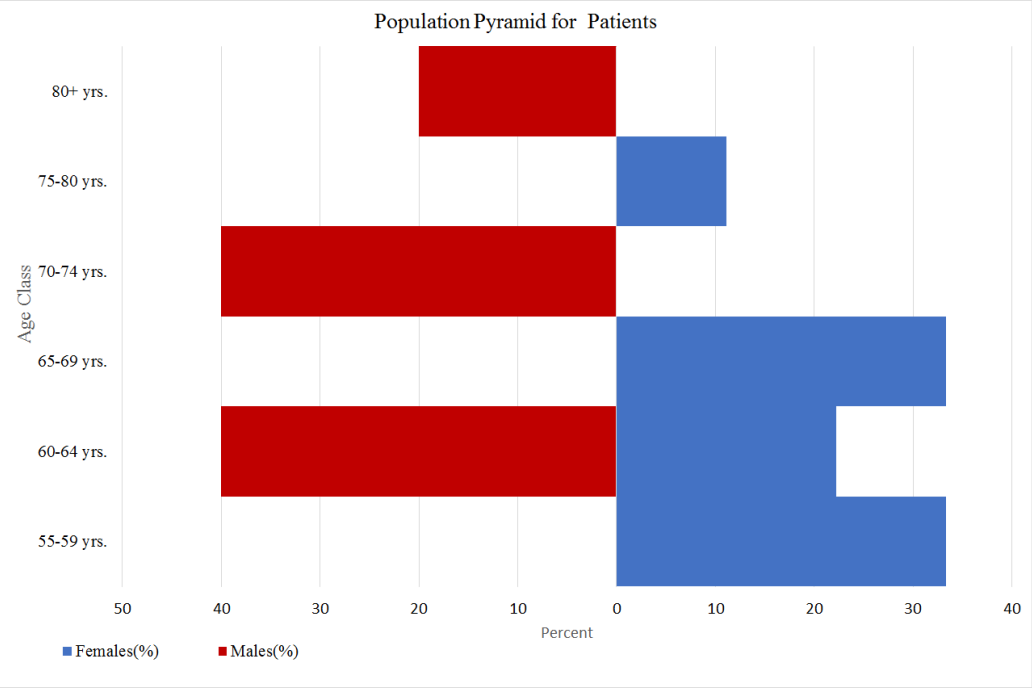


Figure 3.1 : Age-sex graphic of the study population.

Figure 3.2 shows the distribution of body mass index values among the patients. The mean BMI was 30.62 $\pm$ 2.60, while the median value for BMI was 30.30 (25.9-37.5). These values mean that the majority of the patients in the study group were obese.

The following Figure 3.3 demonstrates the fact that being overweight and obese are linked with metabolic syndrome. The factors that have a tendency to occur together in metabolic syndrome, named as metabolic risk factors are;

- A large waistline
- Hypertension
- High fasting blood sugar
- A high blood triglyceride level
- A low blood HDL cholesterol level [179].

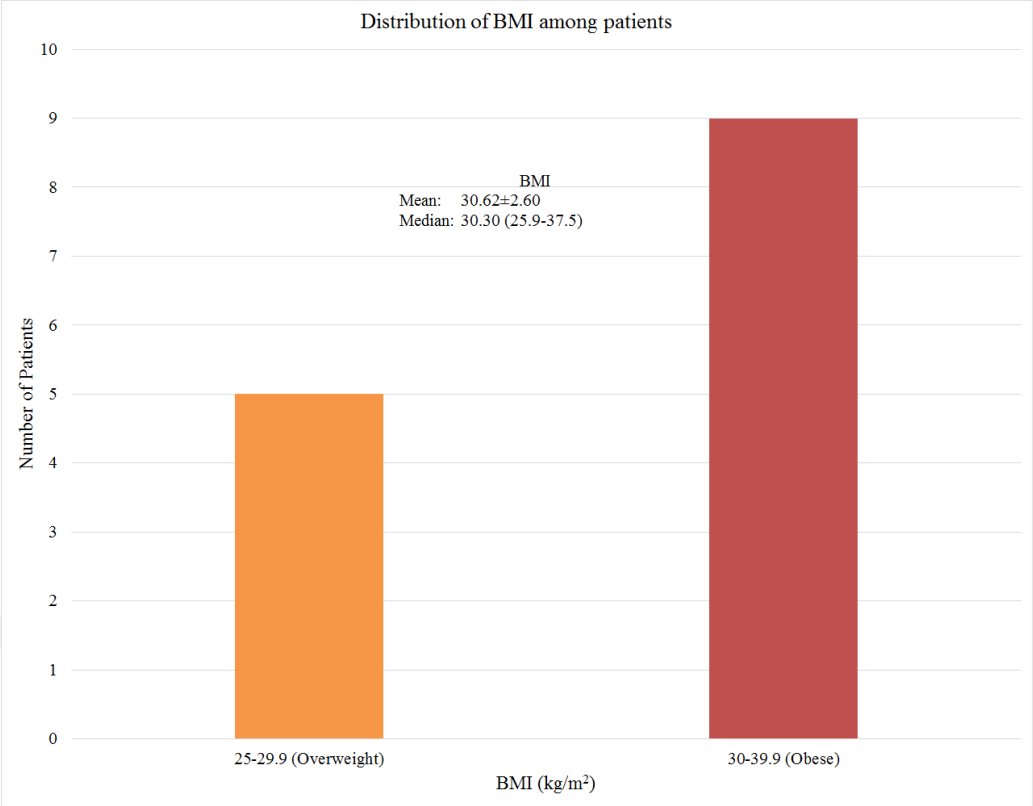


Figure 3.2 : Distribution of BMI among the patients.

The distribution of body mass index values among the patients with hypertension is demonstrated in Figure 3.3. According to the data, 54.4 % of the patients with BMI between 30-34.9 and 9.1 % of the patients with BMI between 35-39.9 (obese patients) were hypertensive. Hypertension was also observed in 9.1 % of patients with BMI between 25-25.9 and in 27.3 % of patients with BMI between 26-29.9 (overweight patients).

Similarly, as can be seen in Figure 3.3, along with hypertension, overweight and obese patients also had diabetes mellitus (DM). 16.7 % of the patients who were overweight had DM, while 16.7 % of the patients within the BMI range of 30-34.9 and classified as obese were diabetic. Also, 16.7 % of the obese patients within BMI range of 35-39.9 had DM.

The overweight and obese patients not only had hypertension and DM, but also suffered from high blood cholesterol or abnormal levels of lipids in blood as well, as in the case of metabolic syndrome explained above. 50 % of the obese patients within the BMI range of 30-34.9 had dyslipidemia, and 12.5 % of the rest of the obese

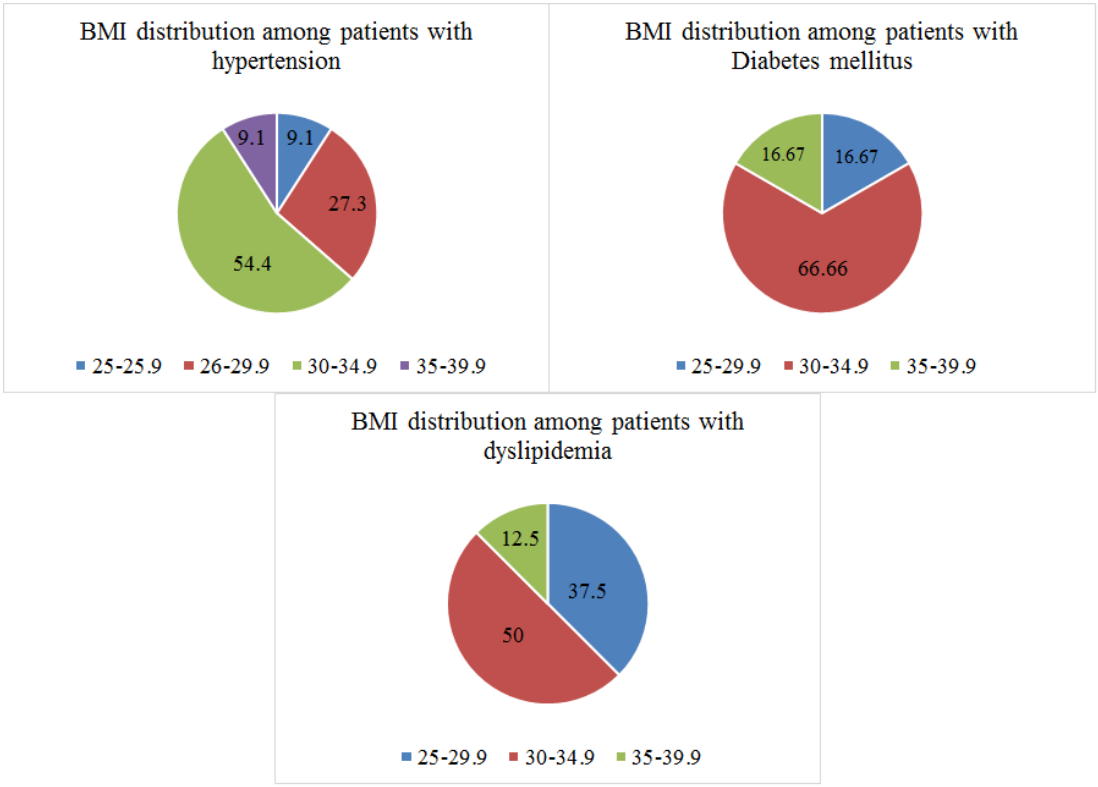


Figure 3.3 : Distribution of BMI among the patients with hypertension, diabetes mellitus and dyslipidemia.

patients within the BMI range of 35-39.9 suffered from dyslipidemia. Dyslipidemia was also observed in 37.5 % overweight patients.

Studies have pointed out to the fact that atherosclerotis is a systemic disease, therefore it has been suggested that the risk factors for its development in carotid arteries and coronary arteries, as well as its manifestations in both arterial systems would share similarities to some extent [180]. In a study in which patients were followed up for three years, it has been observed that myocardial infarction, stroke, vascular death or re-hospitalization rate were 25.5% for patients who had disease in one arterial system, while the same rates were 40.5% for patients with disease in multiple arterial systems [181]. Therefore, the presence of cardiovascular diseases other than carotid artery disease were investigated in patients. In Figure 3.4, it can be seen that 35.7% of the patients who suffered from carotid artery atherosclerosis had also atherosclerotic disease in their coronary arteries. In a similar fashion, 28.6 % of these patients suffered from peripheral artery disease. Because coronary endothelial dysfunction- atherosclerosis- has been associated with an elevated risk of cerebrovascular event, such as stroke [182], the percentage of patients who have

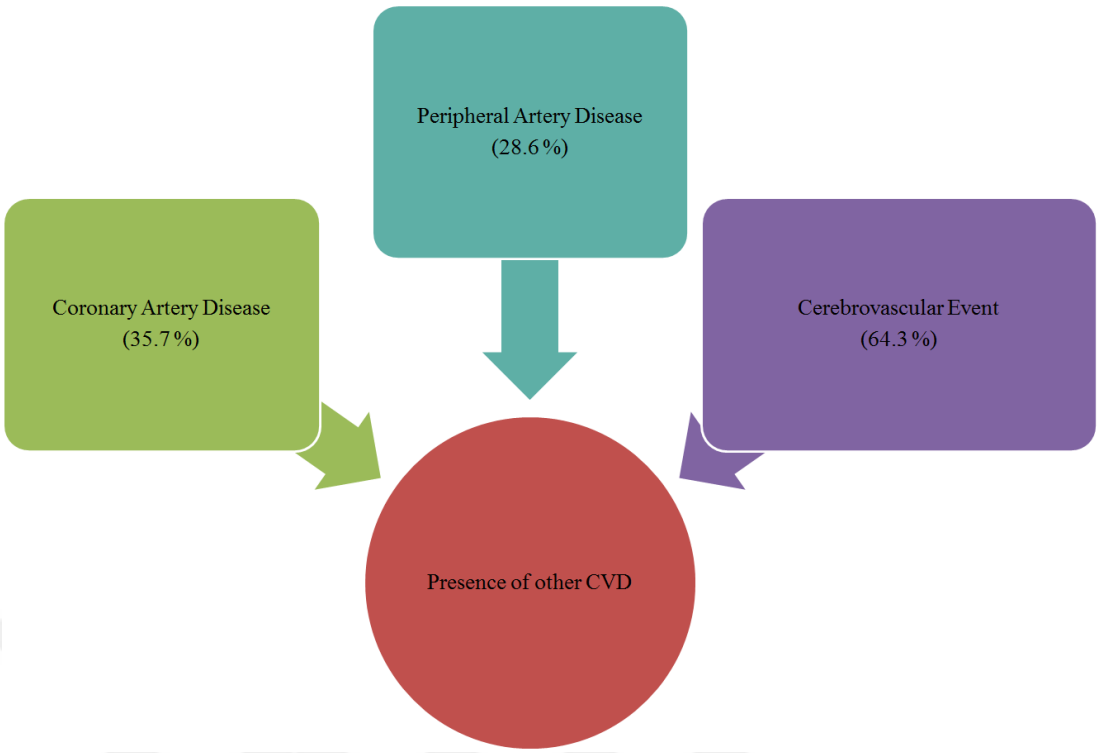


Figure 3.4 : Presence of other cardiovascular diseases in patients.

had a previous cerebrovascular event has also been demonstrated in Figure 3.4. According to the data, 64.3% who underwent carotid endarterectomy have had a previous cerebrovascular event. 1 out of 14 patients who has had a cerebrovascular event had disease in both the carotid and coronary arteries, while 3 patients who has had a cerebrovascular event also had peripheral artery disease. This data demonstrates the correspondence between the atherosclerotic disease in different arterial systems.

3.2 MicroRNA 126 Target mRNA Prediction

Literature review on angiogenesis, on the role of microRNAs in angiogenesis and the so called "angiomiRs" led miRNA 126-3p and its passenger strand -5p to be investigated in carotid atherosclerotic plaques, along with their target genes in this study.

The first and the most comprehensive microRNA database, the mirBase was searched for miRNA 126 [167]. Information on annotation, genome context and its stem loop structure was obtained on miRNA 126 as shown in Figure 3.5. Coinciding with literature information, miRNA 126 was termed as a human microRNA which

is expressed in endothelial cells exclusively and identified as a miRNA taking part in angiogenetic processes.

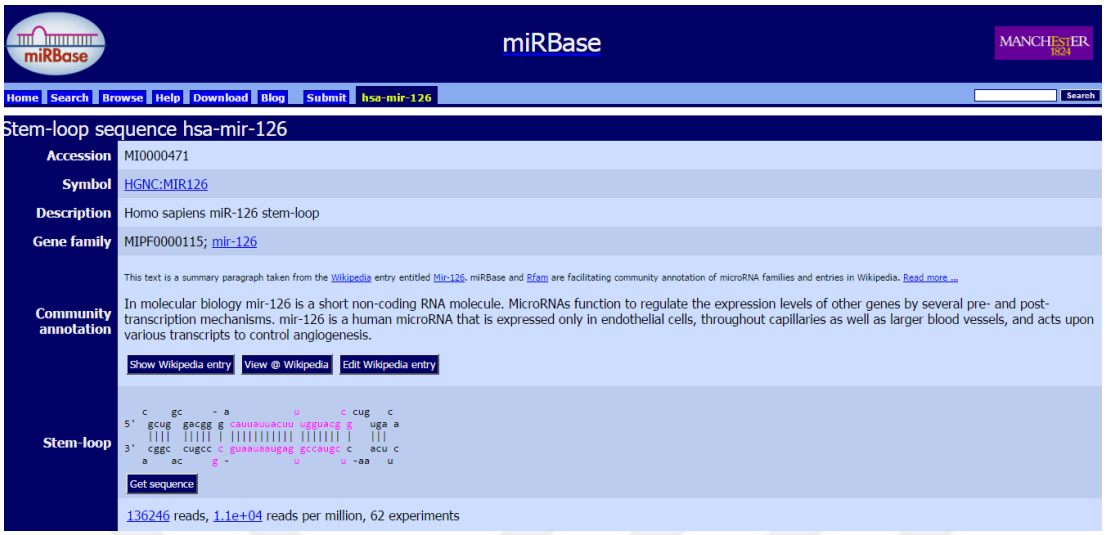


Figure 3.5 : MicroRNA 126 [167].

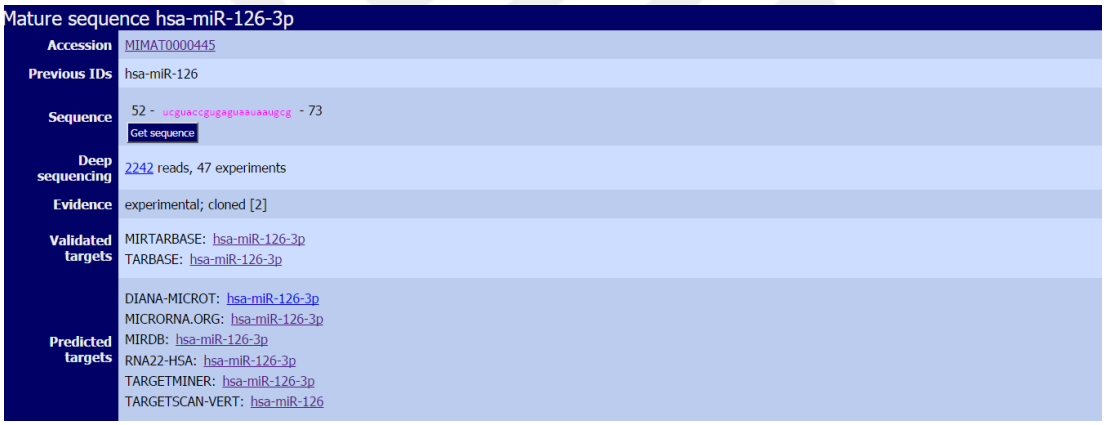
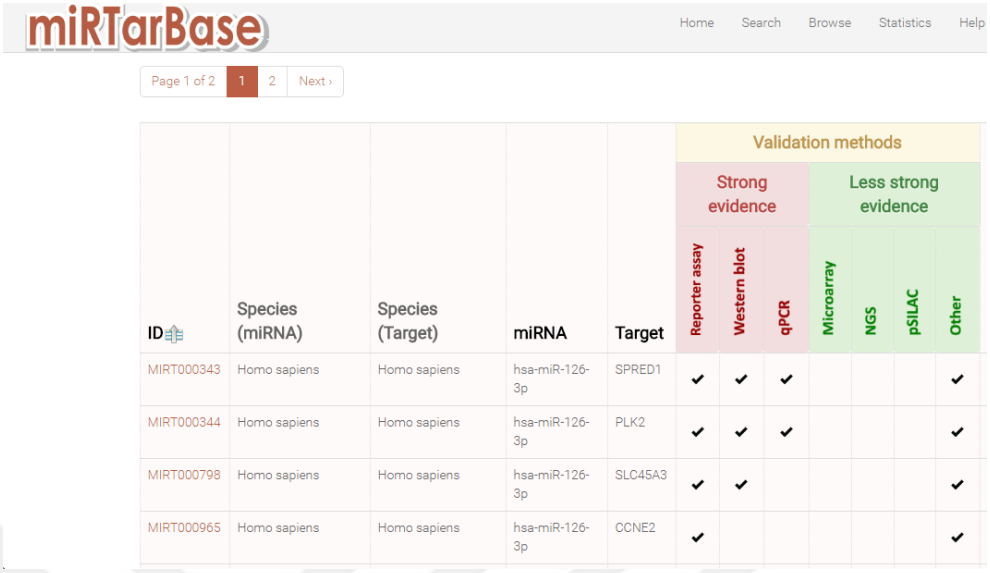


Figure 3.6 : Continued; microRNA 126-3p [167].

Next, mRNA targets for miRNA 126-3p was investigated. For this purpose, mirTarBase and TargetScan databases were used out of the suggested databases in mirBase, which function in investigation of miRNA validated targets as well as predicted targets.

First of all, mirTarBase database was searched for mRNA targets of miRNA 126-3p. This database lists all the mRNA targets of certain miRNAs and the methods that were used for validation. The validation methods, such as reporter assay, Western blot and qPCR are listed under the category "strong evidence". On the other hand, methods including microarray, next generation sequencing and pulsed stable isotope labelling

by/with amino acids in cell culture are categorized as "less strong evidence" [168]. Figures 3.7, 3.8 and 3.9 show all the validated mRNA targets of miRNA 126-3p.



miRTarBase

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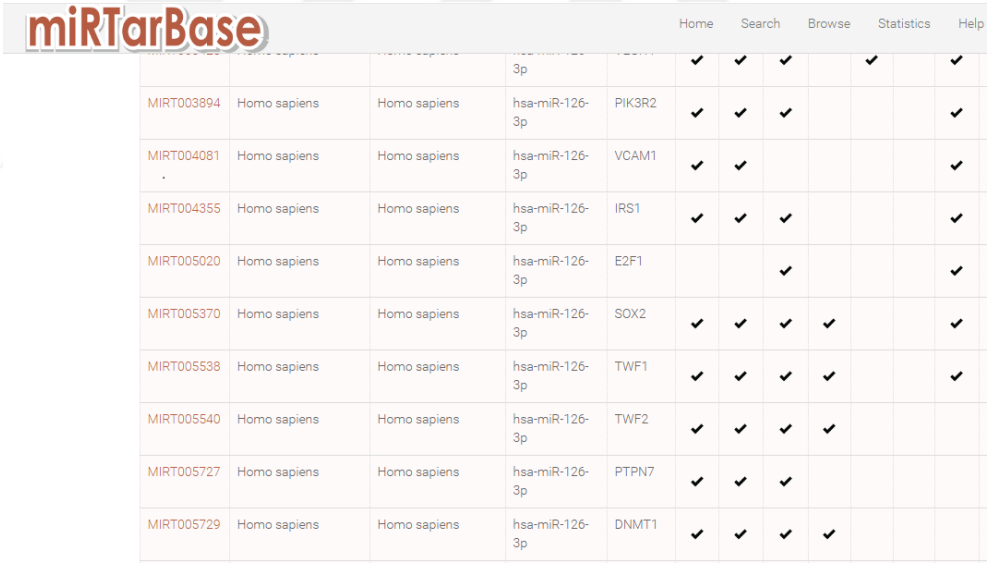
ID	Species (miRNA)	Species (Target)	miRNA	Target	Validation methods: Strong evidence: Reporter assay	Strong evidence: Western blot	Strong evidence: qPCR	Less strong evidence: Microarray	Less strong evidence: NGS	Less strong evidence: pSILAC	Less strong evidence: Other
MIRT000343	Homo sapiens	Homo sapiens	hsa-miR-126-3p	SPRED1	✔	✔	✔				✔
MIRT000344	Homo sapiens	Homo sapiens	hsa-miR-126-3p	PLK2	✔	✔	✔				✔
MIRT000798	Homo sapiens	Homo sapiens	hsa-miR-126-3p	SLC45A3	✔	✔					✔
MIRT000965	Homo sapiens	Homo sapiens	hsa-miR-126-3p	CCNE2	✔						✔

Figure 3.7 : Validated targets of microRNA 126-3p [184].



miRTarBase

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			3p		✔	✔	✔		✔		✔
MIRT003894	Homo sapiens	Homo sapiens	hsa-miR-126-3p	PIK3R2	✔	✔	✔				✔
MIRT004081	Homo sapiens	Homo sapiens	hsa-miR-126-3p	VCAM1	✔	✔					✔
MIRT004355	Homo sapiens	Homo sapiens	hsa-miR-126-3p	IRS1	✔	✔	✔				✔
MIRT005020	Homo sapiens	Homo sapiens	hsa-miR-126-3p	E2F1			✔				✔
MIRT005370	Homo sapiens	Homo sapiens	hsa-miR-126-3p	SOX2	✔	✔	✔	✔			✔
MIRT005538	Homo sapiens	Homo sapiens	hsa-miR-126-3p	TWF1	✔	✔	✔	✔			✔
MIRT005540	Homo sapiens	Homo sapiens	hsa-miR-126-3p	TWF2	✔	✔	✔	✔			
MIRT005727	Homo sapiens	Homo sapiens	hsa-miR-126-3p	PTPN7	✔	✔	✔				
MIRT005729	Homo sapiens	Homo sapiens	hsa-miR-126-3p	DNMT1	✔	✔	✔	✔			

Figure 3.8 : Continued; validated targets of microRNA 126-3p [184].

All of the miRNA 126-3p targets that have been marked under the first category were checked using Pathways from Biosystems under NCBI Gene Database [183]. The genes encoding proteins that are interacting with VEGF signalling pathway have been investigated. As a result, Sprouty-related EVH1 domain-containing protein 1 (SPRED1) and Phosphatidylinositol 3-kinase regulatory subunit beta (PIK3R2) were the chosen miRNA 126-3p targets to be studied.

As is demonstrated in Figure 3.7, SPRED1 was identified as one of the validated miRNA 126-3p targets by miRTarBase.

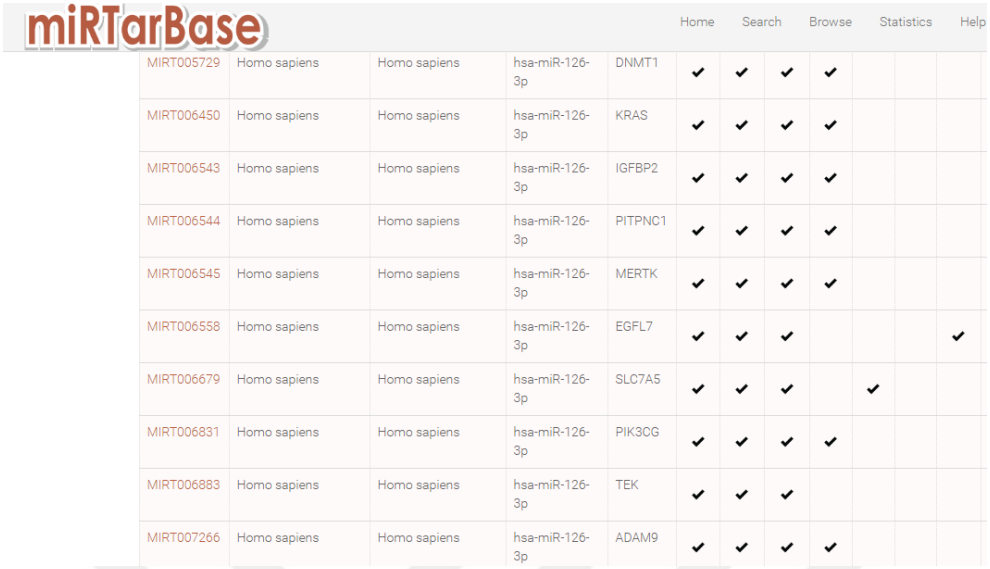

Figure 3.9 : Continued 2; validated targets of microRNA 126-3p [184].

One other validated miRNA 126-3p target among the targets in miRTarBase which was found to take part in VEGF pathway was PIK3R2. As demonstrated in Figure 3.8, similar to SPRED1, strong evidence methods were used to validate the interaction of miRNA 126-3p with PIK3R2.

In addition to SPRED1 and PIK3R2, Epidermal growth factor (EGF)-like domain 7 (EGFL7) was also identified as a validated target of miRNA 126-3p by miRTarBase, as is demonstrated in Figure 3.9. EGFL7 is an angiogenic factor that has important regulatory roles in development of blood vessels during the embryogenic and post-natal stages. Two microRNAs, miR 126-3p and its passanger strand miR 126-5p are encoded from the intron of *Egfl7* gene [160]. *Egfl7* levels are observed to be high during the active proliferating state of the endothelium, such as during embryonic vascular development and physiologic angiogenesis. However, in the adult endothelium, expression of *Egfl7* is mostly down-regulated. The expression levels of *Egfl7* are suggested to increase in cases of pathological angiogenesis, such as atherosclerosis, in adults [161]. Therefore, in addition to the miRNAs it encodes from its intron, *Egfl7* was chosen as a target gene whose expression levels are to be investigated in carotid atherosclerotic plaques.

Human | miR-126-3p.1
100 transcripts with sites, containing a total of **22** conserved sites and **81** poorly conserved sites.
Top 100 predicted targets, irrespective of site conservation, ranked by aggregate P_{CT}. [Sort table by cumulative weighted context++ score] [View only predicted targets with conserved sites]
The table shows at most one transcript per gene, selected for being the most prevalent, based on 3P-seq tags (or the one with the longest 3' UTR, in case of a tie). [Download table]

Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Conserved sites				Poorly conserved sites				6mer sites	Repre- sentative miRNA	Cumulative weighted context++ score
					total	8mer	7mer-m8	7mer-A1	total	8mer	7mer-m8	7mer-A1			
SLC7A5	ENST00000565644.1	solute carrier family 7 (amino acid transporter light chain, L system), member 5	155	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
IRS1	ENST00000305123.5	insulin receptor substrate 1	804	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.35
SPRED1	ENST00000299084.4	sprouty-related, EVH1 domain containing 1	547	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
LRP6	ENST00000261349.4	low density lipoprotein receptor-related protein 6	157	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.19
CRK	ENST00000398970.5	v-crk avian sarcoma virus CT10 oncogene homolog	1841	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.36
SDC2	ENST00000302190.4	syndecan 2	774	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.29
GNA13	ENST00000439174.2	guanine nucleotide binding protein (G protein), alpha 13	190	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.26
CAMSAP1	ENST00000389532.4	calmodulin regulated spectrin-associated protein 1	966	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.35
DIP2C	ENST00000280886.6	DIP2 disco-interacting protein 2 homolog C (Drosophila)	359	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.30

Figure 3.10 : Predicted targets of microRNA 126-3p [187].

Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Conserved sites				Poorly conserved sites				6mer sites	Repre- sentative miRNA	Cumulative weighted context++ score
					total	8mer	7mer-m8	7mer-A1	total	8mer	7mer-m8	7mer-A1			
SLC7A5	ENST00000565644.1	solute carrier family 7 (amino acid transporter light chain, L system), member 5	155	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
IRS1	ENST00000305123.5	insulin receptor substrate 1	804	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.35
SPRED1	ENST00000299084.4	sprouty-related, EVH1 domain containing 1	547	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
LRP6	ENST00000261349.4	low density lipoprotein receptor-related protein 6	157	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.19
CRK	ENST00000398970.5	v-crk avian sarcoma virus CT10 oncogene homolog	1841	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.36
SDC2	ENST00000302190.4	syndecan 2	774	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.29
GNA13	ENST00000439174.2	guanine nucleotide binding protein (G protein), alpha 13	190	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.26
CAMSAP1	ENST00000389532.4	calmodulin regulated spectrin-associated protein 1	966	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.35
DIP2C	ENST00000280886.6	DIP2 disco-interacting protein 2 homolog C (Drosophila)	359	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.30
FBXO33	ENST00000298097.7	F-box protein 33	306	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
PLK2	ENST00000274289.3	polo-like kinase 2	689	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.41
HERPUD1	ENST00000379792.2	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	804	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.43
ADAM9	ENST00000487273.2	ADAM metallopeptidase domain 9	6435	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.44
AKAP13	ENST00000394518.2	A kinase (PRKA) anchor protein 13	53	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.21
TRAF7	ENST00000326181.6	TNF receptor-associated factor 7, E3 ubiquitin protein ligase	1505	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.20
EGFL7	ENST00000371699.1	EGF-like-domain, multiple 7	29	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.38
EFHD2	ENST00000375980.4	EF-hand domain family, member D2	1411	Sites in UTR	1	0	0	1	0	0	0	0	0	hsa-miR-126-3p.1	-0.27

Figure 3.11 : Continued; predicted targets of microRNA 126-3p [187].

ZNF131	ENST00000505606.2	zinc finger protein 131	1015	Sites in UTR	0	0	0	0	1	0	0	1	0	hsa-miR-126-3p.1	-0.38
PMM1	ENST00000216259.7	phosphomannomutase 1	1753	Sites in UTR	0	0	0	0	1	1	0	0	1	hsa-miR-126-3p.1	-1.01
KIAA1715	ENST00000272748.4	KIAA1715	858	Sites in UTR	0	0	0	0	2	0	0	2	1	hsa-miR-126-3p.1	-0.63
KCNAB3	ENST00000303790.2	potassium voltage-gated channel, shaker-related subfamily, beta member 3	35	Sites in UTR	0	0	0	0	1	1	0	0	0	hsa-miR-126-3p.1	-0.72
PTPN9	ENST00000306726.2	protein tyrosine phosphatase, non-receptor type 9	644	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-126-3p.1	-0.09
PARP16	ENST00000261888.6	poly (ADP-ribose) polymerase family, member 16	97	Sites in UTR	0	0	0	0	1	1	0	0	0	hsa-miR-126-3p.1	-0.64
LARP6	ENST00000299213.8	La ribonucleoprotein domain family, member 6	630	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.61
SLC41A2	ENST00000258538.3	solute carrier family 41 (magnesium transporter), member 2	224	Sites in UTR	0	0	0	0	1	1	0	0	0	hsa-miR-126-3p.1	-0.38
MEX3C	ENST00000592416.1	mex-3 RNA binding family member C	5	Sites in UTR	0	0	0	0	1	0	1	0	1	hsa-miR-126-3p.1	-0.60
KCMF1	ENST00000409785.4	potassium channel modulatory factor 1	1285	Sites in UTR	0	0	0	0	2	0	0	2	0	hsa-miR-126-3p.1	-0.01
ZADH2	ENST00000322342.3	zinc binding alcohol dehydrogenase domain containing 2	207	Sites in UTR	0	0	0	0	1	1	0	0	1	hsa-miR-126-3p.1	-0.30
PLXNB2	ENST00000449103.1	plexin B2	82	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-126-3p.1	-0.58
SOX2	ENST00000325404.1	SRY (sex determining region Y)-box 2	506	Sites in UTR	0	0	0	0	1	0	1	0	1	hsa-miR-126-3p.1	-0.57
TNFRSF10B	ENST00000276431.4	tumor necrosis factor receptor superfamily, member 10b	828	Sites in UTR	0	0	0	0	1	1	0	0	0	hsa-miR-126-3p.1	-0.41
ZNF813	ENST00000396403.4	zinc finger protein 813	10	Sites in UTR	0	0	0	0	2	0	0	2	0	hsa-miR-126-3p.1	-0.36
PIK3R2	ENST00000222254.8	phosphoinositide-3-kinase, regulatory subunit 2 (beta)	1484	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.49
KCNJ1	ENST00000392665.2	potassium inwardly-rectifying channel, subfamily J, member 1	9	Sites in UTR	0	0	0	0	1	0	0	1	1	hsa-miR-126-3p.1	-0.49
RGS3	ENST00000374140.2	regulator of G-protein signaling 3	2735	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-126-3p.1	-0.45
F8A2	ENST00000369505.3	coagulation factor VIII-associated 2	5	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.45
F8A1	ENST00000369446.2	coagulation factor VIII-associated 1	5	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.45
F8A3	ENST00000369445.2	coagulation factor VIII-associated 3	5	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.45
TSC1	ENST00000298552.3	tuberous sclerosis 1	838	Sites in UTR	0	0	0	0	1	1	0	0	0	hsa-miR-126-3p.1	-0.41
KANK2	ENST00000586659.1	KN motif and ankyrin repeat domains 2	1735	Sites in UTR	1	0	1	0	0	0	0	0	0	hsa-miR-126-3p.1	-0.42
HARS	ENST00000504156.1	histidyl-tRNA synthetase	34	Sites in UTR	0	0	0	0	1	0	0	1	1	hsa-miR-126-3p.1	-0.05
STX12	ENST00000373943.4	syntaxin 12	1082	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.36
ARMCX1	ENST00000372829.3	armadillo repeat containing, X-linked 1	18	Sites in UTR	0	0	0	0	1	0	0	1	0	hsa-miR-126-3p.1	-0.41
LPAR2	ENST00000586703.1	lysophosphatidic acid receptor 2	45	Sites in UTR	0	0	0	0	1	0	1	0	0	hsa-miR-126-3p.1	-0.41
CKMT2	ENST00000254035.4	creatine kinase, mitochondrial 2 (sarcomeric)	5	Sites in UTR	0	0	0	0	1	0	0	1	0	hsa-miR-126-3p.1	-0.40

Figure 3.12 : Continued 2; predicted targets of microRNA 126-3p [187].

The miRNA targets were checked with TargetScan database as well. TargetScan lists all the predicted mRNA targets of certain miRNAs with their conserved and poorly conserved sites. In addition, values such as, total context score, aggregate probability of conserved targeting (P_{CT}) are also given in results. Context score is identified as the total contributions of site type, 3' pairing, local AU, position, target site abundance and seed pairing stability [144]. TargetScan provides links to sites in untranslated regions (UTRs).

Along with the validated miR 126-3p targets that were found by using miRTarBase, TargetScan database was also used for prediction of miR 126-3p targets. SPRED1, PIK3R2 and EGFL7 were among the predicted targets of miR 126-3p when TargetScan database was used. Figures 3.10, 3.11 and 3.12 demonstrate the results obtained by using TargetScan database for miR 126-3p target prediction.

3.3 Results of RNA Isolations

Following total RNA and miRNA isolations from 14 atherosclerotic plaques and from 14 plaque adjacent control regions, the purity and quantity of all RNA samples were assessed. The purity of RNA samples are evaluated via the ratio of absorbance at 260 nanometer(nm) and 280 nm, which is referred as the 260/280 value. A 260/280 value of approximately 2 is accepted as pure for RNA samples [188].

Table 3.2 shows the concentrations (ng/μl) and purities (260/280) of total RNAs isolated from 14 carotid atherosclerotic plaque samples.

Table 3.2 : The Quantity and Purity of Plaque Total RNA Samples.

Patient Sample No	RNA Concentration(ng/μl)	260/280 nm
1	209.8	2.08
2	65.5	2.03
3	39.8	2.12
4	195.0	1.91
5	60.9	1.98
6	196.0	2.00
7	186.0	2.02
8	201.0	2.07
9	106.6	2.06
10	34.1	1.99
11	79.8	2.02
12	134.8	1.86
13	18.7	2.01
14	22.9	2.05

The total RNA concentrations and purities of 14 control samples are given in Table 3.3.

Table 3.3 : The Quantity and Purity of Control Total RNA Samples.

Patient Sample No	RNA Concentration(ng/μl)	260/280 nm
1	75.5	1.94
2	43.0	1.96
3	49.0	2.00
4	18.1	1.85
5	24.4	1.92
6	124.1	1.99
7	140.8	2.04
8	9.9	1.92
9	28.5	2.08
10	49.0	1.98
11	76.1	1.99
12	274.7	1.99
13	26.2	2.07
14	10.3	1.96

Table 3.4 : The Quantity and Purity of Plaque MiRNA Samples.

Patient Sample No	RNA Concentration(ng/μl)	260/280 nm
1	101.5	1.99
2	52.4	2.02
3	94.6	2.04
4	22.8	1.92
5	21.5	1.96
6	140.8	1.99
7	135.1	2.05
8	50.9	2.01
9	140.3	2.02
10	27.7	2.01
11	36.7	2.02
12	30.8	1.88
13	29.3	1.87
14	86.1	2.03

Table 3.5 : The Quantity and Purity of Control MiRNA Samples.

Patient Sample No	RNA Concentration(ng/μl)	260/280 nm
1	24.8	2.03
2	44.2	1.83
3	48.12	2.04
4	28.3	1.97
5	24.2	1.79
6	43.5	1.87
7	81.6	1.93
8	26.1	1.86
9	44.4	1.88
10	73.6	1.90
11	50.7	1.95
12	26.7	1.84
13	41.5	1.94
14	20.2	1.93

Table 3.4 shows the concentrations (ng/μl) and purities (260/280) of miRNAs isolated from 14 carotid atherosclerotic plaque samples.

Finally, the results of miRNA isolations performed on 14 adjacent to plaque control samples are demonstrated in Table 3.5, with the concentrations (ng/μl) and purities (260/280).

3.4 Results of Real Time Quantitative PCR

Table 3.6 : Analysis of C_T Values for Calculating *Spred1* Relative Gene Expression.

Sample	18S	Spred1				
	C_T Mean	C_T Mean	ΔC_T Mean	$\Delta\,\Delta\,C_T$	$2^{\Delta\Delta C_T}$	Fold Change
Control 1	9.04	26.93	17.89			
Sample 1	7.80	25.29	17.49	-0.40	1.32	0.28
Control 2	13.28	36.01	22.73			
Sample 2	9.33	27.72	18.39	-4.34	20.25	3.01
Control 3	7.71	25.60	17.89			
Sample 3	9.65	28.95	19.30	1.41	0.38	-0.98
Control 4	10.38	27.83	17.45			
Sample 4	13.56	31.53	17.97	0.52	0.70	-0.36
Control 5	11.80	29.23	17.43			
Sample 5	9.37	28.15	18.78	1.35	0.39	-0.94
Control 6	8.39	26.11	17.72			
Sample 6	8.28	26.44	18.16	0.44	0.74	-0.30
Control 7	5.51	24.56	19.05			
Sample 7	7.15	24.91	17.76	-1.29	2.45	0.89
Control 8	10.00	27.18	17.18			
Sample 8	5.51	23.58	18.07	0.89	0.54	-0.62
Control 9	9.89	31.04	21.15			
Sample 9	8.00	27.04	19.04	-2.11	4.32	1.46
Control 10	10.90	32.59	21.69			
Sample 10	15.29	34.19	18.90	-2.79	6.92	1.93
Control 11	13.59	35.10	21.51			
Sample 11	10.83	29.53	18.70	-2.81	7.01	1.95
Control 12	11.75	29.24	17.49			
Sample 12	10.93	29.32	18.39	0.90	0.54	-0.62
Control 13	15.76	38.51	22.75			
Sample 13	15.56	35.26	19.70	-3.05	8.28	2.11
Control 14	14.54	30.81	16.27			
Sample 14	9.41	27.34	17.93	1.66	0.32	-1.15

Following qRT-PCR runs, the raw C_T values were obtained from the Mx3005P Real-Time PCR instrument software. The qRT-PCR runs for genes *Egfl7*, *Spred1*,

Table 3.7 : Analysis of C_T Values for Calculating *Pik3r2* Relative Gene Expression.

Sample	18S	Pik3r2				
	C_T Mean	C_T Mean	ΔC_T Mean	$\Delta\,\Delta\,C_T$	$2^{\Delta\Delta C_T}$	Fold Change
Control 1	9.04	25.28	16.24			
Sample 1	7.80	24.96	17.16	0.92	0.53	-0.64
Control 2	13.28	34.92	21.64			
Sample 2	9.33	26.49	17.16	-4.48	22.32	3.11
Control 3	7.71	24.55	16.84			
Sample 3	9.65	27.35	17.70	0.86	0.55	-0.60
Control 4	10.38	27.14	16.76			
Sample 4	13.56	30.79	17.23	0.47	0.72	-0.33
Control 5	11.80	28.24	16.44			
Sample 5	9.37	26.96	17.59	1.15	0.45	-0.80
Control 6	8.39	26.36	17.97			
Sample 6	8.28	26.29	18.01	0.04	0.97	-0.23
Control 7	5.51	24.04	18.53			
Sample 7	7.15	24.79	17.64	-0.89	1.85	0.62
Control 8	10.00	27.25	17.25			
Sample 8	5.51	23.50	17.99	0.74	0.60	-0.51
Control 9	9.89	30.77	20.88			
Sample 9	8.00	27.72	19.72	-1.16	2.23	0.80
Control 10	10.90	29.26	18.36			
Sample 10	15.29	34.03	18.74	0.38	0.77	-0.26
Control 11	13.59	34.82	21.23			
Sample 11	10.83	29.26	18.43	-2.80	6.96	1.94
Control 12	11.75	29.44	17.69			
Sample 12	10.93	29.10	18.17	0.48	0.72	-0.33
Control 13	15.76	36.25	20.49			
Sample 13	15.56	34.61	19.05	-1.44	2.71	1.00
Control 14	14.54	30.78	16.24			
Sample 14	9.41	26.94	17.53	1.29	0.41	-0.89

Pik3r2 and *18S* in plaques and controls were completed first. Since reactions were run in duplicates, mean C_T values of the duplicates were calculated.

Relative quantification employing the $2^{\Delta\Delta C_T}$ method [175] was used for analyses of *Egfl7*, *Spred1* and *Pik3r2* gene expression levels. The calculated mean C_T values of target genes and the housekeeping gene 18S in controls and in plaque samples were exported to Microsoft Excel for calculation of ΔC_T, $\Delta\Delta C_T$, the relative quantification ratio R ($2^{\Delta\Delta C_T}$) and finally for the log-transformed ratios showing the fold change. The tables 3.6, 3.7 and 3.8 show the calculated ΔC_T, $\Delta\Delta C_T$, $2^{\Delta\Delta C_T}$) and fold change values for *Spred1*, *Pik3r2* and *Egfl7*, respectively.

Table 3.8 : Analysis of C_T Values for Calculating *Egfl7* Relative Gene Expression.

Sample	18S	Egfl7				
	C_T Mean	C_T Mean	ΔC_T Mean	$\Delta\,\Delta\,C_T$	$2^{\Delta\Delta C_T}$	Fold Change
Control 1	9.04	29.21	20.17			
Sample 1	7.80	26.92	19.12	-1.05	2.07	0.73
Control 2	13.28	36.84	23.56			
Sample 2	9.33	28.52	19.19	-4.37	20.68	3.03
Control 3	7.71	29.04	21.33			
Sample 3	9.65	29.24	19.59	-1.74	3.34	1.21
Control 4	10.38	32.47	22.09			
Sample 4	13.56	35.47	21.91	-0.18	1.13	0.12
Control 5	11.80	33.48	21.68			
Sample 5	9.37	34.1	24.73	3.05	0.12	-2.11
Control 6	8.39	27.75	19.36			
Sample 6	8.28	26.59	18.31	-1.05	2.07	0.73
Control 7	5.51	26.53	21.02			
Sample 7	7.15	24.81	17.66	-3.36	10.27	2.33
Control 8	10.00	32.67	22.67			
Sample 8	5.51	23.99	18.48	-4.19	18.25	2.90
Control 9	9.89	32.11	22.22			
Sample 9	8.00	26.25	18.25	-3.97	15.67	2.75
Control 10	10.90	37.37	26.47			
Sample 10	15.29	39.62	24.33	-2.14	4.41	1.48
Control 11	13.59	36.88	23.29			
Sample 11	10.83	30.05	19.22	-4.07	16.80	2.82
Control 12	11.75	30.93	19.18			
Sample 12	10.93	30.12	19.19	0.01	0.99	-0.41
Control 13	15.76	42.14	26.38			
Sample 13	15.56	37.99	22.43	-3.95	15.45	2.74
Control 14	14.54	34.61	20.07			
Sample 14	9.41	30.04	20.63	0.56	0.68	-0.39

Next, miRNA 126-3p and miRNA 126-5p expression levels were calculated by the relative quantification method. After running qRT-PCR experiments for miRNA 126-3P in plaques and controls, miRNA 126-5p was completed as well. Once the raw C_T values were obtained from the instrument software, mean C_T values of the duplicates were calculated. Similar to the target genes, the mean C_T values of miRNA 126-3p and -5p were exported to Microsoft Excel for calculation of ΔC_T, $\Delta\Delta C_T$, $2^{\Delta\Delta C_T}$ and the fold change. The tables 3.9 and 3.10 show the calculated ΔC_T, $\Delta\Delta C_T$, $2^{\Delta\Delta C_T}$) and fold change values for miRNA 126-3p and miRNA 126-5p, respectively.

Table 3.9 : Analysis of C_T Values for Calculating *miR 126-3p* Expression in plaques relative to controls.

Sample	18S	miR 126-3p				
	C_T Mean	C_T Mean	ΔC_T Mean	$\Delta\,\Delta\,C_T$	$2^{-\Delta\Delta C_T}$	Fold Change
Control 1	18.17	20.62	2.45			
Sample 1	19.92	21.98	2.06	-.39	1.31	0.27
Control 2	20.11	22.80	2.69			
Sample 2	22.73	24.93	2.20	-0.49	1.40	0.34
Control 3	20.24	22.66	2.42			
Sample 3	21.61	19.63	-1.98	-4.40	21.11	3.05
Control 4	18.64	24.06	5.42			
Sample 4	20.75	29.19	8.44	3.02	0.12	-2.09
Control 5	20.66	23.22	2.56			
Sample 5	17.29	18.64	1.35	-1.21	2.31	0.84
Control 6	18.42	21.50	3.08			
Sample 6	20.31	21.72	1.41	-1.67	3.18	1.16
Control 7	21.52	24.67	3.15			
Sample 7	21.25	22.04	0.79	-2.36	5.13	1.64
Control 8	20.46	21.08	0.62			
Sample 8	19.77	20.55	0.78	0.16	0.90	-0.11
Control 9	22.11	24.35	2.24			
Sample 9	23.22	22.52	-0.70	-2.94	7.67	2.04
Control 10	21.40	35.15	13.75			
Sample 10	25.54	28.65	3.11	-10.64	1,595.73	7.38
Control 11	23.79	31.47	7.68			
Sample 11	22.20	18.92	-3.28	-10.96	1,992.00	7.60
Control 12	23.21	28.83	5.62			
Sample 12	18.82	18.93	0.11	-5.51	45.57	3.82
Control 13	23.20	34.74	11.54			
Sample 13	23.15	22.19	-0.96	-12.50	5,792.62	8.66
Control 14	24.57	24.54	-0.03			
Sample 14	23.23	29.82	6.59	6.62	0.01	-4.59

3.5 Analyses of Target Gene and MicroRNA Expression Levels in Carotid Artery Atherosclerotic Plaques

Fourteen advanced atherosclerotic plaque samples obtained from carotid artery disease patients and fourteen respective adjacent regions of plaques as control samples were used in this thesis study.

Following RNA isolation from tissue samples and cDNA synthesis steps, real time quantitative PCR analyses were performed to investigate the expression levels of *Eglf7*, miR-126-5p, *Spred1*, *Pik3r2* and miR-126-3p in plaque and control samples.

Table 3.10 : Analysis of C_T Values for Calculating *miR 126-5p* Expression in plaques relative to controls.

Sample	18S	miR 126-5p				
	C_T Mean	C_T Mean	ΔC_T Mean	$\Delta\,\Delta\,C_T$	$2^{\Delta\Delta C_T}$	Fold Change
Control 1	32.01	30.48	-1.53			
Sample 1	29.93	30.36	0.43	1.96	0.26	-1.36
Control 2	26.66	35.79	9.13			
Sample 2	27.52	33.20	5.68	-3.45	10.93	2.39
Control 3	33.70	33.38	-0.32			
Sample 3	36.63	36.70	0.07	0.39	0.76	-0.27
Control 4	22.94	22.60	-0.34			
Sample 4	21.27	21.22	-0.05	0.29	0.82	-0.20
Control 5	26.24	34.42	8.18			
Sample 5	33.31	34.72	1.41	-6.77	109.14	4.69
Control 6	31.46	31.18	-0.28			
Sample 6	31.90	31.14	-0.76	-0.48	1.39	0.33
Control 7	24.30	28.04	3.74			
Sample 7	33.60	34.03	0.43	-3.31	9.92	2.29
Control 8	21.56	22.25	0.69			
Sample 8	27.42	22.88	-4.54	-5.23	37.53	3.63
Control 9	21.99	21.45	-0.54			
Sample 9	20.56	23.49	2.93	3.47	0.09	-2.41
Control 10	21.40	35.15	13.75			
Sample 10	24.60	37.76	13.16	-0.59	1.51	0.41
Control 11	23.79	31.47	7.68			
Sample 11	24.29	29.48	5.19	-2.49	5.62	1.73
Control 12	23.21	28.83	5.62			
Sample 12	21.04	29.83	8.79	3.17	0.11	-2.20
Control 13	23.20	34.74	11.54			
Sample 13	23.98	32.00	8.02	-3.52	11.47	2.44
Control 14	24.57	24.54	-0.03			
Sample 14	23.94	23.94	4.00	0.30	0.98	-0.92

The $2^{\Delta\Delta C_T}$ method was used for comparing *Eglf7*, *Spred1* and *Pik3r2* gene expression levels as well as miR 126 -3p and miR 126-5p expression levels of plaque samples to those of control samples and for normalizing to the levels of the endogenous controls 18S rRNA and snRNA U6.

3.6 *Egfl7* and MiR-126-5p

The expression levels of *Egfl7* in fourteen plaque samples are reported individually with respect to those of the control samples. *Egfl7* was up-regulated in eleven samples as is demonstrated in Figure 3.13.

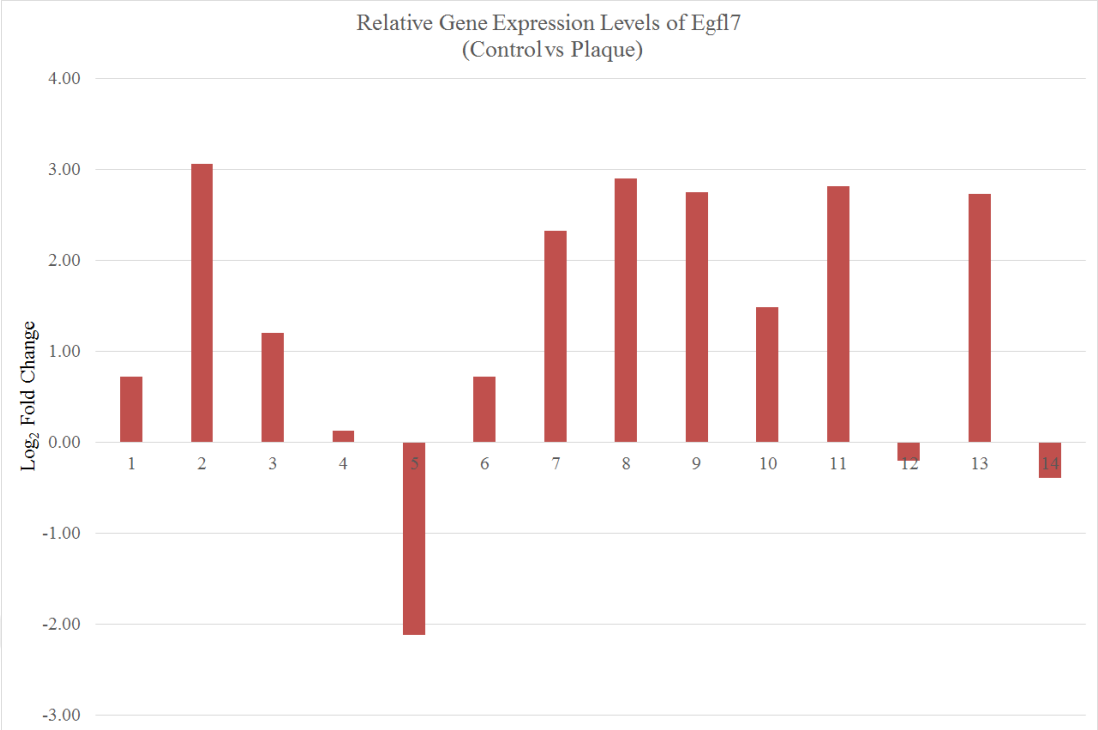

Figure 3.13 : Relative gene expression levels of *Egfl7*.

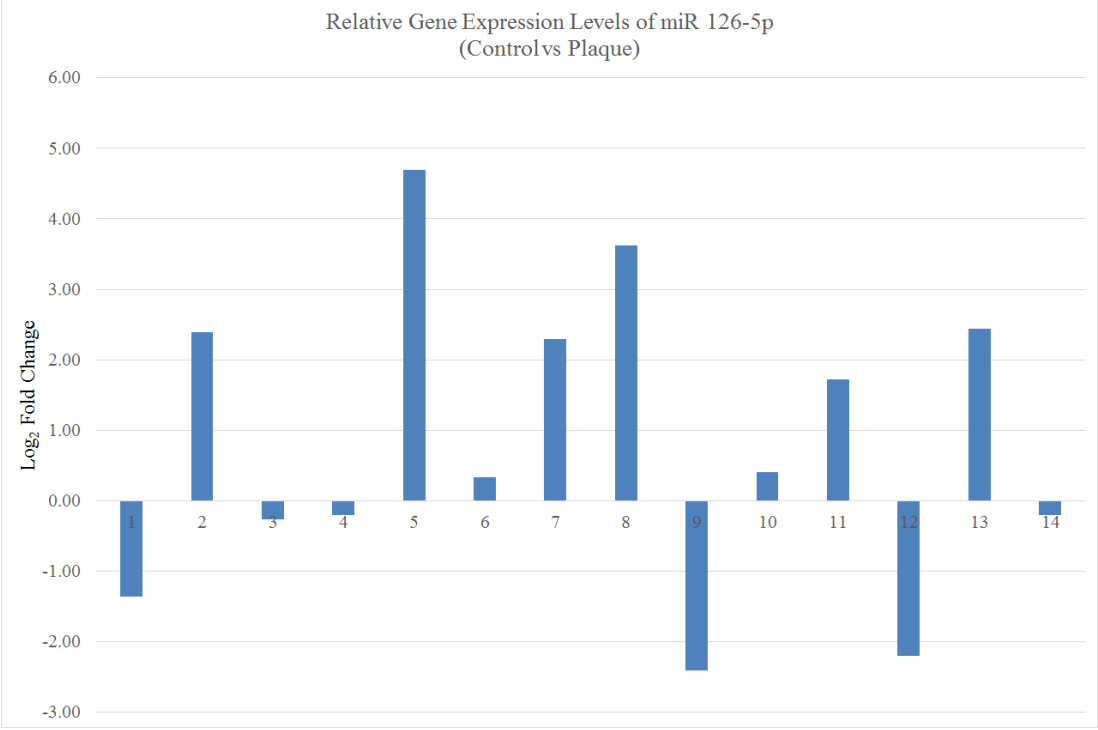

Figure 3.14 : Relative gene expression levels of miRNA 126-5p.

Similarly, miR-126-5p expression levels in fourteen plaque samples are demonstrated individually with respect to those of the control samples. MiRNA 126-5p appeared to

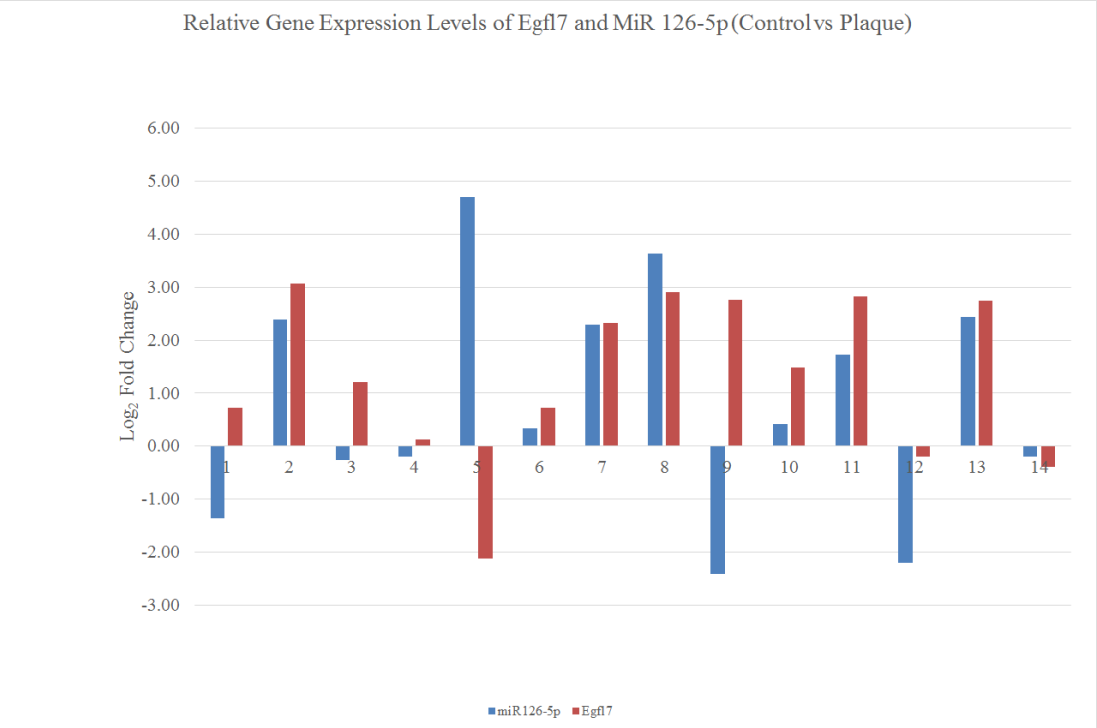

Figure 3.15 : Relative gene expression levels of *Egfl7* and miRNA 126-5p.

be down-regulated in six samples, while up-regulation was observed in eight samples. The results are shown in Figure 3.14.

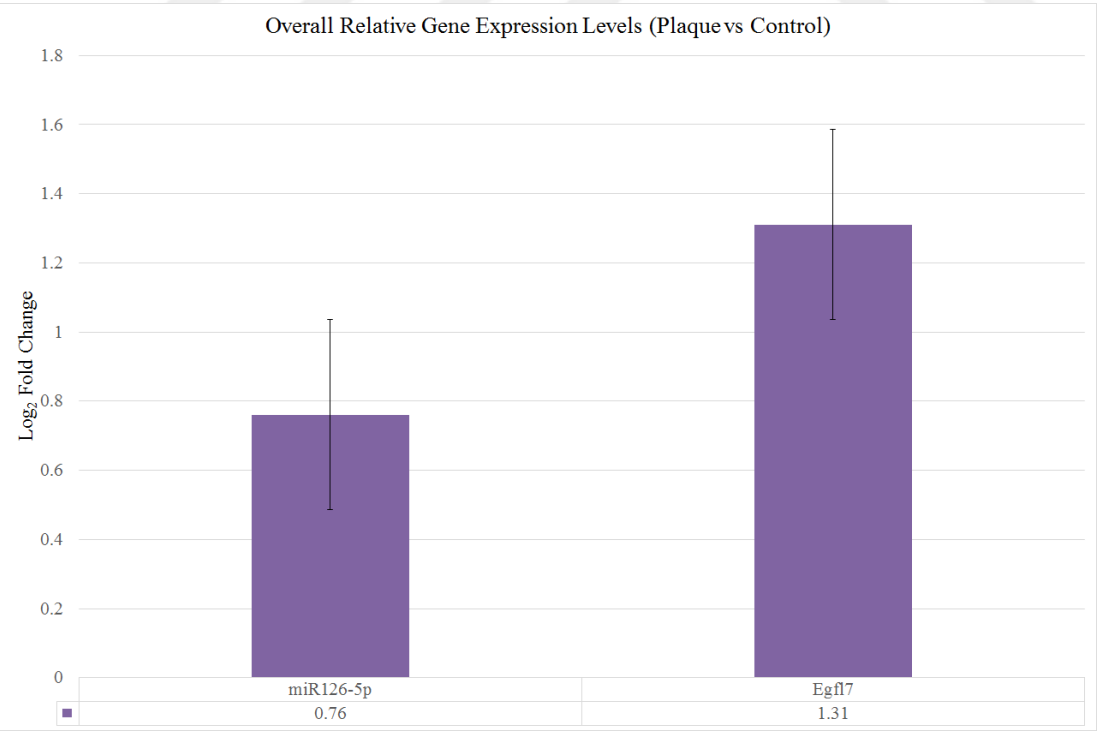

Figure 3.16 : Overall relative gene expression levels of *Egfl7* and miRNA 126-5p.

The expression levels of miRNA 126-5p was expected to coincide with the expression levels of *Egfl7*. Although this was the case in nine samples, *Egfl7* up-regulation was observed in four samples, while miRNA 126-5p was down-regulated in the very same samples. Conversely, miRNA 126-p was up-regulated in one sample, as *Egfl7* was down-regulated in the same sample. The possible reasons for differences in expression levels will be discussed later in the following section. The comparison of expression levels of miRNA 126-5p and *Egfl7* are shown in Figure 3.15.

The overall Egfl7 mRNA expression was higher in plaques (p <0.001) compared to controls. MiR 126-5p expression was also higher in plaques with respect to control regions, but not reaching statistical significance (p= 0.751) as demonstrated in Figure 3.16.

3.6.1 Statistical correlation of *egfl7* and miR-126-5p expression levels with clinical parameters of patients

The correlation between *Egfl7* and miR-126-5p expression levels and clinical parameters of patients was investigated using IBM SPSS Statistics for Windows, version 20.0 software. To to find a correlation between relative gene expression levels of *Egfl7* and miR-126-5p and the clinical parameters of patients, the study population was separated into two groups; those with low expression levels of *Egfl7* and miR-126-5p and those with high expression levels of *Egfl7* and miR-126-5p. For the expression levels of *Egfl7* and miR-126-5p, above 1.32 and 0.82 fold change cut-off values, respectively, were considered as "high" level changes. Expression levels of *Egfl7* and miR-126-5p in patients did not appear to be statistically associated with the tested cardiovascular risk factors such as being female, BMI>25, smoking, family history of cardiovascular diseases, hypertension, diabetes, presence of a cerebrovascular event, coronary artery and peripheral artery disease (Table 3.1). However, a positive correlation was found between the high levels of miR-126-5p and elevated levels of LDL cholesterol (p=0.023) (Table 3.11).

Table 3.11 : Correlation of miR-126-5p and *Egfl7* Gene Expression Levels of Carotid Artery Plaque Samples Compared to Control Samples with Clinical Parameters of Patients.

Parameters	miR-126-5p Expression (n=14)		*Egfl7* Expression (n=14)		[1]P value	[2]P value
	Low <0.82 (n=8)	High >0.82 (n=6)	Low <1.32 (n=7)	High >1.32 (n=7)		
Demographics						
Female Gender (%)	66.7%	33.3%	55.6%	44.4%	0.580	p >0.05
BMI >25 (%)	57.1%	42.9%	50%	50%	p >0.05	p >0.05
Smoking (%)	57.1%	42.9%	42.9%	57.1%	p >0.05	p >0.05
Family History of CVD (%)	62.5%	37.5%	50%	50%	p >0.05	p >0.05
Diabetes (%)	66.7%	33.3%	66.7%	33.3%	0.627	0.592
Hypertension (%)	63.6%	36.4%	54.5%	45.5%	0.538	p >0.05
Cerebrovascular Event, n (%)	66.7%	33.3%	44.4%	55.6%	0.580	p >0.05
Coronary Artery Disease, n (%)	40%	60%	66.7%	33.3%	0.580	0.592
Peripheral Artery Disease, n (%)	50%	50%	50%	50%	p >0.05	p >0.05
Statin Use (%)	80%	20%	80%	20%	0.301	0.266
Laboratory Values						
Total Cholesterol ± SD (mg/dl)	189.5 ± 57.07	296.0 ± 87.0	196.8 ± 71.9	250.2 ± 82.9	0.087	0.223
HDL-Cholesterol ± SD (mg/dl)	38.6 ± 7.9	41.0 ± 13.85	43.7 ± 13.1	35.5 ± 5.02	0.718	0.167
LDL-Cholesterol ± SD (mg/dl)	103.2 ± 44.9	193.33 ± 68.5	109.2 ± 61.6	174.4 ± 68.1	0.023	0.085
Triglycerides ± SD (mg/dl)	175.1 ± 69.1	172.6 ± 132.2	146.8 ± 80.1	201.2 ± 109.5	0.968	0.311

3.7 *Spred1*, *Pik3r2* and MiR-126-3p

The analyses of *Spred1*, *Pik3r2* and miR-126-3p expression levels in plaque and control samples was performed with real time quantitative PCR.

The relative gene expression levels of *Spred1* in fourteen plaque samples is demonstrated in Figure 3.17. *Spred1* up-regulation was observed in six of the atherosclerotic plaque samples, while it was down-regulated in eight samples.

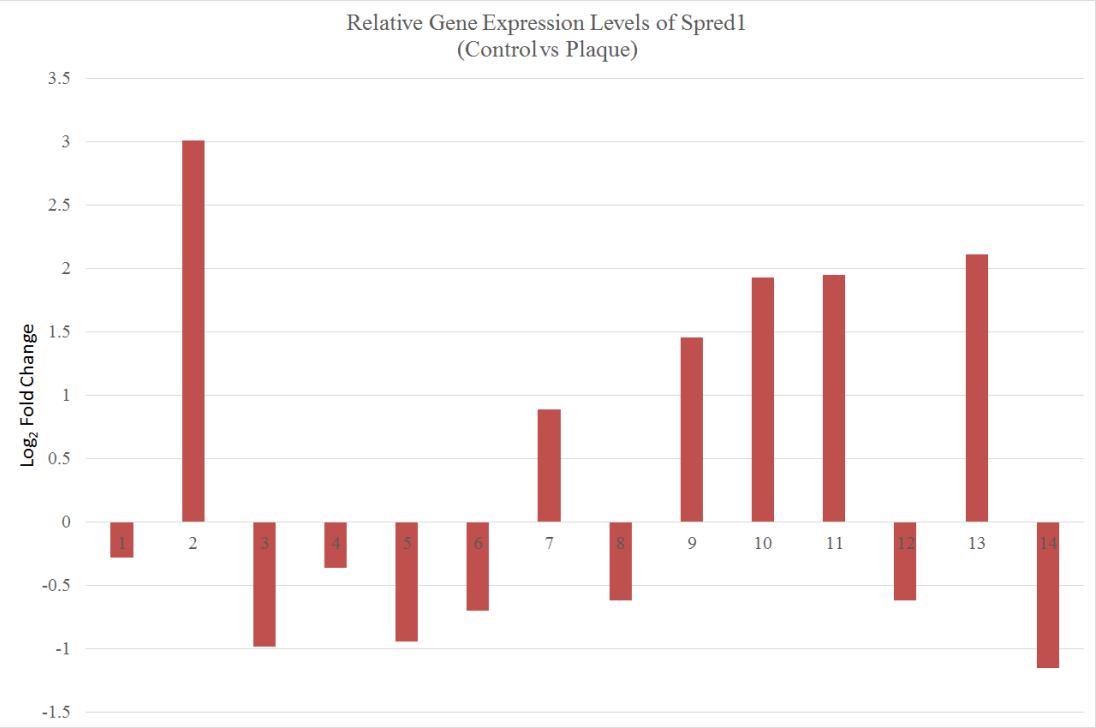

Figure 3.17 : Relative gene expression levels of *Spred1*.

In addition, *Pik3r2* was down-regulated in nine out of fourteen samples. The gene expression levels of *Pik3r2* in each of the samples is shown in Figure 3.18.

In the case of miR-126-3p, up-regulation was observed in eleven samples as demonstrated in Figure 3.19.

As expected, decrease in expression levels of *Spred1* and *Pik3r2* was observed whenever miR-126-3p expression level increased as shown in Figure 3.20.

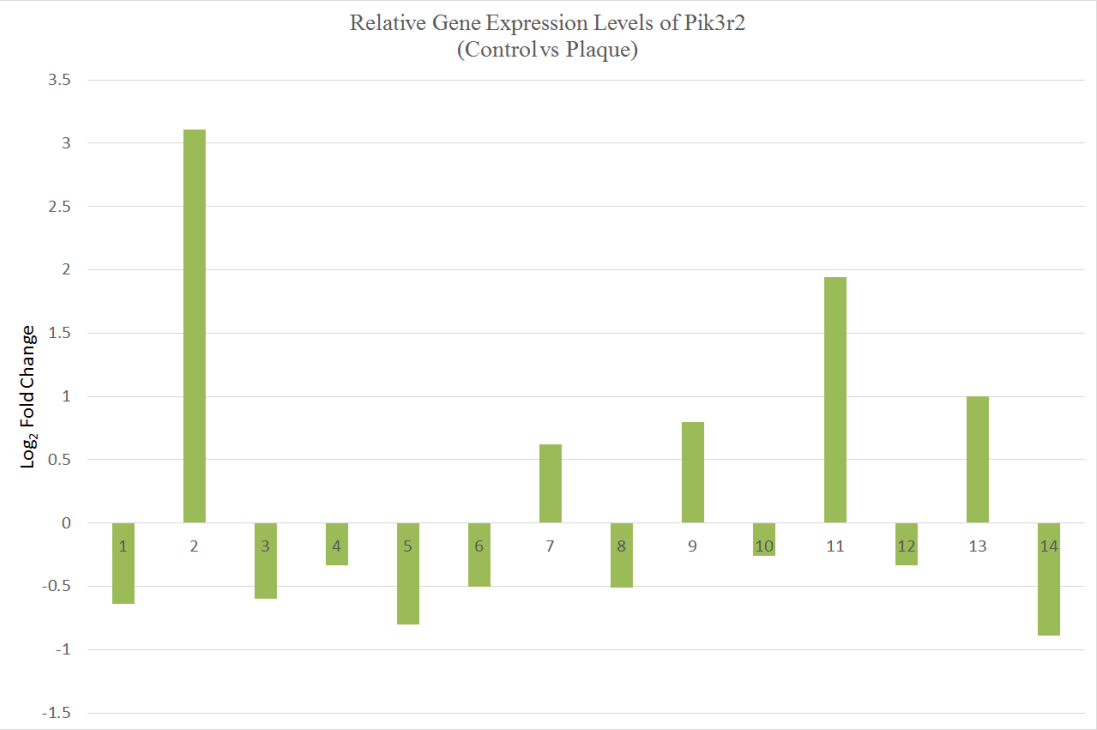

Figure 3.18 : Relative gene expression levels of *Pik3r2*.

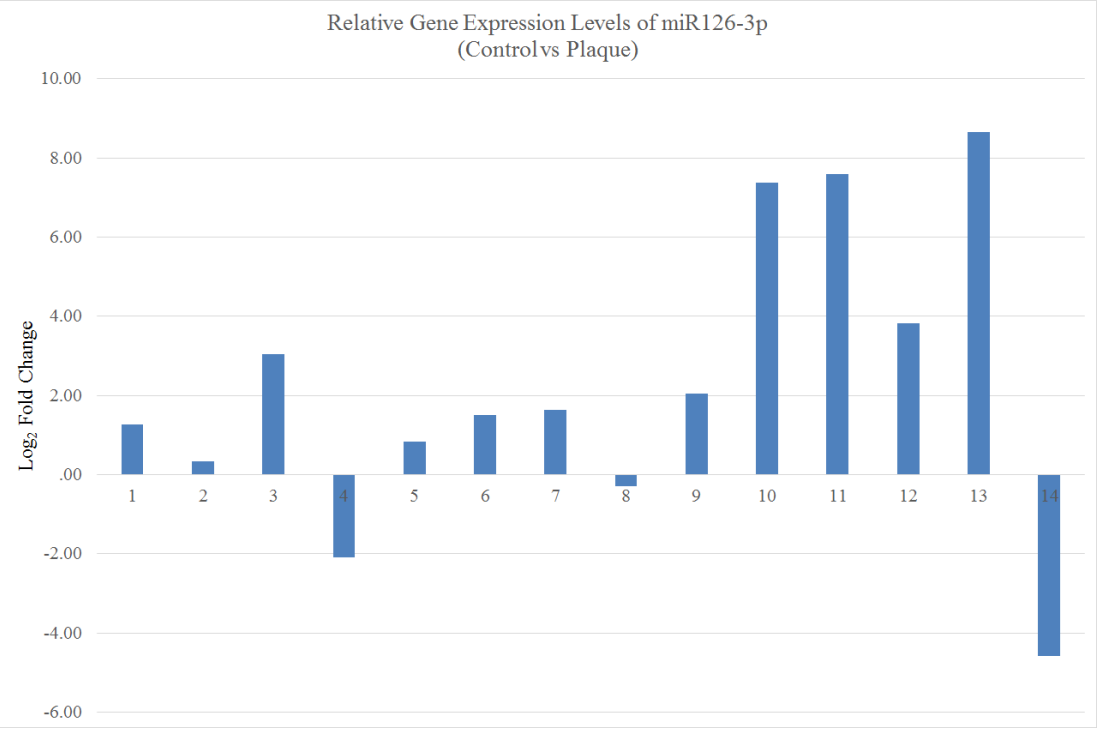

Figure 3.19 : Relative gene expression levels of miRNA 126-3p.

Compared to the control regions, the overall miR-126-3p expression was higher in plaques (p <0.05) as depicted in Figure 3.21. *Spred1* and *Pik3r2* overall expressions

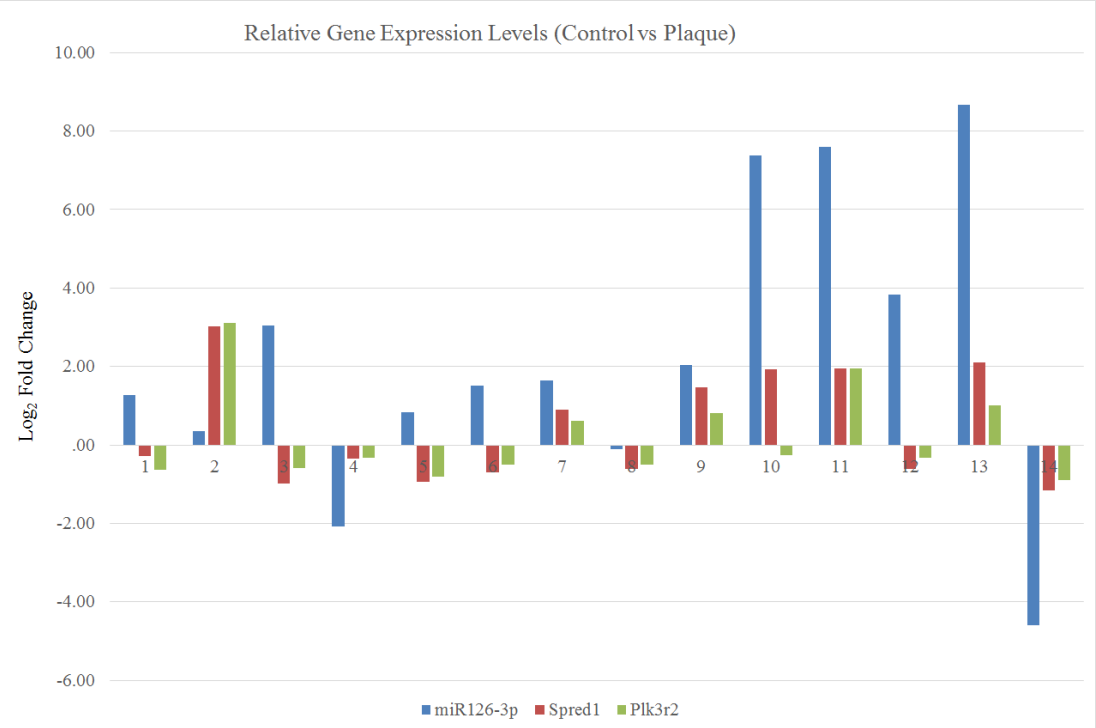

Figure 3.20 : Relative gene expression levels of Spred1, Pik3r2 and miRNA 126-3p.

were also higher in plaques with respect to control regions, but the up-regulations were not found to be statistically significant.

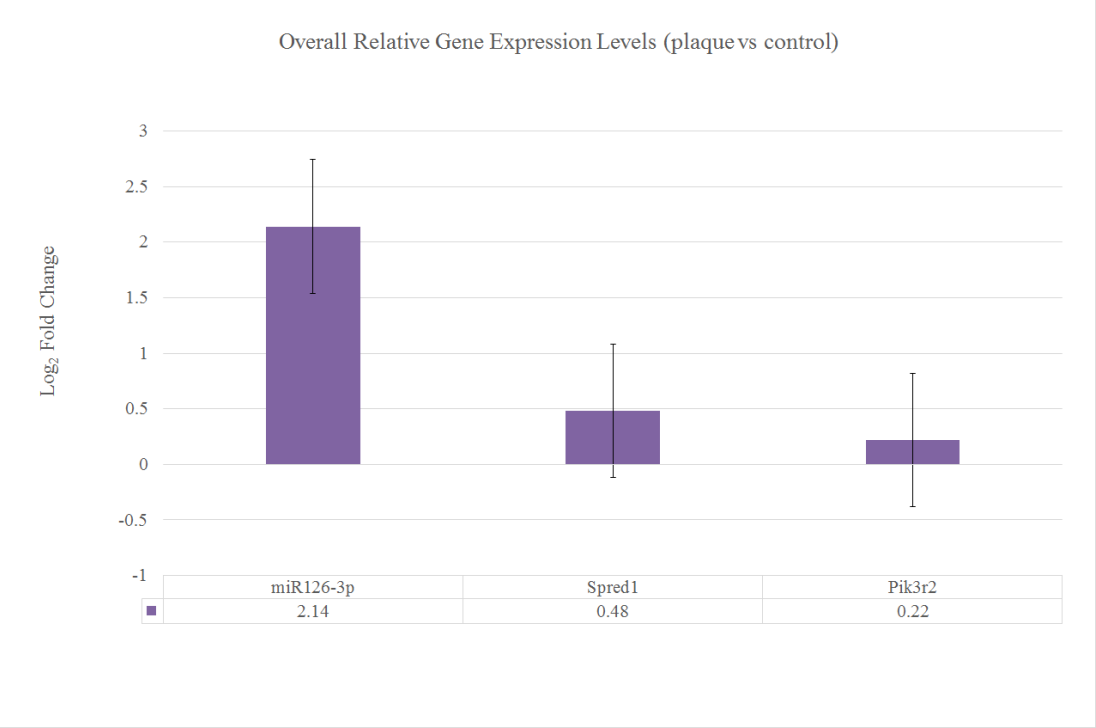

Figure 3.21 : Overall relative gene expression levels of Spred1, Pik3r2 and miRNA 126-3p.

3.7.1 Statistical correlation of *spred1*, *pik3r2* and miR-126-5p expression levels with clinical parameters of patients

The correlation between *Spred1*, *Pik3r2*, miR-126-5p expression levels and clinical parameters of patients was also investigated using IBM SPSS Statistics for Windows, version 20.0 software. To to find a correlation between relative gene expression levels of studied genes as well as the miRNA and the clinical parameters of patients, the study population was separated into two groups; those with low expression levels of *Spred1*, *Pik3r2* and miR-126-3p and those with high expression levels of *Spred1*, *Pik3r2* and miR-126-3p. Expression levels of *Spred1*, *Pik3r2* and miR-126-3p in patients did not appear to be statistically associated with the tested cardiovascular risk factors such as being female, BMI>25, smoking, family history of cardiovascular diseases, hypertension, diabetes, presence of a cerebrovascular event, coronary artery and peripheral artery disease. On the other hand, as demonstrated in Table 3.12, a positive correlation between the up-regulation of *Pik3r2* and elevated levels of LDL cholesterol was found (p=0.044).

Table 3.12 : Correlation of miR-126-3p *Spred1*, *Pik3r2* gene expression levels of carotid artery plaque samples compared to control samples with clinical parameters of patients.

Parameters	miR-126-3p Expression (n=14)		*Spred 1* Expression (n=14)		*Pik3r2* Expression (n=14)		[1]P value	[2]P value	[3]P value
	Downregulation	Upregulation	Downregulation	Upregulation	Downregulation	Upregulation			
Demographics									
Female Gender (%)	11.1%	88.9%	55.6%	44.4%	33.3%	66.7%	0.505	p >0.05	p >0.05
Smoking (%)	28.6%	71.4%	28.6%	71.4%	42.9%	57.1%	0.192	0.286	p >0.05
Family History of CVD (%)	25.0%	75.0%	45.5%	54.5%	75.0%	25.0%	p >0.05	p >0.05	0.580
Diabetes (%)	0.0%	100.0%	50.0%	50.0%	66.7%	33.3%	0.209	p >0.05	p >0.05
Hypertension (%)	18.2%	81.8%	50.0%	50.0%	36.4%	63.6%	p >0.05	p >0.05	p >0.05
Cerebrovascular Event, n (%)	11.1%	88.9%	33.3%	66.7%	44.4%	55.6%	0.505	0.266	0.580
Coronary Artery Disease, n (%)	40.0%	60.0%	80.0%	20.0%	80.0%	20.0%	0.505	0.266	0.580
Peripheral Artery Disease, n (%)	0.0%	100.0%	25.0%	75.0%	50.0%	50.0%	0.505	0.559	0.580
Statin Use (%)	20.0%	80.0%	60.0%	40.0%	100.0%	0.0%	p >0.05	p >0.05	0.086
Laboratory Values									
Total Cholesterol ± SD (mg/dl)	166.00 ± 69.93	239.27 ± 77.39	212.86 ± 67.70	234.29 ± 94.17	200.89 ± 64.58	264.40 ± 94.85	0.166	0.634	0.160
HDL-Cholesterol ± SD (mg/dl)	36.33 ± 12.74	40.55 ± 10.29	43.71 ± 13.18	35.57 ± 5.02	41.10 ± 12.69	37.00 ± 4.58	0.558	0.153	0.504
LDL-Cholesterol ± SD (mg/dl)	100.00 ± 55.10	153.27 ± 72.61	121.00 ± 61.80	162.71 ± 77.98	114.11 ± 55.74	191.80 ± 72.85	0.265	0.289	0.044
Triglycerides ± SD (mg/dl)	177.00 ± 121.05	173.27 ± 95.69	168.99 ± 98.99	179.86 ± 101.35	172.00 ± 87.81	177.80 ± 121,57	0.955	0.833	0.919

4. DISCUSSION

Blood vessel formation during the embryonic and post-natal stages or in the adults takes place by the processes of vasculogenesis and angiogenesis, respectively. Vasculogenesis refers to the generation of blood vessels from scratch, via the differentiation of progenitor cells into the endothelial cells. In vasculogenesis, endothelial progenitor cells in circulation are directed to sites of endothelial damage and differentiate to endothelial cells for damage repair [189]. Conversely, in the process of angiogenesis, new blood vessels are formed from the existing vasculature. Angiogenesis includes the processes of proliferation, sprouting, migration of endothelial cells, as well as vessel pruning and remodelling [190].

Endothelium regulates angiogenesis and responds to extracellular factors, such as vascular endothelial growth factor (VEGF). VEGF regulates proliferation and survival of endothelial cells, by activating many signalling pathways, including the mitogen-activated protein kinase (MAPK) and phosphinositide 3-kinase (PI3K) pathways. On the other hand, angiogenesis can be inhibited as well by extracellular factors. For example, Notch ligand Delta-like 4 functions to antagonize VEGF signalling [191]. There are two types of angiogenesis; physiological and pathological. As opposed to well-functioning vasculature in physiological angiogenesis, the blood vessels formed by pathological angiogenesis have irregular patterns of branching and uneven distribution. The new vessels formed by pathological angiogenesis in conditions, such as wound healing, tumors and atherosclerosis, are also leaky and hyperpermeable [192].

MicroRNAs (miRNAs), small noncoding RNA molecules that regulate gene expression at the post-transcriptional level, take part in every step of atherosclerosis development, including endothelial dysfunction, cellular adhesion, development of plaques and rupture of plaques [148]. MiRNAs have crucial roles in modulating angiogenesis as well [193]. The key role of microRNAs as angiogenic signal transduction pathway modulators has been suggested due to the recently discovered

miRNAs that are exclusively expressed in endothelial cells [194, 195]. Changes in expression of microRNAs as a result of growth factor stimulation, or hypoxia also implicate the role of miRNAs as one of the core components of the angiogenic signalling [196]. MiRNA regulation of the angiogenic pathways in human disease states, such as atherosclerosis, remain to be elucidated.

4.1 MiR-126-3p, *Spred1* and *Pik3r2* expression levels in carotid artery atherosclerotic plaques

The function of many cells that are part of the cardiovascular system are controlled by miRNAs, which either regulate vascular homeostasis or contribute to diseases of the cardiovascular system [197, 198].

The expression of 168 miRNAs were analyzed in human umbilical vein endothelial cells (HUVECs) by Kuehbacher *et al.* (2008), with the aim of understanding the role of miRNAs in functions of endothelial cells. The miRNAs that were found to have high expression levels in HUVECs included miR-21, miR-126, mir-221, and mir-222 [199]. Fish *et al.* (2008) have isolated day 7 and day 14 embryoid bodies from mouse embryonic stem cells and conducted microarray studies for miRNA profiling. They have demonstrated that several miRNAs including, miR-126, miR-146b, miR-197, miR-615 and miR-625 were enriched in these cells. Among them, miR-126 was found to be the miRNA with the highest expression. The expression of miR 126-3p along with the same set of miRNAs were confirmed *in vivo* as well, using qRT-PCR and RNA samples isolated from E10.5 mouse embryos [154].

miR 126-3p is expressed from the intron of *Egfl7* [165], which plays a crucial role in vasculogenesis and angiogenesis [200]. In endothelial cells, angiogenic signalling is modulated by MiR-126.

Two very important study have demonstrated its crucial role in vessel development. The first one that was conducted by Fish *et al.*(2008) have demonstrated the effects of the loss of function of miR-126 *in vitro*. In the study, the levels of miR 126-3p and its passanger strand miR 126-5p were decreased by introducing a morpholino (MO) antisense to miR-126 to HUVECs. Following transfection, levels of both miRNAs were decreased, although miR 126-3p basal level was higher compared to its passanger

strand. In addition, it was observed that the EGFL7 protein levels, encoded by the host gene of both miRNAs, were not affected. The endothelial cells with decreased levels of miR 126 had a higher rate of proliferation and the migration of cells in response to VEGF were inhibited. Knock down of miR 126 affected the formation of stable capillary tubes *in vitro* as well. To see the effects of MiR 126 knock down *in vivo*, zebrafish fertilized eggs were injected with morpholinos. As a consequence of loss of miR 126, defective blood vessels and cranial hemorrhages were observed. The collapsed lumens and defective organization of endothelial tubes revealed in miR-126 morphants suggested the necessity of miR-126 expression for vessel development and integrity [154].

Wang *et al.*(2008) investigated miR 126 functions *in vivo*, by creating miR 126 null mice. Vascular malformations, such as growth failure of the cranial vessels were observed in miR 126 null mice. Systemic edema, multifocal hemorrhages and ruptured blood vessels were present in embryos before they were dead. In addition, decreased response to angiogenic stimuli, as well as delay in angiogenic sprouting were also observed [157]. This study suggested the key role of miR 126 for vessel integrity as well, and led to the further investigation of role of miR 126 in angiogenic signalling.

In the comprehensive study by Fish *et al.*(2008), many miRNA target prediction algorithms were used and based on binding sites, some of the predicted targets of miR 126-3p were validated by cloning parts of their 3 UTR regions into the 3 UTR of a luciferase construct. Then, in HeLa cells, which do not express miR-126, whether miR-126 influnces luciferase expression was checked. As a result, miR-126 was found to target Sprouty-related EVH1 domain-containing protein 1 (SPRED1), vascular cell adhesion molecule 1 (VCAM1), and regulatory subunit 2 of PI3K (PIK3R2). SPRED1 and PIK3R2 were suggested to negatively regulate endothelial cellular signaling by affecting the MAPK and PI3K signaling pathways, while MiR 126-3p, by targeting the negative regulators of VEGF signalling, was suggested to promote angiogenesis. It was also demonstrated that the knock down of miR 126 constricted the phosphorylation of ERK and AKT induced by VEGF [154]. To test whether miR-126 was crucial for the normal endothelial cell response to angiogenic growth factors, Wang *et al.*(2008) transfected HUVECs with miR-126 expressing adenovirus and investigated the activation of MAP kinase by growth factors. The MAP kinase activation was

detected by phosphorylation of ERK1/2. The phosphorylation of ERK1/2 in response to growth factors was increased 2-fold by miR-126 expressing adenovirus transfection, while miR-126 knockdown resulted in decreased ERK phosphorylation [157]. The findings of Wang *et al.* and Fish *et al.* altogether point out to the important role that miR-126 plays in angiogenic signalling, by increasing MAP kinase activation in VEGF pathway. The putative model developed by Fish *et al.*(2008) by the help of their *in vitro* studies and *in vivo* studies in zebrafish is given in Figure 4.1. The model explains how miR 126-3p aids angiogenesis in endothelial cells via its targets *Spred1* and *Pik3r2*, the negative regulators of VEGF pathway.

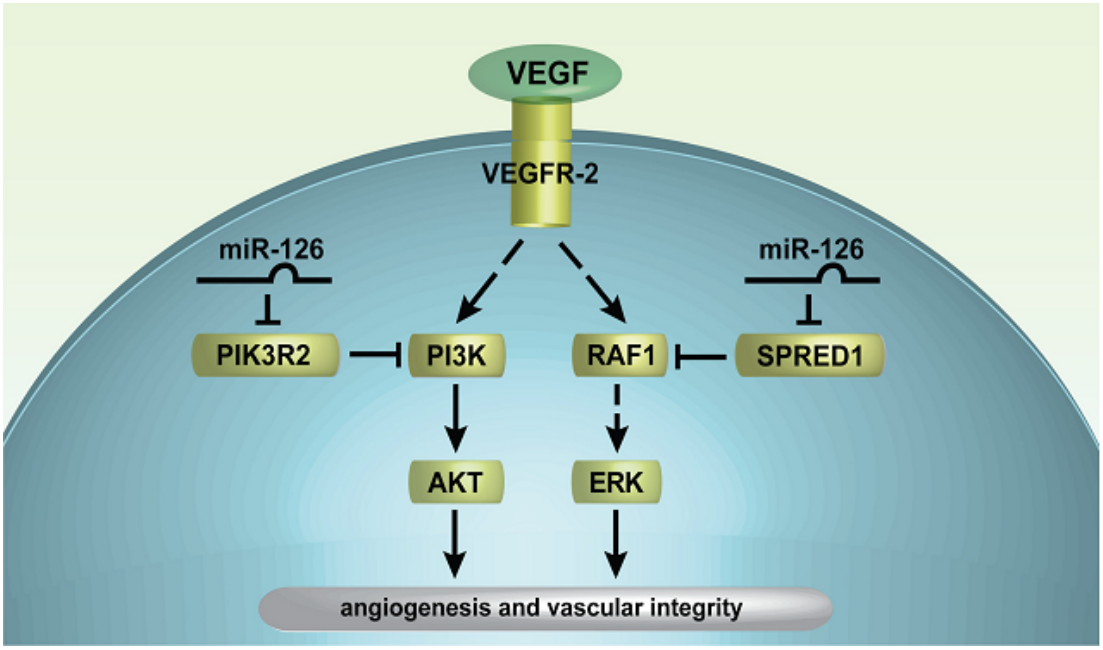

Figure 4.1 : MiR 126-3p and negative regulators of VEGF pathway biogenesis [154].

Based on the hypothesis that vascular cell adhesion molecule 1 (VCAM1) expression and vessel inflammation is regulated by a miRNA, Harris *et al.*(2008) have set out to investigate the miRNA expression profile of endothelial cells via microarray. Alongside with the studies of Fish *et al.* and Wang *et al.*, they too have demonstrated that HUVECs were enriched with miR 126 the most. Using bioinformatic analysis, they have demonstrated that VCAM1 was targeted by miR 126-3p and verified the predicted miR 126-3p target by luciferase assays. When endogenous miR 126-3 levels were decreased in endothelial cells and cells stimulated with tumor necrosis factor- α (TNF-α), expression of VCAM-1 increased. On the other hand, VCAM-1 expression was observed to decrease in respnse to overexpression of miR-126. In

addition, diminished levels miR-126 resulted in an increase in adherence of leukocytes to endothelial cells. All together, their results suggest a miRNA modulation of inflammation in atherosclerosis [201].

Following their study published in 2008, in which they had demonstrated the role of miR 126-3p in inflammation through VCAM1, Harris *et al.*(2010) investigated the roles of E26 transformation-specific sequence (ETS) factors, members of a family of transcription factors that take are known to be expressed in endothelial cells and take part in processes, including vessel formation, remodelling and inflammation. By cloning an upstream regulatory region of the Egfl7/miR-126 locus into a luciferase expression plasmid and transfecting the construct into a variety of endothelial cells, such as HUVECs and human aortic endothelial cells (HAECs), and into non-endothelial cells, they performed luciferase assays. It was observed that the upstream regulatory region of *Egfl7*/miR-126 locus, which preserves an Ets binding site, was crucial for transactivation of miR-126 host gene, *Egfl7*. Many members of ETS family were over-expressed in endothelial cells, with the aim of finding the family members that take part in regulating miR-126 expression. Their data revealed that ETS-1 and ETS-2 take part in regulation of miR-126 expression [202].

Kruppel-like factor 2 (KLF2), a member of the zinc finger family of transcription factors taking part in regulation of processes, such as development and differentiation [203], was found to transcriptionally regulate miR-126 by Nicoli *et al.*(2010). On the other hand, Hergenreider *et al.*(2012) observed in adult endothelial cells that shear stress induced KLF2 did not function to augment miR-126 levels [204, 205].

It has been shown by various studies that angiogenesis is boosted by different population of cells derived from peripheral blood mononuclear cells (PBMC). Cells with different surface markers, identified by the use of cluster of differentiation (CD) system, are separated as different subpopulations. $CD34^+$ or $CD14^+$ surface expression differences have been widely utilized for separation of the PBMC subpopulations [206–208]. Specifically, it has been demonstrated that $CD34^+$ PBMCs help angiogenesis during ischemic conditions [209, 210]. In the study performed by Mocharla *et al.*(2013), differential miRNA expression profiles of $CD34^+$ /$CD14^+$, $CD34^+$ /$CD14^-$, $CD34^-$ /$CD14^+$ and $CD34^-$ /$CD14^-$ PBMC subsets was investigated. In comparison to $CD34^-$ subsets, $CD34^+$ subsets displayed more proangiogenic

properties. In the supernatants of $CD34^+$ subsets of PBMCS, miR 126 expression was higher. MiR 126 was shown to be secreted in microvesicles. MiR 126 expression in $CD34^+$ PBMC fractions of diabetic patients was diminished, leading to the conclusion that the proangiogenic properties of endothelial progenitor cells was abolished in diabetes [211].

Similar studies have been done demonstrating the effects of Diabetes on functions of endothelial progenitor cells which take part in endothelial damage repair. Meng *et al.*(2012) have provided evidence on the effect of the downregulation miR-126 in endothelial progenitor cells obtained from diabetic patients. Their study has shown that, through its target, SPRED1, miR 126 disrupts the endothelial progenitor cell mediated function [212]. Jansen *et al.*(2012) have shown *in vivo*, that endothelial microparticles (EMPs)released from endothelial cells undergoing apoptotis have roles in endothelial repair after vascular injury. The EMPs were assessed for their miRNA content and the highest miRNA expression level was observed with miR 126. It was found that the transport of miR-126 was accomplished by EMP. When diabetic conditions were simulated *in vitro*, the miR 126 levels of EMPs were found to be lower and the capacity of the EMPs to repair endothelial damage was reduced. Same results were obtained when circulating EMPs obtained from 176 coronary artery disease patients with and without diabetes were investigated for their miR 126 expression levels [213]. From all the studies reviewed above, it can be concluded that miR 126 plays an atheroprotective role. In the review by Chistiakov *et al.*(2016), miRNA up-regulation is suggested to enhance angiogenesis by recruiting endothelial progenitor cells to sites with endothelial damage, as a response to vascular injury and ischemic conditions caused by atherosclerosis [153].

While miR 126-3p has atheroprotective roles in endothelial cells under physiological conditions, it has been shown by Zhou *et al.*(2013) to contribute to formation of neointima in vascular smooth muscle cells (VSMCs) in co-cultures with endothelial cells. Mir 126-3p displayed its pro-atherosclerotic affects by increasing the apoptosis and proliferation of VSMCs [214]. A potential function of miR 126-3p was suggested in the arterial remodelling observed in advanced atherosclerosis. Up-regulation of miR 126-3p in the intra-plaque regions due to the hypoxic conditions and static flow inside these regions, is suggested to trigger neoangiogenesis inside atherosclerotic plaques,

as well as proliferation and migration of VSMCs [153]. This thesis study provides the first evidence on miR 126-3p expression in deep intraplaque regions.

Although it has been identified an an angiomiR, miR 126-3p was studied in cell culture or in model organisms, such as Zebrafish or mice. Of the very limited studies performed on samples obtained from cardiovascular disease patients, Tampere Vascular Study conducted by Raitoharju *et al.*(2011) is of importance. In this study, the miRNA expression profile in 12 human carotid, femoral and coronary artery atherosclerotic plaques was investigated and compared to 6 control arteries. MiR-21, -34a, -146a, -146b-5p, and -210 expression levels were found to be significantly up-regulated [215]. In the study by Fichtlscherer *et al.*(2010), 36 coronary artery disease patients and 17 healthy controls were investigated for the levels of circulating miRNAs. Of the 10 endothelial specific circulating miRNAs, miR 126 was observed to be down-regulated in coronary artery disease patients and in coronary artery disease patients with Diabetes, compared to healthy controls [216].

In the present thesis study, the expression levels of miR 126-3p and its targets, *Spred1*, *Pik3r2* and *Egfl7* have been evaluated in human carotid artery atherosclerotic plaques and controls. To the knowledge, this study is the first to evaluate the expression levels of miR 126-3p, its targets Spred1, Pik3r2 and its host gene *Egfl7* in atherosclerotic plaques from human carotid arteries. It was hypothesized that the expression levels of miR 126-3p would be higher in atherosclerotic plaques, as an indicator of ongoing neoangiogenesis in the plaques, while the negative regulators of the VEGF pathway would be down-regulated, due to miR 126-3p mediated repression.

As expected, miR 126-3p expression was higher in 11 out of 14 atherosclerotic plaque samples compared to plaque adjacent controls. *Spred* and *Pik3r2* were down-regulated in 8 and 9 samples compared to controls, respectively. Considering the overall gene expression levels, miR 126-3p was up-regulated 2.14 fold in plaques in comparison to controls, while *Spred1* and *Pik3r2* were also up-regulated by 0.18 and 0.22 fold, respectively.

The correlation of cardiovascular disease risk factors with gene expression levels were investigated, with the aim of determining the factors that affect the levels of miR 126-3p and its targets. Mir 126-3p up-regulation was observed in 88.9 % of female

patients, in 71.4% of smokers, in 75% of patients with a family history of cardivascular disease, in all of the diabetic patients, in 81.8% of hypertensive patients, in 88.9% of patients who have had a cerebrovascular event, in 60% of the patients who have coronary artery disease as well and in all of the patients with peripheral artery disease. The only significance correlation, though, was found between up-regulation of *Pik3r2* and high low-density cholesterol levels(p=0.044).

Larger study groups are needed to confirm these data, since the analysis of single risk factors (such as diabetes or coronary artery disease) is impeded by the relatively small numbers of patients. In addition, underlying molecular mechanisms of miR 126-modulated dysregulation need further investigation, particularly on the phosphorylation status of the downstream signalling molecules, including AKT and ERK, to be able to fully grasp the affect of miR-126 on VEGF regulated angiogenesis in atherosclerosis. The interaction of miR-126 with other miRNAs and signalling molecules needs to be elucidated. Furthermore, larger scale studies must be performed on more than one arterial system, with the aim of determining the specif regulation of angiogenesis by miRNAs in different arterial systems. The potential use of miRNAs as biomarkers to monitor plaque neoangiogenesis and disease progression, as well as the development of miRNA-based therapies are promising areas of research in the battle against the fatal consequences of atherosclerosis.

4.2 MiR-126-5p expression in carotid artery atherosclerotic plaques

What leads to initiation of atherosclerosis and its progression remain to be elucidated. However, endothelial dysfunction plays a key role in the initiation of atherosclerotic process. The arterial branching sites, where there is disturbed laminar flow, are susceptible to endothelial dysfunction, therefore plaque formation [56, 217]. Endothelial cells need laminar flow derived shear stress for differentiating into their specific phenotype, which is non-replicative [218–220]. MiRNAs, in addition to their many other functions, have been investigated for their roles in shear stress. MiR 126-3p has been studied widely and named as an angiomiR, while there are not many studies focusing on the role of its passanger strand, miR 126-5p.

In the comprehensive study by Schober *et al.*(2014), it was shown in rats that in response to injury, high miR-126-5p levels were needed for the regeneration

of endothelial cells. It was demonstrated that miR-126-5p executes its effect by suppressing *Delta like ligand 1 (Dlk1)*. DLK1 is suggested to damage angiogenesis by inhibiting NOTCH1 activation [221]. The role of non-canonical NOTCH ligand DLK1 as an angiogenesis inhibitor had been demonstrated previously by Rodriguez *et al.*(2012). They have demonstrated in many models and organisms, including bovine aortic endothelial cells, porcine aortic endothelial cells, mouse lung endothelial cells, HUVECs, zebrafish and mice, that DLK1 functions as an antiangiogenic molecule via NOTCH pathway [222]. In addition to this non-canonical NOTCH ligand, three canonical ligands, JAGGED 1, DLL1, and DLL4, are known to take on important tasks in angiogenesis in endothelial cells [223]. It was demonstrated by many studies that whenever DLL44 is in, NOTCH pathway functions to inhibit angiogenesis [126]. Rodriguez *et al.* mentioned in their study that the interplay between these canonical NOTCH ligands and the non-canonical ones are complex, therefore requires further investigation. In the study of Schober *et al.*, the impaired angiogenesis results in increased cell permeability and in increased influx of low-density lipoprotein into the vessel wall, causing atherosclerotic lesion formation. It was observed that high levels of miR-126-5p in endothelial cells localized in non-predilection sites resulted in endothelial cell proliferation which managed to overcome the anti-proliferative effects caused by hyperlipidemia, while downregulation of miR-126-5p via upregulation of *Dlk1* in sites with the disturbed flow prevented proliferation of endothelial cells in the hyperlipidemic conditions [221]. At normal arterial sites with laminar blood flow, apoptosis of endothelial cells is limited, therefore endothelial cells do not require proliferation [224]. On the other hand, to be able to keep the endothelial monolayer intact, proliferation of endothelial cells is needed at predilection sites with turbulent flow. Upon disturbed laminar flow, miR 126-5p is suggested to be down-regulated, causing a decrease in the proliferation of endothelial cells residing in such areas. This conditions is worsened in hyperlipidemic conditions [225]. Therefore, expression of miR 126-5p is suggested to have a protective role against endothelial injury [221]. The putative model explaining the atheroprotective effects of miR 126-3p and -5p reviewed by Boon and Dimmeler (2014) is shown in Figure 4.2 [226].

The role of miR 126-5p in leukocyte adhesion and trafficking was investiged in HUVECs and in mice by Poissonnier *et al.*(2014). In the study, it was shown that

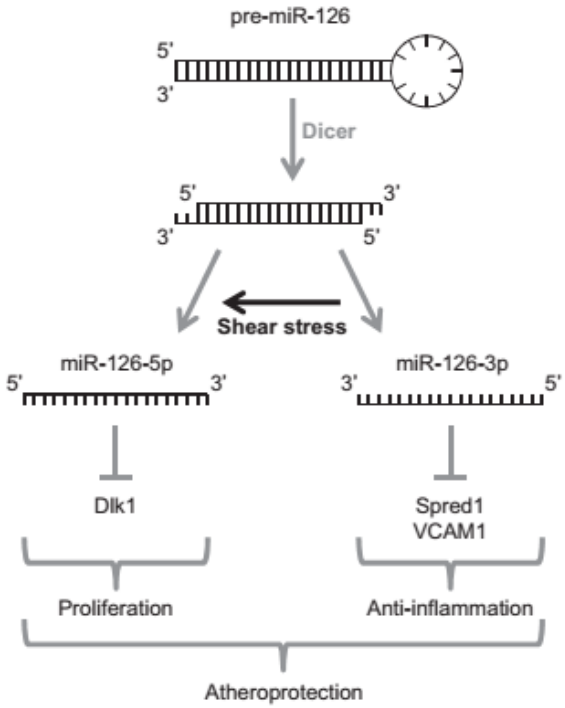

Figure 4.2 : MiR 126-3p, miR 126-5p and their suggested atheroprotective roles [226].

expression of miR126-5p was highest in blood vessels both *in vivo* and *in vitro*. The new roles of promoting adhesion of leukocytes and repressing their transendothelial migration were assigned to miR 126-5p via knock out and over-expression of the miRNA in HUVECs. The activated leucocyte cell adhesion molecule (ALCAM), encoding an adhesion molecule that takes part in leucocyte transendothelial migration and *SetD5* gene with no assigned functions were discovered as novel targets of miR 126-5p. By regulating *SetD5* and *Alcam*, miR 126-5p was suggested to aid in leucocyte adhesion and to repress transendothelial migration, respectively [227].

In this study, which provides the first evidence on the expression levels of miR 126-5p and its host gene, *Egfl7* in human carotid artery atherosclerotic plaques and controls, the levels of miR 126-5p expression were evaluated using real time quantitative PCR and relative gene expression strategy. It was hypothesized that together with its passenger strand miR 126-3p, miR 126-5p would be up-regulated in atherosclerotic plaques due to the intraplague angiogenesis, a hallmark of advanced atherosclerosis.

MiR 126-5p was found to be up-regulated in eight patient plaque samples compared to controls, while down-regulation was observed in six of the patient samples. Considering the overall expression, miR 126-5p expression was higher in plaques

compared to control samples. Moreover, to determine the cardivascular risk factors that influence miR 126-5p expression levels, the correlation between the cardiovascular disease risk factors and gene expression levels were investigated. While performing these analyses, the patients samples were split into two groups; plaque samples with low and high miR 126-5p expression levels. The samples with relative expression fold change values above 0.82 were considered in the high expression level group. Risk factors, including being female, BMI $\geq$ 25, smoking, family history of cardiovascular diseases, hypertension, Diabetes, presence of a cerebrovascular event, coronary artery and peripheral artery disease were not significantly correlated with miR 126-5p expression levels. However, a positive correlation was found between high levels of LDL cholesterol and upregulation of miR-126-5p (p=0.023). The positive correlation between high levels of LDL cholesterol and high levels of miR 126-5p, therefore increased angiogenesis can be explained by the role of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in angiogenesis. LOX-1 is the receptor for facilitation of uptake of oxidized low density lipoprotein [228]. Recently, Jiang *et al.*(2011) have associated LOX-1 with angiogenesis [229]. High levels of LDL cholesterol could be attributed to LOX-1 up-regulation, which also plays a role in angiogenesis, though further investigation is required for linking the angiogenesis related up-regulation of miR-126 to LOX-1 expression.

Carotid artery plaque samples were surgically removed from different locations, and the locations of the plaques is an important parameter, since atherosclerotic plaques are observed more frequently in sites with disturbed laminar flow. The differences in expression levels of miR-126-5p could be attributed to the fact that plaques were removed from sites with high shear stress as opposed to non-predilection sites. Furthermore, the degree of narrowing (stenosis) in the carotid artery is another parameter affecting the expression of miR 126-5p, since miR-126 expression is highly influenced by vascular injury. Further investigations with larger patient samples are needed. Patient samples must be studied considering the different degrees of stenosis, to be able to fully understand the roles of angiogenesis in different stages of atherosclerosis. Moreover, *Dlk1* levels need to be assessed together with miR-126-5p expression in healthy tissues and in atherosclerotic plaques removed from high-shear-stress or non-predilection sites, since these studies have not been conducted

in samples from patients with cardiovascular diseases. Such studies would aid in better understanding of the molecular mechanisms of angiogenesis regulation via miR 126-5p and its targets.

4.3 *Egfl7* expression in carotid artery atherosclerotic plaques

Egfl7 was first identified as a novel gene by Fitch*et al.*(2004). They have demonstrated in mice that *Egfl7* expression begins in early stages of embryonic development, and is restricted to vascular tissues and endothelium in adults [230]. Following this study, the same research group, including Campagnolo *et al.*(2005), have investigated the expression patterns of Egfl7 in the adult vasculature, such as the pregnant uterus, and in two different arterial injury models. Egfl7 expression was observed to be exclusive to the endothelium. Up-regulation of *Egfl7* was shown in the pregnant uterus, where there is increased angiogenesis. In addition, up-regulation was demonstrated in response to vascular injury, both in the balloon injury models in rats and the ferric chloride injury model of mouse carotid arteries [165].

The relationship between *Egfl7* and miR 126 was discovered by the study of Kuhnert *et al.*(2008), in which deletion of MiR-126 in mice (without altering *Egfl7* expression) caused major defects in blood vessel development, such as delays in angiogenesis, haemorrhage and early embryonic lethality [156]. As reviewed by Nikolic, Plate and Schmidt (2010), *Egfl7* encodes miR 126-3p and -5p, along with the 30 kD protein specific to vascular endothelium. In contrast to its high levels of expression during development, *Egfl7* is downregulated in the vascular system with birth and kept at low levels only in vessels on the lung, heart, kidney, spleen and uterus under normal physiological conditions. In tissues that require high levels of angiogenesis, such as uterus during pregnancy, or in conditions of pathological angiogenesis, including neoangiogenesis in atherosclerosis or tumor angiogenesis, *Egfl7* is up-regulated in adults [160].

As strongly suggested by the data available, EGFL7 functions as a suppressor of NOTCH activity in the endothelium, which negatively regulates the proliferation of endothelial cells and inhibition of endothelial cell sprouting [231]. Nichol *et al.*(2010) have over-expressed *Egfl7* in transgenic mice and observed partial lethality and hemorrhaging in mice as a consequence of the over-expression of *Egfl7*. The

defects observed were suggested to be resulting from excessive angiogenesis. On the other hand, in human primary endothelial cells, the knockdown of *Egfl7* had an inhibitory effect on endothelial cell proliferation, sprouting, and migration, as a result of NOTCH inhibition. Their results provided evidence on the physical interaction of EGFL7 and NOTCH, suggesting the inhibitory role of EGFL7 on NOTCH activity [161].

The expression of miR-126 host gene, *Egfl7* was studied together with miR 126-3p, miR 126-5p, *Spred*1 and *Pik3r2* in human carotid atherosclerotic plaques in this thesis work. To the knowledge, this study represents the first report to assess the levels of *Egfl7* and miR-126-5p in human carotid atherosclerotic plaques.

Egfl7 expression is exclusive to the endothelium. EGFL7 acts only on endothelium and plays key roles in angiogenesis. It was hypothesized that *Egfl7* would be up-regulated in atherosclerotic plaques in response to the vascular injury caused by progressive atherosclerosis. As expected, *Egfl7* up-regulation was observed in eleven plaque samples out of fourteen.

Although the role of new blood vessel development within advanced atherosclerotic plaques remains to be understood, its presence in advanced atherosclerosis is considered as a marker of ongoing disease activity and a factor that produces high-risk plaques. Neo-angiogenesis causes stenosis, hemorrhage and thrombosis within the plaques, contributing to atherosclerosis progression. Conversely, an atheroprotective role for neo-angiogenesis was proposed. The proangiogenic miRNAs, cytokines and endothelial progenitor cells play roles in the repair of the endothelium via angiogenesis. Despite the opposing roles of *Egfl7*, its higher expression levels could possibly be contributing to disease progression via boosting neo-angiogenesis in diseased vessels, which is already a hallmark of atherosclerosis.

Further studies elucidating the role of *Egfl7* in pathological angiogenesis might potentially help atherosclerotic plaque regression and stabilize vulnerable plaques in order to prevent the devastating effects of thrombosis. In addition, Egfl7 can be considered as a promising prognostic marker of intraplaque neoangiogenesis, therefore aid in monitoring the unstable plaques, as well as preventing vulnerable plaque rupture. Moreover, *Egfl7* based anti-angiogenesis therapy could be offered as a promising

strategy in eliminating intraplaque neoangiogenesis, and stabilization of plaques that are prone to rupture, therefore preventing the devastating consequences of advanced atherosclerosis.

4.4 The use of microRNAs as prognostic biomarkers atherosclerosis

An molecule that is to be used as a biomarker must be expressed in high levels in the tissue of interest. It needs to be present at low concentrations in the blood and other body fluids as well [233, 234]. These biomarkers are released into the blood following tissue injury, therefore their detection is facilitated via a blood-based assay [235, 236]. For example, biomarkers used to diagnose heart disease, such as creatine kinase–MB isoenzymes and cardiac troponins, have been developed via targeted analyses of myocardial proteins [237]. For diagnosing acute myocardial infarctions and prognosis after coronary artery bypass surgery, cardiac troponins are the 'gold standard' [238].

It was shown by many studies that biomarkers are valuable tools for monitoring progression of atherosclerosis. Such widely used biomarkers include, proinflammatory biomarkers or biomarkers of plaque destabilization and rupture, which are very important in prediction of up-coming cardiovascular events. Some of the biomarkers which are under investigation include, c-reactive protein, cytokines interleukin 6 (IL-6) and IL-8, oxidized LDL, glutathion peroxidase, myeloperoxidase, matrix metalloproteinases and monocyte chemoattractant protein-1 [239].

The role of miRNAs in cardiovascular development and modulation, as well as in diseases of the vascular system has been highlighted by several studies. In the recent years, the functions of miRNAs in the regulating vessel biology and pathophysiology have been widely investigated [240]. Currently research suggests the use miRNAs as sensitive and early biomarkers for cardiovascular diseases. MiRNA expression is often specific to tissues. They represent high sensitive and specific biomarkers, because they can be detected by real-time PCR. Furthermore, there is evidence that upon tissue injury, they are released into blood stream via microvesicles, therefore they can be found in circulation at consistent and reproducible levels [241]. However, their low amount in blood constitutes a problem in concentration and quality measurements, therefore is a challenge that needs further work [242].

The miRNAs 126-3p and -5p, as well as their host gene *Egfl7* can be considered as promising prognostic markers for monitoring neoangiogenesis in advanced atherosclerosis. Further work investigating the levels of circulating miR 126-3p and miR 126-5p in peripheral blood of patients with cardiovascular diseases is needed to assess the use of these circulating miRNAs as biomarkers for advanced atherosclerotic disease.

5. CONCLUSIONS

Cardiovascular diseases are the leading cause of death in the world and in our country today. The underlying pathological condition of cardiovascular diseases is atherosclerosis.

Atherosclerosis is the chronic, progressive and immunoinflammatory disease of the medium- and large-size arteries. It is a complex disease which involves interactions of multiple genetic and environmental factors. Starting with birth, atherosclerotic process progresses slowly, causing diseases, such as coronary artery disease, stroke, peripheral artery disease. In the inflammatory process of atherosclerosis, the outer layer of arteries, the intima layer, gets thicker with lipid rich material (atheroma) and connective tissue (sclerosis). According to the "Response to Injury" hypothesis of atherosclerosis, potential sources of injury, such as hyperlipidemia, hypertension, metabolites, infections and mechanical factors such as shear stress are suggested to cause the initiation of atherosclerosis. The pathological changes in atherosclerosis occur due to molecular events of endothelial dysfunction, monocyte adherence and entry into the vessel wall, monocyte development into foam cells, smooth muscle cell migration and proliferation, advanced plaque formation with necrotic core and neoangiogenesis, and thrombosis as a result of plaque rupture. Following rupture of a vulnerable plaque, thrombus formation resulting in stroke or a myocardial infarction causes deaths or disabilities. Atherosclerosis would possibly be a more benign disease if thrombus formation could be prevented. The investigation of molecular mechanisms resulting in angiogenesis in advanced plaques, a hallmark event of atherosclerosis, has been the main focus of this thesis study.

Angiogenesis is the process of new vessel formation from existing ones. The main signalling pathways that control angiogenesis in cells are the vascular endothelial growth factor (VEGF) pathway and Notch signalling. Intraplaque neoangiogenesis has been linked with progressive atherosclerotic disease. The signalling pathways contributing to angiogenesis represent potential targets for therapies aiming to prevent

intraplaque neoangiogenesis. This thesis study focuses on the role of angiogenic mechanisms related to vascular endothelial growth factor pathway and the mechanisms that interact with this pathway, such as microRNAs, the small, non-coding RNAs that post-transcriptionally regulate many cellular processes, including angiogenesis. *Egfl7*, a major player of angiogenesis that is expressed exclusively in embryonic and adult vasculature, encodes two endothelial-specific miRNAs: miR 126-3p and miR 126-5p. This thesis study was conducted with the aim of understanding the role of miR-126 signalling in angiogenesis and its contribution to carotid artery disease progression. For this purpose, the expression levels of miR 126-3p, the targets of miR 126-3p, miR 126-5p and *Egfl7* were investigated in 14 human carotid artery atherosclerotic plaques and control samples.

The results have shown higher miR 126-3p expression in 11 out of 14 atherosclerotic plaque samples compared to plaque adjacent controls. *Spred* and *Pik3r2* were down-regulated in 8 and 9 samples compared to controls, respectively. Considering the overall gene expression levels, miR 126-3p was up-regulated 2.14 fold in plaques in comparison to controls ($p < 0.05$). MiR 126-5p was found to be up-regulated in eight patient plaque samples compared to controls, while down-regulation was observed in six of the patient samples. Considering the overall expression, miR 126-5p expression was higher in plaques compared to control samples. *Egfl7* up-regulation was observed in eleven plaque samples out of fourteen. The overall Egfl7 mRNA expression was higher in plaques ($p < 0.001$) compared to controls.

This work, supported by data on expression levels of miR 126-5p, miR 126-3p and its targets *Spred1*, *Pik3r2* and *Egfl7*, demonstrates the contribution of miR-126 signalling molecules and neoangiogenesis driven by these molecules to atherosclerotic plaque progression in advanced atherosclerosis, despite their known atheroprotective roles in endothelial progenitor cells. This thesis study provides the first evidence on miR 126-3p expression in deep intraplaque regions. To the knowledge, the expression levels of miR 126-3p, its targets *Spred1*, *Pik3r2*, miR 126-5p and *Egfl7* in atherosclerotic plaques from human carotid arteries were evaluated for the first time.

The molecular mechanisms of miR 126-modulated dysregulation need further investigation, particularly on the phosphorylation status of the downstream signalling molecules, to be able to fully grasp the affect of miR-126 on vascular endothelial

growth factor regulated angiogenesis in atherosclerosis. The interaction of miR-126 with other miRNAs and signalling molecules needs to be elucidated. To be able to fully understand the roles of angiogenesis in different stages of atherosclerosis, the study of patient samples considering the different degrees of stenosis is suggested. Furthermore, larger scale studies must be performed on more than one arterial system, with the aim of determining the specific regulation of angiogenesis by miRNAs in different arterial systems.

The potential use of miRNAs as biomarkers to monitor plaque neoangiogenesis and disease progression, as well as the development of miRNA-based therapies are promising areas of research in the battle against the fatal consequences of atherosclerosis. The results of this thesis study constitutes preliminary bases for future miRNA biomarker studies to be used in monitoring plaque progression and intraplaque neoangiogenesis. The miRNAs 126-3p and -5p, as well as their host gene *Egfl7* can be considered as promising prognostic markers for monitoring neoangiogenesis in advanced atherosclerosis. Further work investigating the levels of circulating miR 126-3p and miR 126-5p in peripheral blood of patients with cardiovascular diseases is needed to assess the use of these circulating miRNAs as biomarkers for advanced atherosclerotic disease.

The results of miRNA expressions in atherosclerotic plaques also provide useful information that can lead to future miRNA based therapies for preventing intraplaque neoangiogenesis, therefore thrombosis. In addition, Egfl7 can be considered as a promising prognostic marker of intraplaque neoangiogenesis, to aid in monitoring the unstable plaques, as well as preventing vulnerable plaque rupture. *Egfl7* based anti-angiogenic therapy could be offered as a strategy in eliminating intraplaque neoangiogenesis and stabilization of plaques that are prone to rupture, therefore preventing the devastating consequences of advanced atherosclerosis.

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APPENDICES

APPENDIX A : Chemicals and Commercially Available Kits
APPENDIX B : TaqMan® Assay ID Numbers
APPENDIX C : Laboratory Equipment

APPENDIX A: Chemicals and Commercially Available Kits

Table A.1 : Chemicals and Commercially Available Kits.

Name	Brand	Supplier
mirVana miRNA Isolation Kit with phenol	Ambion	MedSanTek
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems	MedSanTek
TaqMan® MicroRNA Reverse Transcription Kit	Applied Biosystems	MedSanTek
TaqMan® Universal PCR Master Mix, 2-pack	Applied Biosystems	MedSanTek
Ultrapure DNASE/RNASE-Free Water	Applied Biosystems	MedSanTek
TaqMan® MicroRNA Assay, hsa-miR-126-3p	Applied Biosystems	MedSanTek
TaqMan® MicroRNA Assay, hsa-miR-126-5p	Applied Biosystems	MedSanTek
TaqMan® MicroRNA Assay, U6 snRNA	Applied Biosystems	MedSanTek
TaqMan® Gene Expression Assay, SPRED1	ThermoFisher Scientific	MedSanTek
TaqMan® Gene Expression Assay, PIK3R2	Applied Biosystems	MedSanTek
TaqMan® Gene Expression Assay, EGFL7	Applied Biosystems	MedSanTek
TaqMan® Gene Expression Assay, 18S	Applied Biosystems	MedSanTek
RNaseZap	Sigma-Adrich	LABOMED
Ethanol Absolute	Merch	LABOMED

APPENDIX B: TaqMan® Assay ID Numbers

ID numbers of the commercially available TaqMan®miRNA and Gene Expression Assays are as follows:

- miRNA 126-5p: 000451
- miRNA 126-3p: 002228
- U6 snRNA: 001973
- SPRED1: Hs01084559_ m1
- PIK3R2: Hs00178181_ m1
- EGFL7: Hs00211952_ m1
- 18S: Hs99999901_ s1

APPENDIX C: Laboratory Equipment

Table C.1 : Laboratory Equipment.

Name	Brand-Model
Analytical Balance	Precisa LT120A
Micropipettes	Eppendorf® Research Plus 4-pack
Vortex mixer	Scilogex MX-S
Microcentrifuge	ThermoFisher Scientific Eppendorf® 5424
Thermomixer	ThermoFisher Scientific Eppendorf® Comfort
Spectrophotometer	ThermoFisher Scientific Nanodrop 2000
Thermal Cycler	Bio-Rad T100
Refrigerator	Arçelik 4263
Freezer	Arçelik 2052 DY
Ultra Low Freezer	VWR® Symphony
qPRC System	Agilent Mx3005P

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Sezer-Zhmurov Ç.**, Timirci-Kahraman Ö., Zeybek Ü, Fazlıogulları O., Catal T., Tunoglu S., Isıkoren G.E and Bermek H., An Angiogenic Cooperation: Epidermal Growth Factor Like Domain 7 (EGFL7) and MicroRNA-126. *V. International Conference of Molecular Medicine*, May 20-22, 2015 İzmir, Turkey.

- **Sezer-Zhmurov Ç.**, Timirci-Kahraman Ö., Amadou Z.F., Fazlıoğulları O., Başaran C., Catal T., Zeybek Ü and Bermek H., (2016). Expression of *Egfl7* and

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- **Sezer-Zhmurov Ç.**, Timirci-Kahraman Ö., Amadou Z.F., Fazlıoğulları O., Başaran C., Catal T., Zeybek Ü and Bermek H., (2016). Expression of *Spred1, Pik3r2* and miR 126-3p in human carotid artery plaques (In preparation).