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ASPECTS OF ARTERIAL WALL HEALING - RE-ENDOTHELIALIZATION, INTIMAL HYPERPLASIA AND VASCULAR REMODELING

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Aspects of Arterial Wall Healing – Re-endothelialization, Intimal Hyperplasia and Vascular Remodeling
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To my family and friends

POPULÄRVETENSKAPLIG SAMMANFATTNING

Åderförkalkningssjukdomarna innefattar flertalet olika sjukdomstillstånd såsom kärlkramp, hjärtinfarkt, stroke och fönstertittarsjuka (perifer kärlsjukdom). Dessa sjukdomar beror på att åderförkalkningen orsakar förträngningar i blodkärlen. Förträngningarna gör att blodtillförseln till ett organ eller kroppsdel hämmas vilket resulterar i lokal syrebrist och symptom i form av smärta och nedsatt funktion. Kirurgisk behandling vid kärlförträngning syftar till att återställa blodflödet genom att lokalt vidga blodkärlet eller leda om blodet förbi ett trångt område (bypass). Dessa behandlingar orsakar en skada i kärlväggen vilket ger upphov till en läkningsprocess. Komplikationer efter behandling är vanligt och beror ofta på en överdriven läkningsreaktion i kärlväggen. Detta leder till förträngning av blodkärlet med återkomst av symptom, vilket orsakar ökat lidande för patienten och i värsta fall död.

Kärlväggen består av tre lager: *intima* – den tunna innersta delen som skiljer blod från kärlväggen, *media* – den muskulösa mittendelen och *adventitia* – den stödjegivande yttre delen. Läkningsprocessen innefattar kärlväggens alla lager och kan delas upp i tre delar: läkning av intiman (re-endotelialisering), ärrbildning (intimal hyperplasi) och förändringar i kärlväggens stödjevävnad (vaskulär remodellering).

Lokal behandling av förträngningar görs idag framför allt genom kateterburen teknik. Denna teknik innebär att man genom ett litet hål i blodkärlet kan föra in olika instrument, som exempelvis ballonger, för att lokalt vidga kärlförträngningar från insidan av blodkärlet, så kallad ballongsprängning. För att minska risken för komplikationer efter dessa ingrepp används expanderande metallnät (stent) som utsöndrar cellgifter vilket hämmar krympning av blodkärlet och minskar ärrbildningen. Tyvärr gör cellgifterna att läkningen av intiman försenas eller rent av uteblir. Avsaknaden av detta innersta kärllager medför att det blir ett öppet sår i kärlväggen som kan ge upphov till bildning av blodproppar. Dessa blodproppar kan försämra blodflödet och i värsta fall ge upphov till akut stopp i blodkärlet. Det finns därför ett stort behov av att finna metoder för att selektivt kunna påverka ärrbildningsprocessen.

Studierna i denna avhandling belyser de olika delarna av kärlväggens läkningsprocess och hur man kan påverka denna. I första studien visar vi att det går att åskådliggöra läkningen av intiman med icke-invasivt högupplöst ultraljud i en experimentell kärlskademodell. Denna teknik kommer vara användbar för att utvärdera effekten av läkemedel på kärlväggsläkning i framtida experimentella studier. Den andra studien undersöker hur behandling med diabetesmedicinen linagliptin påverkar kärlväggsläkningen. Tidigare studier har visat att behandling med liknande diabetesmediciner kan minska ärrbildningen efter kärlskada. Vi

kunde inte se någon effekt av behandling med linagliptin på kärlväggens läkningsprocesser varken under normala eller diabetiska förhållanden. I den tredje studien kartlägger vi genuttrycket i den läkande kärlväggen över tid och har även upprättat en biobank med vävnadsprover. Denna biobank kommer att vara en tillgång för forskningsfältet, möjliggöra samarbeten och utgöra en viktig del i jakten på nya mekanismer och behandlingsvägar. I den fjärde studien undersöks hur en tidigare okänd mekanism påverkar kärlväggens läkning. Vi kan visa att avsaknad av enzymet PCSK6 (proprotein convertase subtilisin/kexin 6) bidrar till strukturella förändringar i kärlväggen och hämmar de celler som bidrar till ärrbildning. Dessa resultat antyder att hämning av PCSK6 kan vara en potentiell behandlingsmetod för att minska risken för komplikationer efter kärlkirurgisk behandling.

Sammanfattningsvis har denna avhandling bidragit med en metod för att uppskatta intimans läkning med hjälp av ultraljud (Studie I), gett ökad förståelse kring linagliptins effekt vid kärlskada (Studie II), studerat förändringen i genuttryck över tid genom läkningsprocessen (Studie III) och identifierat en potentiellt ny behandlingsväg för selektiv hämning av ärrbildning efter kärlskada (Studie IV).

ABSTRACT

Cardiovascular disease is the leading cause of mortality in the world. Despite prevention, the need for interventions remains high. Patients with type 2 diabetes mellitus have an increased cardiovascular burden and are at higher risk of complications following invasive vascular interventions. Complications related to an excessive healing response are a major clinical problem, which results in increased morbidity and possibly death. The arterial wall healing response consists of re-endothelialization, intimal hyperplasia (IH) formation and vascular remodeling. Current pharmacological treatment relies on non-selective anti-proliferative drugs, which reduces IH formation but increases the risk of thrombosis due to a delayed re-endothelialization. Hence, there is a need for development of selective treatments. Evaluation of the re-endothelialization process in the rat carotid balloon injury model has previously been limited to histological staining and invasive imaging techniques. We demonstrate that it is possible to estimate the re-endothelialization process in ultrasound biomicroscopy using IH morphology as a surrogate marker. This technique will be a useful tool for non-invasive real-time evaluation of the re-endothelialization process in pharmacological studies. Incretin-modulating drugs is a group of antidiabetic drugs, which targets the glucagon-like peptide-1 (GLP-1) receptor by either direct activation or suppressing breakdown of native GLP-1 with dipeptidylpeptidase-4 (DPP-4) inhibitors. GLP-1 receptor activation has been shown to reduce IH formation by selective inhibition of smooth muscle cell (SMC) proliferation. However, we show that treatment with linagliptin, a DPP-4 inhibitor, does not influence the arterial wall healing in normal or type 2 diabetic conditions. Large-scale transcriptomic analysis is an important tool for confirmation and identification of novel molecular mechanisms in experimental research. In Study III, we generated an encyclopedia of the transcriptomic landscape over time in the rat carotid balloon injury model. We could detect three separate phases of the healing process and contribution of novel molecular mechanisms. This resource includes a biobank of tissue samples, which will be a powerful tool for validation and identification of novel treatment targets. The utilization of transcriptomic data to identify new biological pathways in the arterial wall healing process can be exemplified with proprotein convertase subtilisin/kexin 6 (PCSK6). Previously, we could identify an increased expression of PCSK6 in patients with symptomatic carotid artery stenosis. PCSK6 has been associated with tumor invasiveness and extracellular matrix modulation in cancer but its function in the vasculature remains elusive. We demonstrate that PCSK6 deletion increases outward remodeling, reduces SMC differentiation and influences contractility in a murine model of flow-mediated remodeling. These results indicate that PCSK6 could be a potential target to reduce the risk of constrictive remodeling and restenosis.

LIST OF SCIENTIFIC PAPERS

I. Noninvasive in vivo Assessment of the Re-endothelialization Process Using Ultrasound Biomicroscopy in the Rat Carotid Artery Balloon Injury Model.
Röhl S, Eriksson L, Saxelin R, Lengquist M, Östenson CG, Hedin U, Caidahl K, Razuvaev A
Journal of Ultrasound in Medicine. 2018 Nov 13, DOI: 10.1002/jum.14858 [Epub ahead of print]

II. Effects of Linagliptin on Vessel Wall Healing in the Rat Model of Arterial Injury Under Normal and Diabetic Conditions.
Eriksson L, **Röhl S**, Saxelin R, Lengquist M, Kronqvist M, Caidahl K, Östenson CG, Razuvaev A
Journal of Cardiovascular Pharmacology. 2017 Feb;69(2):101-109

III. Transcriptomic Profiling of Experimental Arterial Injury Reveals New Mechanisms and Temporal Dynamics in Vascular Healing Response.
Röhl S*, Rykaczewska U*, Seime T*, Suur EB, Gonzales Diez M, Gårdin RJ, Lengquist M, Kronqvist M, Bergman O, Odeberg J, Lindeman HNJ, Roy J, Hamsten A, Eriksson P, Hedin U, Razuvaev A[#], Matic L[#]
(*,# equal contributions)
In manuscript.

IV. The Role of PCSK6 in Flow-mediated Arterial Remodeling in Mice.
Röhl S, Suur EB, Lengquist M, Caidahl K, Hedin U, Arner A, Matic L, Razuvaev A
In manuscript.

Publications not included in this thesis:

Phenotypic Modulation of Smooth Muscle Cells in Atherosclerosis is Associated With Downregulation of LMOD1, SYNPO2, PDLIM7, PLN and SYNM.
Perisic Matic L, Rykaczewska U, Razuvaev A, Sabater-Lleal M, Lengquist M, Miller CL, Ericsson I, **Röhl S**, Kronqvist M, Aldi S, Magné J, Paloschi V, Vesterlund M, Li Y, Jin H, Diez MG, Roy J, Baldassarre D, Veglia F, Humphries SE, de Faire U, Tremoli E, Odeberg J, Vukojević V, Lehtiö J, Maegdefessel L, Ehrenborg E, Paulsson-Berne G, Hansson GK, Lindeman JH, Eriksson P, Quertermous T, Hamsten A, Hedin U
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Journal of Vascular Research. 2015;52(1):41-52

CONTENTS

LIST OF ABBREVIATIONS

B-mode	Brightness mode
CCA	Common carotid artery
cDNA	Complementary DNA
CVD	Cardiovascular disease
DES	Drug-eluting stent
DPP-4	Dipeptidyl peptidase-4
EC	Endothelial cell
ECM	Extracellular matrix
FSS	Fluid shear stress
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
IEL	Internal elastic lamina
IH	Intimal hyperplasia
IHC	Immunohistochemistry
IL	Interleukin
IMT	Intima-media thickness
MMP	Matrix metalloprotease
MT-MMP	Membrane-type matrix metalloprotease
NO	Nitric oxide
PCSK6	Proprotein convertase subtilisin/kexin 6
PDGF-B	Platelet-derived growth factor-beta
PI	Pulsatility index
qRT-PCR	Quantitative real-time polymerase chain-reaction
RI	Resistive index
RNA-seq	RNA-sequencing

SD	Sprague-Dawley
SEM	Scanning electron microscopy
SMC	Smooth muscle cell
T2DM	Type 2 diabetes mellitus
TEM	Transmission electron microscopy
TGF-B	Transforming growth factor-beta
TIMP	Tissue inhibitor of matrix metalloprotease
UBM	Ultrasound biomicroscopy
WT	Wild-type
ZDF	Zucker diabetic fatty

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the major cause of mortality worldwide and estimated to be responsible for 31% of the global mortality (17.9 million deaths per year) in 2016.[1] The major causes of cardiovascular deaths (85%) are coronary heart disease and stroke.[2] The distribution of cardiovascular mortality varies in different geographical regions and because of the regional socioeconomic status, the majority of the cardiovascular deaths (approximately 75%) occurs in the low- or middle-income countries.[1] The term "CVD" is defined as the diseases of the heart and blood vessels including diseases such as coronary artery disease, cerebrovascular disease and peripheral artery disease, which all share the same pathology, atherosclerosis. During the past decades, extensive research has identified several risk factors for atherosclerotic CVD development, which can be divided into non-modifiable and modifiable. The non-modifiable risk factors include gender, age and genetic inheritance while the modifiable risk factors include smoking, diabetes mellitus, hypertension, obesity, dyslipidemia, physical inactivity and depression.[3,4] Despite extensive efforts on preventive lifestyle changes and medical treatment the need for vascular surgical interventions remains high.[5,6]

1.2 STRUCTURE OF THE ARTERY

The artery is a multilayered structure and is generally divided into the *tunica intima*, *tunica media* and *tunica adventitia* (Figure 1). The *tunica intima* is the innermost layer of the arterial wall and consists of a mono-cellular layer of endothelial cells (ECs), the endothelium, and the basal lamina.[7,8] The endothelium regulates the vascular wall homeostasis and provides a protective barrier between the blood and the arterial wall.[9] The *tunica intima* is separated from the *tunica media* by the internal elastic lamina (IEL), a conglomerate of elastin, collagen fibers and microfibrils.[8,10] The *tunica media* consists of circumferentially arranged contractile vascular smooth muscle cells (SMCs), which are surrounded by a basement membrane and embedded in the medial extracellular matrix (ECM), consisting of elastin, collagen and proteoglycans, and is responsible for the vascular tone and pulse wave propulsion.[8,11] *Tunica media* and *tunica adventitia* are separated by the collagen-rich external elastic lamina. The *tunica adventitia* is the outermost layer of the artery and consists of collagen-rich ECM, *vasa vasorum*, nerves and perivascular cells (fibroblasts, progenitor cells and tissue resident inflammatory cells).[8,12] The adventitia provides mechanical support and contributes to the morphological adaptions in the arterial wall seen upon physiological alterations.[12]

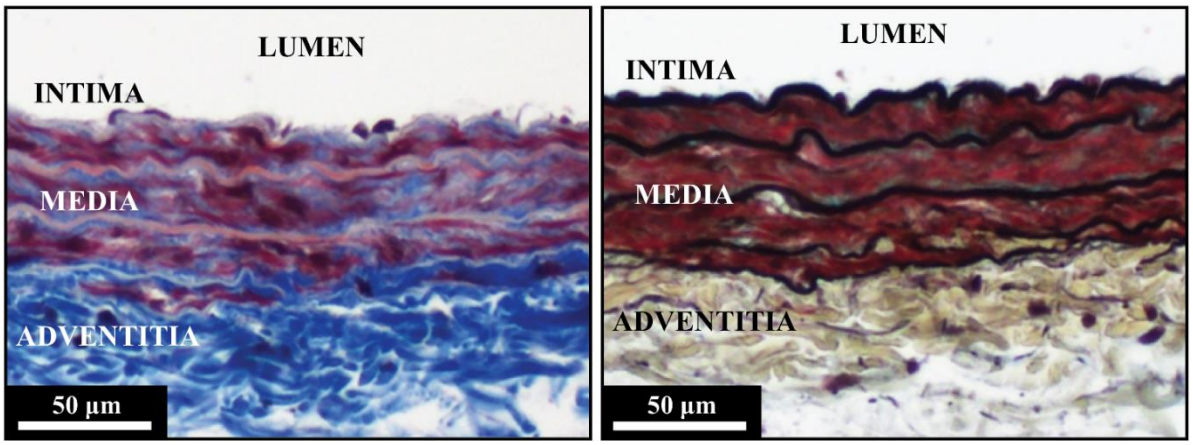

Figure 1. Structure of the arterial wall. Histochemical staining of a rat carotid artery, with Masson trichrome (left) and Movat pentachrome (right). Masson trichrome: dark blue – nuclei, blue – collagen, red – muscle fibers. Movat pentachrome: black – elastic fibers and nuclei, blue – mucin, bright red – fibrin, red – muscle fibers, yellow – collagen.

1.3 ATHEROGENESIS

The formation of an atherosclerotic plaque occurs in areas of disturbed blood flow and is initiated by inflammation and infiltration of circulatory lipids to the sub-intimal layer of the arterial wall.[13,14] Disturbance in the blood flow induces a focal thickening of the arterial wall (described in detail below). Presence of lipids in the vessel wall triggers a local inflammatory response, which activates ECs resulting in leucocyte recruitment from the blood stream. Continuous accumulation of lipids, inflammatory cells and cellular debris forms the fatty streak, a symptomless preceding form of the atherosclerotic plaque.[15–17] As the atherosclerotic process progresses a focal lesion is formed, which can further develop into an atherosclerotic plaque with a necrotic core formed by accumulation of lipids, tissue debris and dying cells. Activation of medial SMCs by inflammatory stimuli triggers a phenotypic switch, from a non-proliferative contractile to a proliferative synthetic state, and initiates a transmigration to the luminal surface of the plaque where they proliferate and secrete ECM components, forming a fibrous cap that shields the necrotic core from the lumen.[18] Plaques with thick and stable fibrous caps rarely rupture but can cause luminal stenosis of the artery with subsequent ischemia of the tissue in the perfused organ such as the myocardium. Inflammatory processes, from within the plaque, in the surrounding tissue or alterations in blood flow initiates ECM degradation, SMC apoptosis and thinning of the fibrous cap forming an unstable or vulnerable plaque prone to rupture.[13,18–20] Endothelial erosion and thinning or rupture of the fibrous cap expose the highly thrombogenic core to the bloodstream, which triggers thrombus formation and embolic precipitation resulting in arterial occlusion and ischemia in the tissue distal to the occlusion.[13,21]

1.4 INVASIVE TREATMENT OF ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

Surgical management of atherosclerotic cardiovascular diseases can be divided to open and endovascular techniques. The open techniques include endarterectomy and bypass surgery. Endarterectomy consists of a surgical removal of atherosclerotic plaques and is commonly performed to treat unifocal stenosis of the carotid and femoral arteries. Bypass surgery is generally performed in order to treat multifocal atherosclerotic disease and encompasses an arterial reconstruction passed the stenotic areas using an autologous vein or synthetic graft as conduit. The minimally invasive endovascular treatments rely on a controlled dilation of the stenotic area (balloon-angioplasty) with or without placement of an expandable mesh (stent). Hybrid treatments with a combination of open and endovascular techniques may be performed in selected cases.[22]

1.5 DIABETES MELLITUS IN CARDIOVASCULAR DISEASE

The prevalence of diabetes mellitus is increasing worldwide and diabetic patients have an increased atherosclerotic burden and cardiovascular mortality.[23,24] Diabetes mellitus is roughly divided into two subtypes, type 1 and type 2, both associated with an increased cardiovascular morbidity and mortality. Type 1 diabetes account for 5-10% of the diabetic patients and is considered an autoimmune disease caused by destruction of the beta cells in the pancreatic Langerhans islets resulting in insulin deficiency. Type 2 diabetes mellitus (T2DM) accounts for approximately 90% of the diabetic patients and differs from type 1 in etiology and clinical presentation. T2DM is caused by an increased insulin resistance resulting in hyperglycemia and hypersecretion of insulin from the beta cells.[25] The etiology is related to a combination of environmental, genetic, lifestyle and dietary factors. Apart from hyperglycemia, these patients often present with an altered metabolic profile and an increased prevalence of CVD risk factors such as dyslipidemia, obesity and hypertension.[26] Despite strict glycemic control, the cardiovascular mortality in these patients remains high.[27,28] The diabetes-related vascular complications are commonly divided into micro- and macrovascular. The microvascular affects small arteries and *arteriolii* in the vascular networks in the retina, kidneys and nervous system while the macrovascular affect the arteries of the cardiovascular system.[29,30]

1.5.1 The macrovascular effects of diabetes mellitus type 2

The altered metabolic status in type 2 diabetic patients, with hyperglycemia and hyperinsulinemia, induces irreversible metabolic and molecular modulations in the arterial

wall.[31] Hyperglycemia causes formation of advanced end-glycation products, reactive oxygen species accumulation, increased glucose to sorbitol conversion and an activation of the protein kinase C signaling pathways.[29,32] Presence of advanced-end glycation products in the arterial wall induces cross-linking between extravascular proteins and the ECM resulting in arterial stiffening.[33–35] Hyperinsulinemia is associated with an inflammatory and pro-atherogenic response in the macrovasculature. Insulin mediated insulin-growth factor-1 receptor activation in SMCs induces phenotypic switch, proliferation and migration.[32] Insulin resistance impairs intracellular glucose metabolic pathways and modulates the extracellular-receptor kinase 1/2 pathway resulting in increased pro-inflammatory and mitogenic signaling.[29] Combined, these alterations induce a chronic inflammation in the arterial wall, which causes modulation in ECM composition, increased prevalence of synthetic SMCs, endothelial dysfunction, increased arterial stiffness and atherogenesis.[29,32,36] Apart from having an increased CVD prevalence, patients with T2DM have a higher risk of complications following invasive vascular interventions, such as restenosis, vein graft failure and late in-stent thrombosis.[37–39]

1.5.2 Glucagon-like peptide-1

The incretin glucagon-like peptide-1 (GLP-1) is an insulinotropic hormone mainly secreted by intestinal enteroendocrine L-cells upon ingestion. GLP-1 stimulates insulin secretion and exerts glucagonostatic effects in a glucose dependent manner. Due to these effects, the incretin modulating drugs were developed as a pharmacological treatment of T2DM with low risk of therapy-induced hypoglycemia. The drugs act by stimulating the GLP-1 receptor or inhibiting the dipeptidyl peptidase-4 (DPP-4) activity, an enzyme responsible for the rapid degradation of endogenous GLP-1.[40,41] Apart from their effect on the glucose homeostasis, these drugs have direct beneficial effects on the cardiovascular system and arterial wall healing. Treatment with GLP-1 receptor agonists have been shown to reduce CVD mortality in patients with T2DM.[42] Also, GLP-1 receptor agonists have been shown to lower systolic blood pressure and reduce diabetes-induced endothelial dysfunction in type 2 diabetic patients. *In vitro* studies have shown that GLP-1 receptor agonists stimulate EC proliferation and inhibit SMC proliferation. *In vivo* models of arterial injury have shown that GLP-1 receptor agonists decrease SMC proliferation, reduce intimal hyperplasia (IH) formation and improve arterial distensibility.[43,44] Similar studies of DPP-4 inhibitors are less conclusive regarding its cardioprotective and beneficial effects on the arterial wall healing.[45,46]

1.6 ARTERIAL WALL HEALING

In response to arterial wall injury, caused by vascular disorder, trauma or iatrogenic damage, a complex healing process is initiated, aiming to repair and restore vascular homeostasis. This process involves an intricate interplay between the cells in the arterial wall and the biomechanical forces from the blood stream. Inadequate or excessive arterial healing reactions may cause clinical complications such as restenosis and thrombosis.[47,48]

1.6.1 The re-endothelialization process

The vascular endothelium exerts a household function on the vascular wall and provides a protective barrier between the thrombogenic subintimal layer and the blood stream. Endothelial-mediated signaling, such as the nitric oxide (NO) signaling pathway, regulates arterial tone, structure, cellular proliferation, inflammation and coagulation.[9] Upon arterial injury, traumatic or inflammatory, the continuity of the endothelial layer is disrupted. The loss of endothelial coverage with exposure of thrombogenic substrates to the blood flow induces a local inflammatory response with platelet aggregation, leucocyte recruitment, SMC activation and subsequent IH formation.[9,49,50] In response to endothelial disruption, ECs adjacent to the injured area become activated and proliferative and begin to migrate in order to cover the denuded areas of the arterial wall. Once re-endothelialized, the newly formed ECs will mature and stabilize the arterial wall by reducing inflammation, inhibiting SMC proliferation and initiate an IH modulation process.[9,51] Inadequate re-endothelialization or inability of proper endothelial maturation causes a local chronic inflammation, which increases the risk of restenosis and thrombosis following invasive vascular interventions.[51]

1.6.2 Intimal hyperplasia formation

Intimal hyperplasia formation (or neointima formation) occurs in response to vascular wall injury, endothelial denudation, inflammation and alterations in the fluid shear stress (FSS) exerted on the vessel wall. The intimal hyperplastic response is directly related to the degree of injury inflicted to the arterial wall.[52–54] This healing process involves the medial SMCs, platelets, leucocytes and to a lesser extent adventitial progenitor cell and mesenchymal stem cells.[12,55,56] The IH formation has been extensively studied in animal models of arterial injury and can be viewed upon as process of three phases; the SMC activation, the migratory and the intimal hyperplastic phase (Figure 2).

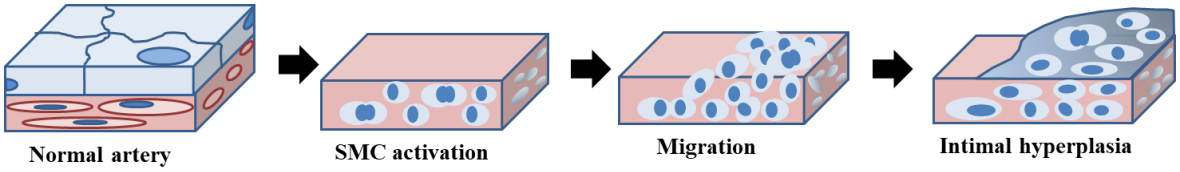

Figure 2. Schematic illustrations of the phases in intimal hyperplasia formation.

The SMC activation phase is induced upon injury and is characterized by platelet adhesion, leucocyte recruitment, SMC activation and proliferation. SMCs are normally embedded in the ECM of the *tunica media* in a spindle-shaped non-proliferative contractile state. Upon injury to the arterial wall, with tearing of the *tunica media*, IEL and concomitant endothelial disruption, the medial SMC become activated and stimulated into a phenotypic switch.[57] Endothelial disruption inhibits the EC-mediated anti-proliferative NO-signaling and exposes the subendothelial layer to the blood flow, which causes platelet adhesion, thrombus formation and leucocyte recruitment.[58] Mechanical stretch, decreased NO-signaling, apoptosis of injured SMCs, local secretion of growth factors, such as platelet-derived growth factor-β (PDGF-B), fibroblast-growth factor 2 and insulin-growth factor-1, and cytokines, such as interleukin-1(IL-1), IL-6 and tumor necrosis factor-α, stimulate a downregulation of the contractile SMC specific genes. This induces a transformation of the differentiated SMCs into a rhomboid-shaped non-contractile synthetic SMCs. The synthetic SMCs have proliferative potential, may activate matrix metalloproteases (MMPs) and transmigrate to the *tunica intima.*[59–62]

Following the initial response, a phase characterized by migration of the activated SMCs from the *tunica media* to the *tunica intima* is initiated. SMC migration is a complex process which depends on integrin-mediated adhesion, activation of MMPs and the bioavailability of growth factors, such as PDGF-B.[63] The integrin-mediated adhesion, mainly mediated through $\alpha_v\beta_3$-integrin, facilitates anchoring between the SMCs and the components of the ECM during migration.[64] Activation of MMPs, such as MMP2, MMP9 and membrane-type-MMP1 (MT-MMP1), also known as MMP14, enables cleavage of collagen type IV with subsequent detachment of the SMCs from the basement membrane and surrounding ECM. The MMPs also facilitate the ECM degradation with concomitant release of ECM-bound substances, which further stimulates proliferation and migration of the SMCs.[65–67] Secretion of growth factors from activated SMCs, adherent platelets and leucocytes induces a chemotactic migration of medial SMCs to the *tunica intima.*[63]

The intimal hyperplastic phase is initially characterized by proliferation of the migrated SMCs. With time, the intimal SMC proliferation gradually subsides and an increase in ECM component secretion is seen. The formed intimal ECM may account for up to 60-80% of the IH and consists of proteoglycans, hyaluronan and fragmented collagen.[68,69] The regulation of SMC dependent intimal ECM accumulation in IH formation is related to re-endothelialization, biomechanical forces and pro-fibrotic signaling pathways, such as transforming growth factor-β (TGF-B). Regeneration of the endothelium with subsequent restoration of the NO-mediated signaling has been shown to modulate and decrease the IH.[70] Reductions in FSS, the force generated from the friction of blood flow to the arterial wall, has been shown to increase the IH formation.[71] Furthermore, downregulation of TGF-B_1 has been shown to decrease the IH formation *in vivo* following arterial injury.[72]

1.6.3 Vascular remodeling

In response to arterial wall injury or mechanical stress, such as increased blood flow, FSS or stretch, a vascular remodeling process is initiated. This process is characterized by MMP activation, ECM modulation and collagen deposition, which causes increased wall thickness and arterial stiffening.[73,74] The remodeling process may result in an outward remodeling, an adaptive enlargement of the vessel circumference. Absence of adaptive enlargement or presence of inward remodeling, a reduced vessel circumference, causes luminal narrowing with an increased risk of restenosis (Figure 3). MMPs are commonly secreted to the extracellular space as inactive proproteins, or proMMPs, which requires proteolytic cleavage to become biologically active.[75,76] However, membrane-bound MMPs, such as MT-MMP1, can be activated intracellularly by furin and serine proprotein convertase proteinases.[77] Activation of proMMPs may also be conducted by activated members in the MMP family, for example MT-MMP1 can proteolytically cleave and activate proMMP2.[65,76,77] In addition, expression of certain MMPs, MMP2 and MMP9, increases in response to alterations in the biomechanical forces exerted on the vessel wall.[76] The MMPs activity is closely regulated by tissue inhibitors of metalloproteinases (TIMPs), which can be subdivided according to the affinity for specific MMPs.[75,76] It has been speculated that

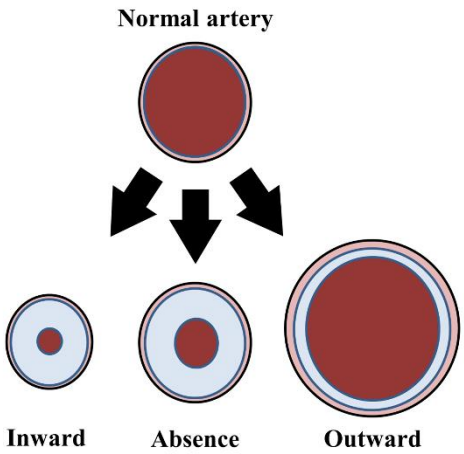

Figure 3. Illustration of the outcomes in vascular remodeling.

pathological vascular remodeling is related to shifts in the MMP/TIMP ratio, in which an increased ratio causes outward remodeling while decreased ratio results in constrictive remodeling.[78,79] The TGF-B signaling pathway has been identified as a key modulator of the vascular remodeling process. TGF-B exists in three isoforms (TGF-B_{1-3}) of which TGF-B_1 have been shown to be associated with the fibroproliferative effects seen in vascular remodeling. In the arterial wall TGF-B exists in an inactive ECM-bound form, which is activated upon matrix degradation by MMP2 and MMP9. TGF-B may also be produced and secreted by platelets, leucocytes, SMCs, fibroblasts and ECs. The release of active TGF-B reduces collagen degradation and induces a differentiation of adventitial fibroblasts into myofibroblasts which may migrate, proliferate and secrete collagenous ECM components.[80,81] Inward remodeling with concomitant excessive IH formation is the major cause of treatment failure in patients treated with autologous venous by-pass grafting and arteriovenous dialysis fistulas.[73,82,83] Vascular remodeling is a common feature in atherosclerotic plaque destabilization in which the sudden onset of inflammation stimulates ECM degradation resulting in thinning of the fibrous cap and outward remodeling.[13,84]

1.6.4 Effects of wall shear stress on arterial wall healing

As previously described, the FSS in laminar blood flow is the friction force generated by the blood flow to the endothelium of the arterial wall (Figure 4). FSS, expressed as dyne/cm^2, can be calculated as: $FSS=4nQ/\pi r^3$. In which n is blood viscosity, Q is the volume flow rate and r is lumen radius of the vessel.[85] In the uninjured artery, the endothelium protects the arterial wall from the mechanical forces of the blood flow. Hence, FSS does not directly affect the SMCs and adventitial fibroblasts of the arterial wall.[86] However, alterations in biomechanical forces exerted by the blood flow on the arterial wall may induce a vascular remodeling response mediated through endothelial mechanosensors. Reduced FSS in the uninjured artery may induce an increased arterial wall thickness whereas increased FSS have been shown to modulate and reduce the IH formation in vascularized synthetic by-pass grafts.[87–90] Upon arterial injury with subsequent endothelial denudation an increase in arterial wall permeability and transmural flow is seen. The altered transmural flow increases the FSS exerted on the cells of the arterial wall. Results from *in vitro* studies suggest that the increased transmural flow induces SMC activation, proliferation and

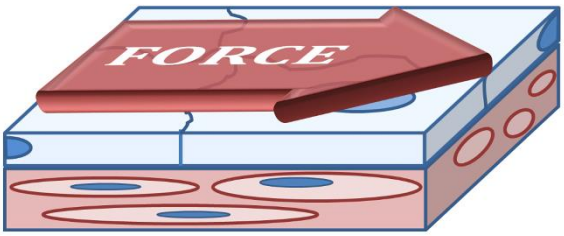

Figure 4. Exemplification of fluid shear stress in laminar blood flow.

migration. The increase in IH reduces the transmural flow, which decreases the SMC proliferation. The contribution of adventitial fibroblasts in IH formation in relation to transmural flow activation still remains elusive.[86] A clinical manifestation of the impact of FSS on IH formation is seen in arteriovenous dialysis fistulas, where areas of low FSS display an excessive IH formation and inward remodeling resulting in stenosis and fistula failure.[82,83]

1.6.5 Restenosis and late in-stent thrombosis

Restenosis is a major clinical problem and the main limiting factor following any open and endovascular surgical procedure. The restenotic process consists of an excessive IH formation with simultaneous inward or insufficient outward remodeling. Combined, these processes decrease the luminal diameter resulting in an impaired blood flow with subsequent ischemia of the distal tissue.[91,92] The introduction of the minimal invasive endovascular techniques has revolutionized the surgical treatment of patients suffering from cardiovascular disorders. However, upon its introduction patients treated with endovascular balloon angioplasty displayed a high frequency (50%) of post-interventional restenosis due to arterial recoil, negative remodeling and IH formation.[92] The introduction of bare-metal stents reduced the risk of arterial recoil and negative remodeling, which decreased the failure rate to 30%. The development of drug-eluting stents (DES) has further reduced the risk of restenosis to 10%.[48,92] DES decreases IH formation by inhibition of SMC proliferation through local secretion of non-selective anti-proliferative drugs. Although reducing IH driven restenosis, large register and clinical cohort studies have shown a DES associated increase in late in-stent thrombosis attributed to a drug-induced impairment of the re-endothelialization process.[93,94] The absence of endothelial coverage results in a local chronic inflammation and exposure of the subendothelial layer to the blood stream, which increases the risk of a thrombotic event.[51] Hence, there is a great need for novel treatment strategies with selective SMC inhibition and simultaneous stimulation of EC proliferation.

1.6.6 A novel target for modulation of arterial wall healing

Proprotein convertase subtilisin/kexin 6 (PCSK6), also known as PACE4, is a serine protease, which acts by cleaving and activating biologically inactive target proteins.[95,96] The function of PCSK6 in relation to cancer has been extensively investigated whilst its function in vascular wall healing and disease remains elusive. PCSK6 has been associated with enhanced tumor invasiveness, MMP-activity and cytokine release.[97,98] Polymorphisms in PCSK6 have been associated with congenital heart disease and aortic dissection.[99–102] Previously, PCSK6 was shown to influence blood pressure in PCSK6$^{-/-}$ mice subjected to sodium-chloride enriched diet.[103] We have recently reported that PCSK6 was highly upregulated in atherosclerotic plaques and associated with plaque instability in patients with carotid artery stenosis.[104] Combined, these results suggest that PCSK6 could be a potential target for modulating IH formation and vascular remodeling.

2 METHODOLOGICAL CONSIDERATIONS

2.1 ANIMALS MODELS

Several experimental animal injury models have been developed in order to study the arterial wall healing processes. The complex biology and effects of hemodynamics in vessel wall healing is yet to be recreated in an *in vitro* setting, which is why the *in vivo* models is the preferred methodology for this purpose. However, animal models have some drawbacks, such as the genetic difference from humans, timeline of the injury response and differences in preexisting or induced pathology.[105]

2.1.1 Rat strains

The Sprague-Dawley (SD) and Wistar rats are two commercially available and commonly used wild-type (WT) rat strains in experimental research. Male animals are more commonly used then females since they are, in general, less aggressive and more tolerant to be housed with other individuals in the same cage.

Several rat models with altered metabolic profiles have been developed by selective breeding, such as the Zucker Diabetic Fatty (ZDF) rat and the Goto-Kakizaki (GK) rat. The ZDF rat is an obese hypertensive type 2 diabetic rat strain, whereas the GK rat is a non-obese normotensive type 2 diabetic rat strain.[106] The ZDF have homozygous mutation in the leptin hormone receptor, which results in hyperphagia. Exposure to high-fat diet in male ZDF rats results in development of hyperlipidemia, hyperinsulinemia, insulin resistance and glucose intolerance.[107] The GK strain was generated in Japan in the 1970s through selective inbreeding of Wistar rats with increased glucose tolerance. Over the last decades, several colonies have been established in multiple locations across the globe. These rats display an early-life onset of mild hyperglycemia, dyslipidemia and insulin resistance due to an impaired Beta-cell function and progressive selective pancreatic islet fibrosis.[106,108] Despite the difference in pathology, GK rats display similar diabetes-related complications as seen in humans such as neuropathy, retinopathy, vascular associated cognitive impairment, decreased kidney function and endothelial dysfunction.[109] In comparison to the ZDF rat, the GK strain displays polygenic mutations in multiple chromosomes, which also vary between the different colonies.[109,110] Interestingly, the characteristics of this metabolic model remains similar across the colonies.[111] Utilization of the GK model, using Wistar as control, allow for investigation of the influence of T2DM without the influence of hypertension or obesity, in complex biological processes such as the arterial wall healing process.[106]

The SD rat, used in **Study I** and **III**, is commonly used as WT in experimental vascular injury models. This strain was selected based on our previous experiences with this strain in the common carotid artery (CCA) balloon injury model, availability and comparability to previously published studies. The GK and Wistar rats, used in **Study I** and **II**, were selected in order to investigate the influence of T2DM on vessel wall healing. The GK rats were chosen based on availability and were bred at Karolinska Institutet[112] and provided through a collaboration with Prof. Claes-Göran Östenson. Wistar rats were used as WT control since GK rats originate from the Wistar strain. Despite presentation of metabolic characteristics resembling the human disease, the pathophysiology in rodent models of T2DM is different and therefore translational conclusions should be made with caution. Mouse models of T2DM, such as the db/db mice[113], were not considered since our aim was to investigate the influence of T2DM on vascular biology using the established experimental injury models available in our research group, the rat CCA balloon injury model and primary aortic cell cultures.

2.1.2 Mouse strains

Murine models are often utilized in experimental animal research due to the relatively low cost of genetic modification and high reproductive rate in comparison to other mammals. Hence, the mouse models allow investigation of the influence of specific genes during normal and abnormal conditions in an *in vivo* environment. The C57Bl/6 mouse strain is commonly used in experimental research as a WT, or control, and may also serve as a carrier of induced mutations using the backcrossing technique. In **Study IV**, the experiments were performed on PCSK6$^{-/-}$ mice, which had been backcrossed onto a C57Bl/6 background for at least 10 generations, using C57Bl/6J mice as controls.[114,115] The PCSK6$^{-/-}$ mice were chosen since there were no other available models at the time, such as conditional knock-outs or pharmaceutical methods to inhibit the PCSK6 activity *in vivo*.

2.2 EXPERIMENTAL MODELS OF ARTERIAL INJURY

2.2.1 Rat common carotid balloon injury

The CCA balloon injury model was originally described by Clowes AW et al and is one of the most frequently used *in vivo* models for studying IH formation and the re-endothelialization process.[116,117] In this model, a mechanical stretch injury with concomitant endothelial denudation is inflicted to the whole length of the CCA using a 2F Fogarty balloon embolectomy catheter (Figure 5). The mechanical injury with tearing of the elastic lamina induces an intimal hyperplastic response with SMCs activation, migration and proliferation with subsequent IH formation. Endothelial denudation induces a rapid re-endothelialization process which decreases and ultimately ceases 6 weeks after the injury leaving one third of the artery un-endothelialized.[117–119] The incomplete re-endothelialization is related to the inflammatory response but also to the lack of branches in the CCA to contribute to the re-endothelialization process. Hence, the carotid balloon injury model is ideal for the investigation of SMCs and IH formation during the arterial wall healing process. However, there are differences in biological reactivity between rats and humans, which is reflected in the temporal range of the injury response. In rats, the arterial healing process occurs within the first weeks after injury while the healing process may continue for months in humans.[120]

Injury models with complete re-endothelialization have also been developed for investigating the influence of ECs on IH formation. Partial aortic balloon injury has been shown to result in full re-endothelialization due to the contribution of ECs from aortic branches such as the lumbar arteries.[121,122]

The wire injury model is commonly used for investigating the different aspects of vessel wall healing.[123,124] In this model, a surgical denudation of the endothelium is performed without mechanical stretch of the *tunica media*, commonly to the femoral artery, resulting in an arterial healing response. This model is attractive due to the anatomic accessibility of femoral artery and the possibility to use animals with altered genetics.[124] However, utilization of non-invasive visualization techniques for longitudinal assessment of vessel wall structures in murine models is limited due the resolution and small size of the anatomical structures.

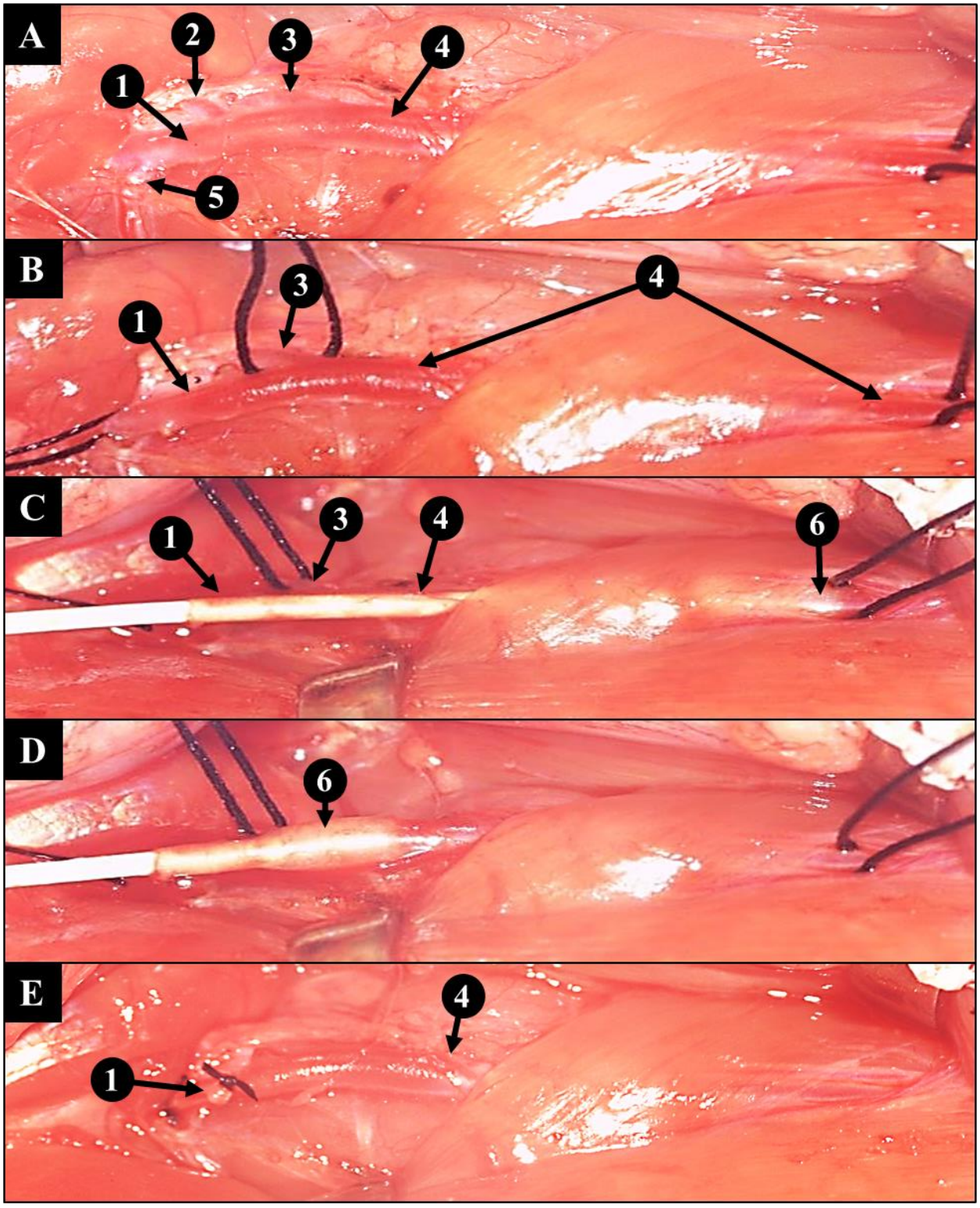

Figure 5. Intraoperative images of the rat carotid balloon injury.
A) Exposure of the carotid bifurcation, B) distal control, C) and D) balloon injury to the common carotid artery, E) ligation of the external carotid artery. 1. External carotid artery, 2. Occipital artery, 3. Internal carotid artery, 4. Common carotid artery, 5. Superior thyroid artery, 6. Inflated balloon.

The CCA balloon injury model, used in **Study I-III**, was selected based on the availability of in-house expertise, experiences from previous studies, comparability to the existing literature and possibility to combine with non-invasive imaging modalities, such as ultrasound biomicroscopy (UBM). Also, the CCA balloon injury model has the advantage of being a well-established model, which is less invasive compared to aortic injury models.

2.2.2 Mouse carotid ligation

The carotid ligation model was originally developed by Kumar A and Lindner V and utilizes alterations in FSS in order to induce IH formation and vascular remodeling.[125] Complete ligation of the CCA proximal to the carotid bifurcation or ligation of the CCA branches except for the occipital artery, results in a reduced of the blood flow and FSS in the CCA.[126] The reductions in FSS induces an intimal hyperplastic response with concomitant inward remodeling.[125] In addition, the IH formation is also influenced by the traumatic dissection and foreign body effect of the suture material. Also, the intimal hyperplastic response is influenced by the genetic background and differs between different murine strains.[127] Unilateral carotid ligation induces a redirection of the blood flow to the un-ligated contralateral CCA, which is increased by 40-70%. The elevated blood flow increases the FSS exerted to the arterial wall, which induces a non-inflammatory flow-mediated outward remodeling response.[126,128] Flow-mediated remodeling may also be investigated using the aortic banding model in which a partial ligation of the aortic arch distal to the innominate artery is performed. This method causes a dramatic increase in pulse pressure in the right CCA and induces a left ventricular hypertrophy without affecting the systemic mean arterial pressure.[129,130] In comparison to the aortic banding model, the carotid ligation is a more suitable model for investigation of vascular remodeling since it is less invasive and does not induce left ventricular hypertrophy.

In **Study IV**, complete ligation of the CCA proximal to the carotid bifurcation was performed using monofilament non-resorbable suture (Surgipro 8-0, Auto Suture Company, Norwalk, CT, USA). Compared to the partial ligation, the complete ligation method is performed using a smaller surgical incision with reduced dissection of the surrounding tissue. Also, an inert suture material was selected in order to reduce the inflammatory response in the artery and surrounding tissue. All carotid ligations were performed by the author.

2.2.3 Primary aortic cell cultures

In general, the cell culture technique is dependent on the proliferative potential and dedifferentiation capacity of the cell line of interest. Certain cell types, such as neurons and myocardiocytes, are terminally differentiated and have a limited capacity for dedifferentiation and proliferation while other cells, such as SMCs, may dedifferentiate and have a high proliferative potential. During the dedifferentiation process, cells may lose cell-line specific function and morphology while gaining characteristics typically not related to the differentiated cell. Therefore, the translational value of cell culture experiments is dependent on the number of passages.[131,132]

The SMCs response in the acute proliferative phase of arterial wall healing can be studied *in vitro* using primary cell cultures of aortic SMCs. Primary cell cultures refers to cells that originate from the harvested tissue without any previous passage. Secondary cell cultures refers to cultures from passage 1 and onwards. At later passages, over 7-8, the translational value decreases due to dedifferentiation and loss of the differentiated phenotypic characteristics. Also, immortalized cell lines are commonly used in pharmacological studies due to their proliferative potential and maintained phenotype. Upon harvest and preparation SMCs display similar characteristic cellular response as seen *in vivo* with SMC activation, proliferation and dedifferentiation.[133,134] Treatment with serum-free medium on a substrate of basement membrane components reduces the proliferative response and maintains the cells in a more differentiated state.

In **Study II**, primary aortic SMC cell cultures from GK rats were used to evaluate the influence of linagliptin on phenotypic transition. Secondary aortic SMC cultures (passage 3-7), generated from primary aortic SMCs, from GK rats were used to investigate the effects of linagliptin on SMC proliferation *in vitro*. This model was chosen based on the availability of GK rats, previous experiences with aortic SMC cultures and presence of an established standardized protocol for tissue harvest and processing. In addition, utilization of this rat strain for investigation of the pharmacological effects *in vitro* enabled us to compare the data with the *in vivo* experiments. A limitation of this model is the risk of contamination with pericytes and adventitial fibroblasts. Also, the translational value decreases at later passages due to selective generation of dedifferentiated SMCs with increased proliferative potential.

2.3 VASCULAR ANATOMY AND PHYSIOLOGY

2.3.1 Vascular ultrasound

Ultrasonic imaging is an important diagnostic tool for the assessment of vascular wall morphology and physiology. The technique utilizes the piezoelectric effect in which electrical energy is converted to soundwaves. In general, ultrasonic soundwaves are created by the vibration of the piezoelectric crystals in the ultrasound transducer upon electrical stimulation. The amount of soundwave reflection is dependent on the difference in acoustic impedance of the tissue, while the difference in return time is dependent on the distance to the reflection site. Since the piezoelectric effect is bidirectional, the crystals in the transducer may convert the reflected soundwaves to electrical impulses, which can be processed to generate an ultrasound image. A higher frequency increases the resolution at the expense of tissue penetration resulting in a reduced image depth.[135] Clinical vascular ultrasound is commonly performed using 8-13 MHz transducers for peripheral arteries and 4-8 MHz for visualization of the abdominal aorta. Spectral and color Doppler is extensively used to determine blood flow direction, velocity and flow pattern. Since the introduction of ultrasound in medicine, the technological advancements have generated numerous ultrasonic applications, such as contrast-enhanced ultrasound, 3D-ultrasound and speckle tracking, which has increased the morphological and physiological assessment.[136,137] The advantage with ultrasound in comparison to other imaging modalities is that it is fast, does not require use of contrast agents and does not expose the subject to radiation. However, ultrasound image acquirement and image analysis have an increased observer variability.

2.3.2 Assessment of vessel wall anatomy

Structural assessment in vascular ultrasound is performed according to the leading-edge principle in which the location of an anatomical structure is defined by the upper demarcation line of the echo. Measurements of vessel wall structures, such as intima-media thickness (IMT), is performed in diastole on the most distant arterial wall in relation to the transducer, the far wall (Figure 6). Visualization and assessment of IMT and lumen diameter is commonly performed in a longitudinal brightness mode (B-mode) image.[138,139] The carotid IMT is increased in diabetic patients and has been identified as an early marker of atherosclerotic disease.[17,140–142] Measurements of lumen diameter can be used to diagnostically grade luminal stenosis and determine presence of pathological vascular remodeling.[73,143]

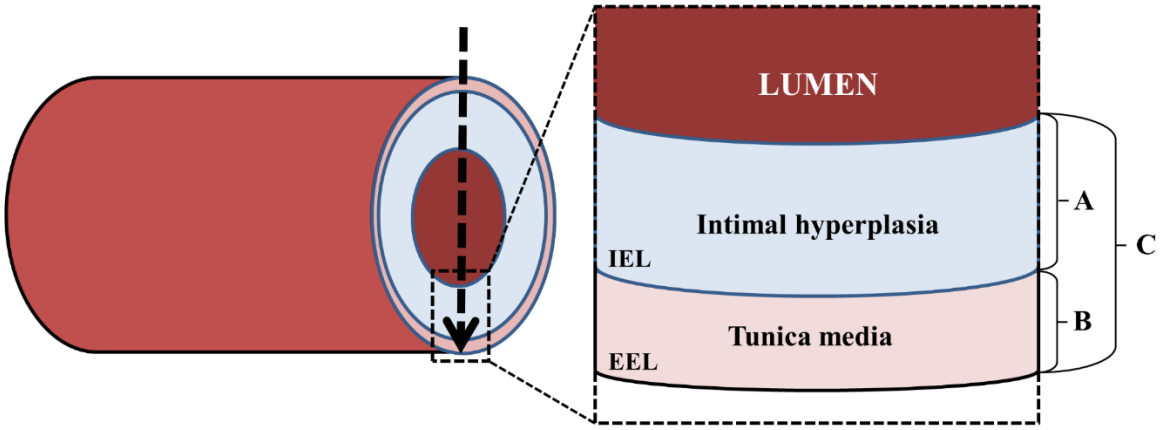

Figure 6. Schematic illustration of the vessel wall layers in an injured artery assessed in vascular ultrasound. The arrow represents the ultrasonic beam. The box represents the far wall in an artery. Measurements of A) intima-thickness, B) media-thickness and C) intima-media thickness. The *tunica adventitia*, constituting the outer surface of *tunica media*, is not included in the measurements and not shown in this figure.

2.3.3 Assessment of vascular physiology

Strain is a non-invasive rough estimate of the elastic properties of the artery in which a standard blood pressure is assumed.[144] Increased arterial stiffening measured as pulse wave velocity is a well-documented risk factor for future cardiovascular events.[144,145] Utilizing the Doppler technique in ultrasound it is possible to estimate the blood flow velocity by indirect measurement of the difference in wavelength between the emitted and returned soundwaves. The velocity is calculated using the Doppler equation, which is dependent on the angle between the emitted soundwave and the measured object, known as the angle of insonation (Figure 7). The angle of insonation should be kept below 60 degrees since greater angles results in increasing errors in the estimated velocity.[146] From the calculated blood velocity profiles it is possible to identify and extract velocities corresponding to different time points during the cardiac cycle, but also estimations of the mean velocity over time and the velocity time integral. Combining dimensional measurements of lumen area with the time-averaged velocity of the blood it is possible to non-invasively estimate the amount of blood flow.[147,148] Cardiac output may be calculated from aortic or pulmonary artery using lumen area, velocity time integral and heart rate.[148] Furthermore, using the calculated blood flow it is possible to estimate the FSS (Table 1).[147,148]

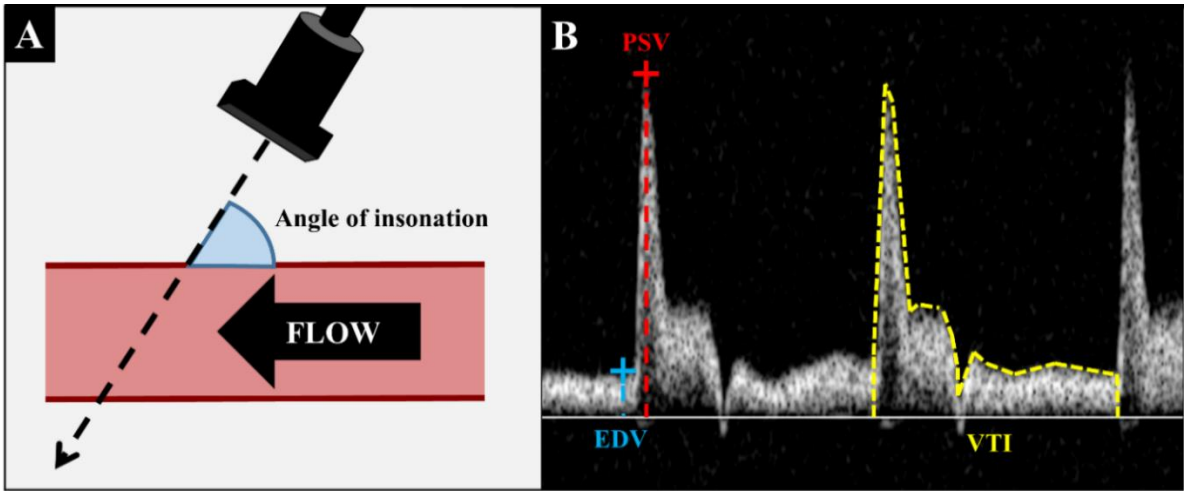

Figure 7. Assessment of carotid artery blood flow velocity in ultrasound. A) Schematic figure of angle of insonation. B) Velocity measurements in ultrasound biomicroscopy. EDV= End diastolic velocity, PSV= Peak systolic velocity, VTI= Velocity time integral.

Resistive index (RI) is an estimated measurement of the resistance in the vascular bed distal to the point of measurement and has been used clinically to evaluate perfusion in renal transplants and the placenta.[149–151] RI is dependent on the peak systolic velocity and the end diastolic velocity and is calculated as: *RI= (Peak Systolic Velocity –End Diastolic Velocity)/ Peak Systolic Velocity*. Pulsatility index (PI) is a measurement of the resistance in the distal vasculature, but also the elastic property of the proximal vasculature and is calculated as: *PI= (Peak Systolic Velocity-End Diastolic Velocity)/Mean Velocity*. PI is used for estimations of the fetal circulation[152] and to evaluate vascular remodeling in experimental research.[130,153] PI and RI are non-dimensional ratios, which reduces the risk of observer variability related to lumen geometry assessment (**Study IV**) (Table 1). However, assessment of blood velocity is also observer dependent since the estimated velocities are influenced by the angle of insonation and sample volume size.[146]

Table 1. Physiological parameters used for assessment of arterial function.

Parameter	*Formula*	*Unit*
Strain	(SD-DD)/DD x 100	Ratio
Blood flow	(HR x VTI x LA) /1000	mL/min
Fluid shear stress (FSS)	$4nQ/\pi r^3$	Dyne/cm^2
Resistive index (RI)	(PSV-EDV)/PSV	Ratio
Pulsatility index (PI)	(PSV-EDV)/MV	Ratio

EDV= end-diastolic velocity, DD= diastolic diameter, HR= heart rate, LA= lumen area (mm^2), MV= mean velocity, n= blood viscosity, PSV= peak systolic velocity, Q= volume flow rate (mL/s), r= radius (cm), SD= systolic diameter, VTI= velocity time integral (mm).

2.3.4 Ultrasound biomicroscopy in experimental research

Ultrasound biomicroscopy, or ultrahigh-frequency ultrasound, was originally developed in 1980's and the first reported use on human tissue was in 1990, by Pavlin CJ, Sherar MD and Foster FS, for visualization of the ophthalmic anatomy.[154,155] The technique utilizes the similar principle as conventional ultrasound with the differences being smaller distance between the piezoelectrical crystals, higher frequency of the emitted soundwaves and advanced software processing resulting in an increased image resolution.[154] Visualsonics Inc. was founded in 1999 by Foster FS and has since focused on development of UBM systems for preclinical and clinical settings. In 2002, Foster FS et al reported on the first use of UBM for imaging with simultaneous non-invasive estimation of blood flow using duplex Doppler in mice.[156] Recently, their latest UBM system (Vevo3100, 50MHz) was approved for usage on humans by the United States Food and Drug Administration. Over the last decade, several applications dedicated for experimental cardiovascular research have developed, such as ECG-gated imaging and advanced offline analysis software. However, these have yet to be validated for the use in vascular biological research.

Image acquirement in UBM is performed as in conventional ultrasound, which allows for non-invasive longitudinal assessment of anatomical and physiological alterations in response to injury or blood flow manipulation. Previous studies have shown that UBM can be used for accurate assessment of the IMT and lumen diameter in experimental rodent models (Figure 8).[157–159]

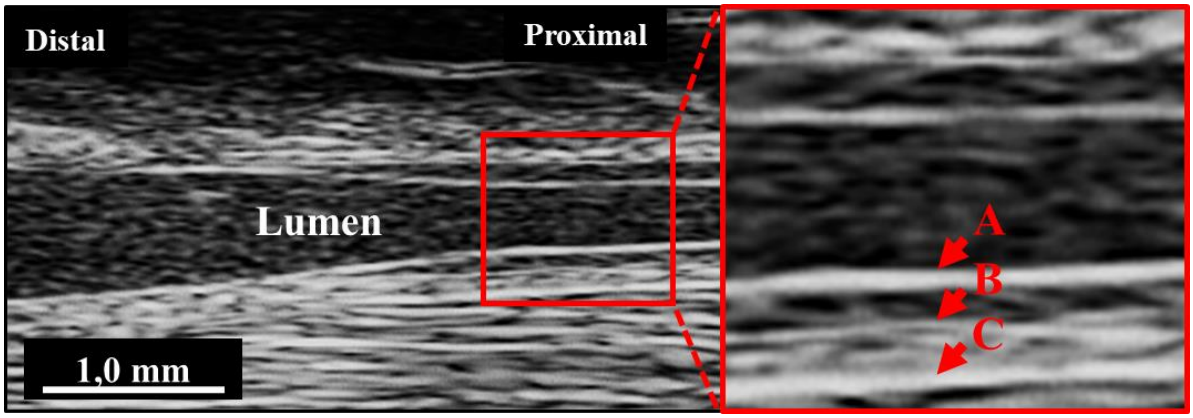

Figure 8. Ultrasound biomicroscopy image of a rat common carotid artery 4 weeks after balloon injury. A) Blood-intimal hyperplasia interface, B) intimal hyperplasia-internal elastic lamina interface and C) media-external elastic lamina interface.

In **Study I**, **II** and **IV**, a UBM system from Visualsonics Inc. (Vevo 2100) equipped with a 30-70 MHz probe (MS700) was used, which allows for a spatial resolution of 30 μm according to the manufacturer. Prof. Kenneth Caidahl utilized this technique early on for visualization of anatomical structures but also for assessment of the cardiac physiology in research animals. Further refinement of the technique by Dr. Anton Razuvaev revealed that this method can be used for accurate assessment of the arterial wall structures in balloon injured rats CCA.[157] Based on our previous experiences, we chose to further explore the potential of this technique.

2.3.5 Ex vivo assessment of vascular physiology

The wire myography method was originally described by Mulvani MJ and Halpert W and has been extensively used for assessment of arterial physiology in vascular remodeling.[160,161] Upon tissue harvest the *tunica adventitia* is macroscopically removed and the vessels are then mounted onto jaws (or pins) attached to a micrometer and a force transducer which allow assessment of vessel circumference (mm), applied tension (passive wall tension (mN/mm)) and contractile tension (active wall tension (mN/mm)) (Figure 9). Similar to the Frank-Starling phenomena in cardiac physiology, increased stretch or tension results in a stronger contraction due to an increased possibility for actin/myosin interaction.[161,162] The circumference at which

maximum active wall tension is achieved is referred to as the "optimal stretch" and can be visualized in a length-tension curve. A length-tension curve is generated by repeated potassium-induced contractions at increasing circumference and passive wall tension until the active wall tension decreases.[161] Vascular remodeling induces alterations in the circumference, passive- and active wall tension at optimal stretch which may generates shifts in the length-tension curve.[163] In addition, combining myography with histological and electron microscopy evaluation it is possible to calculate the force generated per *tunica media* area.[164] The myography technique may also be used in experimental pharmacological studies to assess contractile function and vascular reactivity in arteries fixed at optimal stretch.[165]

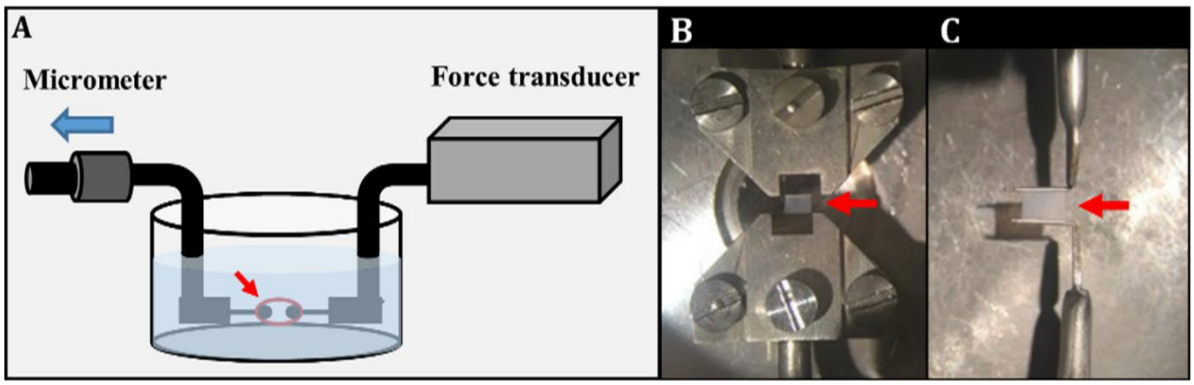

Figure 9. Vascular function measured in myography. A) Schematic illustration of the myography technique. Myography images of mouse B) common carotid artery and C) aorta at optimal stretch. Red arrows indicate location of the artery.

Assessment of the vessel wall function may also be performed using other myography methods such as pressure myography. In this method, vessel segments are mounted onto a perfusion system, which allow for intraluminal pressurization to physiologic conditions. The vessel segments may then be exposed to drugs, flows or pressures and assessment of the geometric alterations is monitored using a digital camera.[166] This method is suitable for evaluation of endothelial function but does not provide qualitative measurement of the contractile properties of the vessel wall.[166,167]

In **Study IV**, the wire myography method was selected in order to investigate the physiologic properties of the arterial wall. This method was chosen since it allowed for characterization of the influence of PCSK6 ablation on the functional and contractile properties of the arterial wall in a model of flow-mediated vascular remodeling.

2.3.6 Macro- and microscopic assessment of arterial wall healing

The morphological aspects of the arterial wall healing process may also be assessed using different staining techniques, such as *en face* and histochemistry. *En face* staining is commonly performed on unprocessed biological tissues, such as an artery, and allow for macroscopic evaluation. Evans-blue dye is a commonly used *en face* staining for visualization of areas with increased endothelial permeability, such as organ damage in ischemia reperfusion experiments, and non-endothelialization areas following balloon injury (**Study I-II**).[118,168] Prior to euthanization, the dye is injected to the systemic circulation where it binds to albumin forming an Evans-blue/albumin complex. In areas with increased permeability, such as un-endothelialized, inflamed or ischemic areas, the Evans-blue/albumin complex extravasates to the vessel wall, where Evans-blue dissociates from albumin and binds to the ECM resulting in a blue staining. Hence, the Evans-blue staining indicates absence of an intact endothelium rather being a specific staining for the presence of ECs.[169,170]

Histochemical staining is a valuable tool for microscopic evaluation of arterial wall morphology and composition (**Study I-IV**). Staining of tissue requires several processing steps with fixation, embedment and sectioning prior to performing the actual staining. Tissue processing and methodology for cryo-sectioning will not be considered since this method was not used in the thesis included studies. In general, the following tissue harvest the arteries were put in fixative, commonly Zn-formaldehyde, and embedded in paraffin blocks prior to sectioning. The sectioned slides are then deparaffinized prior to performing the actual staining. Morphometric evaluation of IH thickness and intima/media ratio is commonly performed using image analysis software. Also, using pressure fixation prior to tissue harvest allow for assessment of lumen dimensions and arterial remodeling.[171] Estimation of tissue composition may be performed using Masson Trichrome staining, which allow for separation of collagen, cytoplasm and cellular nuclei, or Movat pentachrome staining, which allows for visualization of nuclei/elastic fibers, collagen, mucin, fibrin and muscle. Quantitative analysis of arterial wall composition may be performed using image analysis software. In general, the histochemical staining may identify components of a biological tissue but lacks specificity in regards to identifying molecular targets.

Immunohistochemical (IHC) staining allows for detection and visualization of specific antigens in a biological tissue (**Study I-IV**). In short, the initial tissue processing steps are similar to the histochemical staining. Following deparaffinization the tissue needs to be further

processed for antigen-retrieval, commonly by adding heat through pressure-boiling. Once the antigens are retrieved, the primary antibody is added, which binds to the specific antigen of interest, followed by addition of the secondary antibody, which binds to the primary antibody and commonly have an attached enzyme reporter to which chromogens can adhere in order to amplify the staining signal. Furthermore, histochemical counterstain is used in order to visualize the anatomical structures. Quantification of IHC staining is traditionally performed using subjective scoring but may also be performed using image analysis software. However, the IHC staining intensity is sensitive to minor alterations in the protocol, resulting in an intra- and inter-observer variability, and interpretation of automated image analysis software should be made with caution.

2.3.7 Ultrastructural evaluation

The electron microscopy imaging technique allows for visualization of the ultra-structures in the material of interest, such as biological tissues. This technique requires preparation of the tissue in a specific manner which includes fixation in glutaraldehyde followed by further preparation depending on the type of electron microscopy being performed, such as thin metal coating or embedment with subsequent ultra-thin sectioning. Scanning electron microscopy (SEM) utilizes the reflection of emitted electron beam to visualize the topography of the material of interest (**Study I**) (Figure 10A). Transmission electron microscopy (TEM) relies on detection of scattering of the emitted electron beam when it passes through the tissue or material of interest (**Study IV**) (Figure 10B). Compared to SEM, TEM allows for higher resolution and visualization of the interior of a biological tissue at the expense of a more technically demanding tissue preparation process.

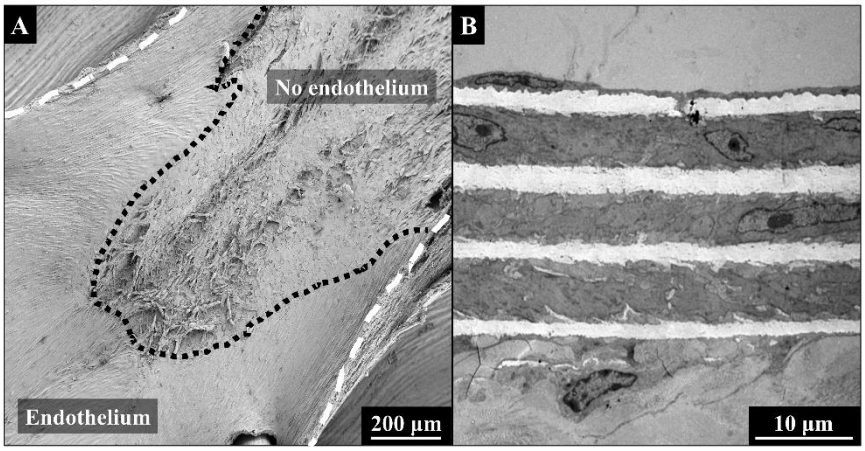

Figure 10. Representative electron microscopy images of the carotid artery.
A) Scanning electron microscopy of a rat common carotid artery following balloon injury. The white line represents the arterial border and the black line represents the re-endothelialization border. B) Transmission electron microscopy of a mouse common carotid artery.

2.4 BIOINFORMATICS

Large-scale transcriptome analyses, such as microarray and RNA-sequencing (RNA-seq), has revolutionized the understanding of alterations in gene expression and its relation to downstream biological effects. Compared to traditional polymerase chain reaction, in which the expression levels of a small number of pre-selected genes are assessed, this approach allows for an unbiased description of the gene expression landscape.[172] Microarray analysis of gene expression is performed by measuring the hybridization of specific nucleic acid strands in a large number of targets. In general, each probe-sequence corresponds with a specific genetic transcript, such as messenger RNA and microRNA.[172,173] Following tissue harvest with subsequent RNA extraction, the RNA needs to be further processed with reverse transcription to create the more stable complementary DNA (cDNA).[172] The cDNA is then loaded onto a chip containing a vast number of preselected probes, such as cDNA or oligonucleotides representing a specific gene. Following hybridization, or binding of the cDNA to the target probe, fluorescent dyes is added for detection of the amount of hybridized cDNA at each probe by measuring the differences in fluorescent emission. Normalization of the data is performed prior to analysis using "control"-probes.[174,175] Further analysis is performed in an unbiased manner by statistically comparing the relative gene expression and identifying significantly dysregulated sets of genes. Combining gene ontology classification with publicly available databases, it is possible to investigate the relationship between the gene expression and its associated downstream biological effects by using gene set enrichment analyses for associated pathways and prediction of key transcription factors and network drivers.[176–181] Validation of the microarray analysis is commonly performed using quantitative real-time polymerase chain reaction (qRT-PCR) with a small number of preselected genes using "house-keeping" genes as control.

The RNA-seq technique allows for quantitative assessment of the whole transcriptome. In comparison to microarrays, this technique allows for detection of small alterations in the transcriptome and identification of novel transcripts, since this technique does not depend on predetermined hybridization probes.[182] Similar to the microarray, RNA-seq data may be used for the computational analyses as described above. In comparison to microarrays, this technique has a higher reliability and reproducibility at the expense of being expensive and also requiring specific technical and data analysis expertise.[172,183]

In the **Study III**, we utilized the microarray technique, with approximately 31 000 probes (Affymetrix GeneTitan Rat Gene ST v1.1), to evaluate the temporal alterations in gene expression in the arterial wall healing process. This technique was selected in order to perform analysis of the relative changes in gene expression over time rather than quantification and identification of novel transcripts at different time points. IHC staining was used for validation of alterations in gene expression, for a selected number of genes.

2.5 ETHICAL CONSIDERATIONS

Studies of the vascular wall healing processes can only to some extent be considered in *in vitro* experiments due to the complex biology of the arterial wall, the contribution of blood components and the effects of hemodynamic forces. Therefore, the experimental *in vivo* models are the only available methods for this purpose. Prior to initiation of each experiment a careful literature review was conducted. In general, *in vivo* studies are preceded by *in vitro* experiments in order to reduce the number of animals needed. However, *in vivo* experiments may generate hypotheses which can be further investigated *in vitro* (**Study II**). Pilot studies and power calculation were performed when applicable in order to reduce the number of animals needed. Also, the use of UBM for morphological assessment of the vascular wall healing further reduced the number of animals needed. The injury models used have been refined in our research group and display a low rate of complications. Also, the animals were housed together with other similar animals in cages with a stimulating environment. The animals were monitored by professional animal caretakers for signs of pain and discomfort on a daily basis. Analgesia and anesthesia were given upon surgery and post-surgical analgesia was administered upon signs of suffering or pain. During the course of the studies, the UBM technique has been refined which has further reduced the number of animals needed in each study. In **Study I**, a retrospective analysis of UBM images from previously performed experiments was performed which has improved our methodology. Also, the use of non-invasive UBM has replaced the morphological assessments of the arterial wall which was previously performed in histology *ex-vivo*. Creation of the biobank in **Study III** will be a valuable tool for the research community and reduce the number of animal experiments needed. All animal experiments were approved by the Regional Ethical Review Board in Stockholm and conducted according to the laws and regulations applicable to experimental *in vivo* experiments.

2.6 STATISTICAL ANALYSIS

In general, the statistical analysis was performed using two-tails and a significance level of $p<0.05$. The statistical calculations were performed using GraphPad Prism (version 5 and 6). Distribution of the data was assessed using Sharpio-Wilks normality test. Comparative statistics between two groups was performed using Students t-test for parametric data and Mann-Whitney U-test for non-parametric data. Paired analysis was performed using paired t-test or Wilcoxon matched-paired signed rank test on parametric and non-parametric data. One-way ANOVA with Bonferroni multiple comparison test and Kruskal-Wallis with Dunn's multiple comparison test was used for comparison of parametric and non-parametric data containing more than two groups. Two-way ANOVA was used for comparison of data with more than two groups and multiple observations. To investigate existence of correlation between two or more groups we used Pearson or Spearman's rank correlation test, for parametric and non-parametric data respectively. Evaluation of the relation between different methodologies to assess the similar structure or function may result in statistically significant correlation. However, the correlation analysis does not provide information regarding potential systematic errors, such as under- or overestimations. The Bland-Altman method is a graphical and statistical method used to visualize and evaluate existence of potential systematical differences and was used when applicable.[184] In order to assess the intra- and inter-observer variation, a variability coefficient was calculated according to the following formula: *mean standard deviation/mean average x 100*. Statistical evaluation of gene expression data was performed using Student's t-test with Bonferroni multiple comparison test. Prediction of transcription factors was performed using a false discovery rate<0.05 as cutoff value.

3 AIMS

3.1 GENERAL AIM

The overall purpose of this thesis was to investigate the different aspects of the arterial wall healing process; re-endothelialization, intimal hyperplasia and vascular remodeling.

3.2 SPECIFIC AIMS

Study I: To evaluate the use of ultrasound biomicroscopy as a method to assess the re-endothelialization process using intimal hyperplasia morphology as a surrogate marker in the rat carotid balloon injury model.

Study II: To investigate the influence of treatment with linagliptin on arterial wall healing in normal and diabetic conditions.

Study III: To describe the transcriptomic landscape of the arterial wall healing process in the rat carotid balloon injury model.

Study IV: To elucidate the influence of PCSK6 on flow-mediated vascular remodeling in the mouse carotid ligation model.

4 RESULTS AND DISCUSSION

4.1 STUDY I

The re-endothelialization process is a crucial component of the arterial wall healing process and is needed in order to restore the vessel wall homeostasis.[185,186] This is exemplified by previous experiences from large registry studies, in which DES with non-selective anti-proliferative effects has been shown to increase the risk of myocardial infarction due to a delayed or non-existing re-endothelialization process.[187,188] Assessment of the re-endothelialization in experimental animal studies have traditionally been limited to *ex vivo* methodologies, such as *en face* staining and histology.[116,171] Previous studies have shown that presence of an endothelium influences and may even reduce the neointima.[9,51] Therefore, we hypothesized that the influence of the re-endothelialization process on the IH formation can be detected in UBM. Hence, we aimed to investigate the use of morphological alterations in the IH thickness as a surrogate marker for the re-endothelialization process. A retrospective analysis was performed on UBM images from 93 rats, three different strains (SD, GK and Wistar), which all had been subjected to carotid balloon injury and examined with UBM, using handheld probe or a probe-holding rail-system, at 2 and 4 weeks after injury. At sacrifice 4 weeks after injury, *en face* staining for endothelial permeability with Evans-blue was performed and was tissue harvested for histological and SEM evaluation.

From the 93 rats, 66 were included for further analysis. Comparative analysis of all included animals revealed a significant correlation between the UBM assessed re-endothelialization length and the *en face* measurements. Subgroup analysis revealed the similar pattern for SD and GK rats but not for the Wistar rats, which displayed a borderline significant pattern with a reduced correlation coefficient. Further, we could identify presence of a systematic underestimation of the re-endothelialization length assessed in UBM. Also, a significant increase in re-endothelialization length over time was observed in the Wistar rats. Histochemical staining confirmed the morphological pattern seen in UBM and IHC staining for von Willebrand factor revealed presence of endothelium in the re-endothelialized areas detected in UBM. In addition, SEM provided topographical confirmation of the *en face* staining. The intra- and inter-observer variability of ultrasound assessed re-endothelialization length were found to be at an acceptable level (Figure 11).

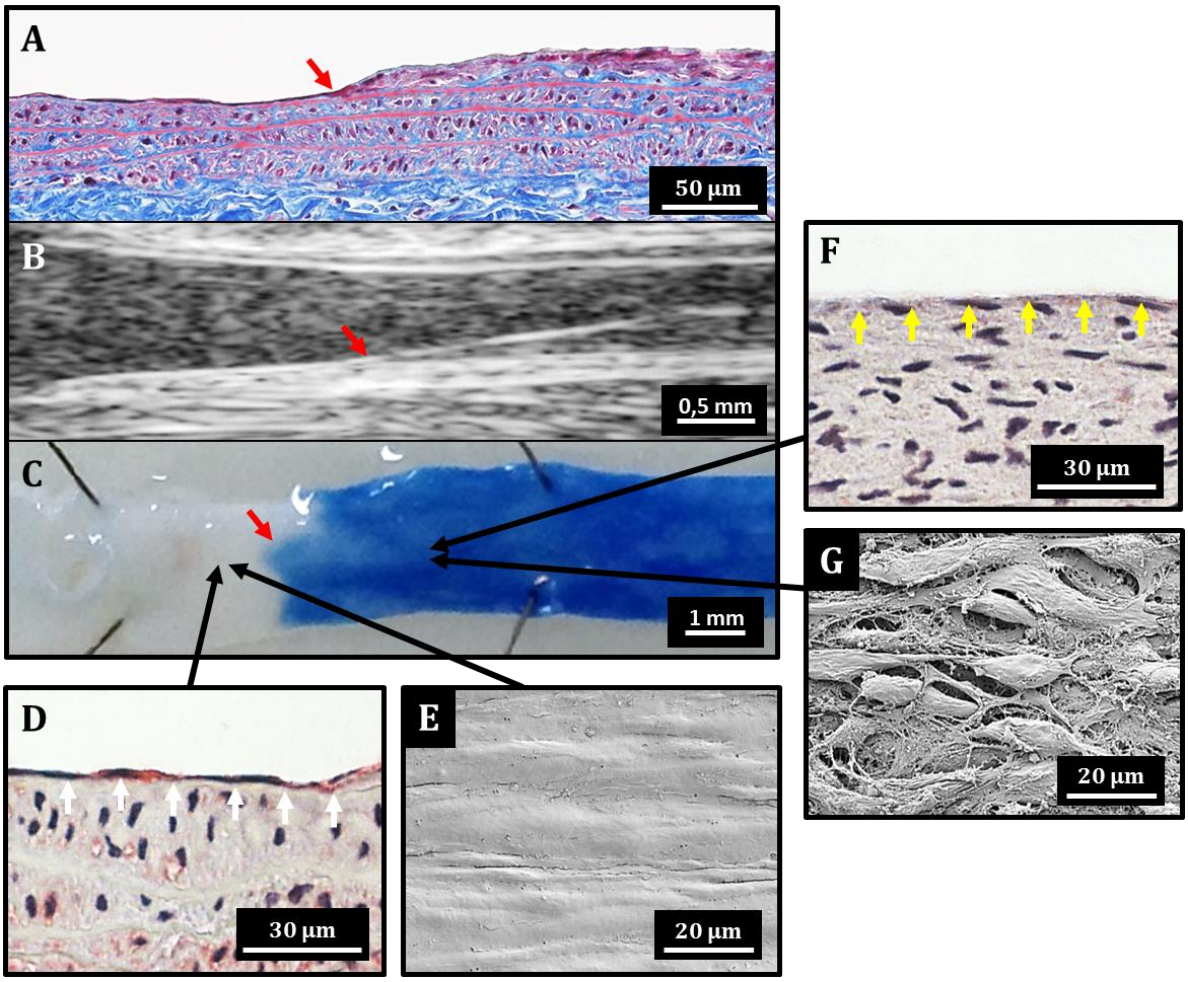

Figure 11. Visualization of the re-endothelialization process following arterial injury. Images of endothelial regrowth with different methods and magnification. A) Histochemistry, B) ultrasound biomicroscopy, C) Evans-blue, immunohistochemical staining and scanning electron microscopy of endothelialized, D) and E), and non-endothelialized areas, F) and G). Red arrows indicate the endothelialization border, white arrows indicate positive staining and yellow arrows indicate absence of staining for von Willebrand factor. Reproduced from *J Ultrasound Med* with permission.[189]

The comparative analysis revealed a systematic difference in re-endothelialization length between our methods. This could be explained by the difference in methodology since the *en face* staining visualizes areas permeable to albumin[116] and these measurements included the bifurcation area while the ultrasound measurements rely on morphological differences in IH thickness and does not include the bifurcation. Furthermore, it is possible that differences in arterial longitudinal tension, between the *ex vivo* and *in vivo* methods, could have influenced our results. No direct comparative validation between *en face* and ultrasound in regards to the position of the edge of endothelium was performed.

The difference in correlation detected in the Wistar rats could be related to the increased body weight of the animals upon surgery. Since the arterial lumen size is dependent on animal body weight and length, it is possible that an increased lumen diameter could have reduce the injury inflicted to the artery upon surgery. Also, we did not use any pressure-control device during the surgical procedures, which could have influenced our results since the intimal hyperplastic response is directly associated with the pressure in the balloon upon surgery.[53] Intriguingly, our results indicate that the re-endothelialization length increases over time in the Wistar rats. Previously, it has been reported that Wistar rats have an increased re-endothelialization process and IH formation compared to SD rats following carotid air-drying injury.[190] However, we could not detect any difference in IH thickness between the different rat strains (Figure 12). Whether our results are influenced by strain specific differences in the re-endothelialization process remains to be fully elucidated. Also, these results should be interpreted with caution since we did not detect significant correlation at 4 weeks for Wistar rats and did not validate UBM for estimation of the re-endothelialization process at 2 weeks.

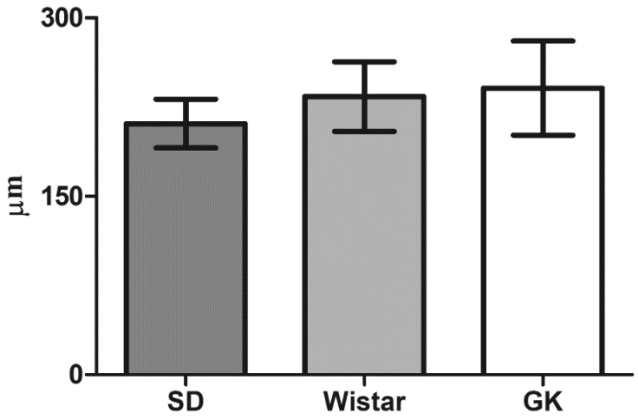

Figure 12. Intima-media thickness at 10-13 mm proximal to the carotid bifurcation 4 weeks after injury. Data expressed as mean± 95% CI. GK= Goto-Kakizaki, SD= Sprague-Dawley. No significant differences in intima-media thickness could be detected.

The major reason for exclusion was poor visualization of the carotid bifurcation, which was related to the ultrasound image acquirement method. The probe-holding rail-system had a higher exclusion rate, 48.0% compared to 11.8%, which could be explained by a reduced flexibility for proper angulation of the ultrasound probe and suboptimal positioning of the rat. The ultrasound images used in this study were acquired in order to visualize the IH formation rather than the re-endothelialization process. Hence, it is possible that utilization of a dedicated

image acquirement protocol with optimal probe angulation and rat positioning would reduce the exclusion rate.

Specific staining for endothelium (von Willebrand factor) confirmed presence of a uniform endothelium in the UBM defined re-endothelialized areas. However, in the presences of IH the ECs displayed a non-uniform gradual decrease and ultimately absence of staining. These results implicate that the UBM detectable morphology of IH formation serves as a valid surrogate marker for the proximal endothelial border.

Previous studies have reported on visualization of the injured endothelium by targeting the albumin permeability using modified Evans-blue molecules labeled with gadolinium or gadofosveset for detection of endothelial permeability in magnetic resonance imaging.[191,192] Similar studies has been performed using contrast-enhanced ultrasound with microbubbles targeted for constituents of the subendothelial layer, such as $\alpha_V\beta_3$-integrin and vascular cell adhesion molecule-1.[193,194] These methods are invasive, time consuming and rely on vascular access and contrast agents. In comparison, our method is non-invasive, fast and reproducible with the drawback of being unspecific and relying on surrogate morphological measurements. The applicability of our methodology to other experimental models of arterial injury may be limited since the endothelial thickness lies below the resolution of the UBM system and our surrogate measurement rely upon detectable differences in IH thickness. Previous experiences from an unpublished pilot study reveals that full re-endothelialization with concomitant IH formation is achieved following pressure-controlled balloon injury (1,5-2,0 ATM) to half of the CCA. Therefore, assessment of the re-endothelialization process using IH morphology could not be performed. Despite these limitations, our methodology has the advantage of being fast, non-invasive, reproducible, does not rely on contrast agents and is applicable on a commonly used experimental model of arterial injury.

4.2 STUDY II

Patients with T2DM have an increased cardiovascular disease burden and display a higher frequency of complications following invasive vascular interventions.[37–39] It has been suggested that T2DM induces phenotypic modulation of SMCs, which results in an increased intimal hyperplastic response.[32] The influence of T2DM on the re-endothelialization process remains elusive.[195,196] Modulation of the incretin system, with GLP-1 agonists, has been shown to reduce the cardiovascular mortality in type 2 diabetic patients and decrease the IH formation following balloon injury in rats.[43] Previous studies have revealed GLP-1 independent beneficial effects of DPP-4 inhibitors on SMCs and ECs *in vitro*.[197,198] We hypothesized that treatment with DPP-4 inhibitors reduces the risk of complications following invasive vascular interventions by improving the arterial wall healing process in type 2 diabetic conditions. Therefore, we aimed to investigate the effect of treatment with linagliptin, a DPP-4 inhibitor, in an experimental model of vessel wall healing in normal and diabetic conditions. Wistar and diabetic GK rats were subjected to CCA balloon injury and randomized to treatment with linagliptin or placebo through daily gavage. The healing process was monitored at 2 and 4 weeks using UBM and *en face* staining for assessment of re-endothelialization process was performed upon euthanization at 4 weeks. An additional short-term experiment was performed on GK rats in which the animals randomized to treatment and sacrificed at 24h after injury. Secondary aortic SMC cell cultures from GK rats were used for investigation of the pharmacological effects of linagliptin on SMC proliferation.

Treatment with linagliptin improved the glucose tolerance and reduced the DPP-4 activity in serum after 4 weeks of treatment. However, no effect of linagliptin on arterial wall morphology could be detected in UBM, which could be confirmed in histomorphometry. Furthermore, linagliptin did not influence lumen diameter or SMC proliferative rate in the vessel wall at 4 weeks after injury. In addition, no difference in the re-endothelialization process could be detected. Interestingly, we could detect a linagliptin associated reduction of CD44 expression in the early response to arterial injury *in vivo*. Presence of linagliptin, in absence of GLP-1, retained SMCs in a contractile state and reduced SMC proliferation in a concentration dependent manner *in vitro*.

The discordance between the *in vitro* and *in vivo* experiments could have been influenced by differences in concentration of linagliptin during the healing process. We did not measure the concentration of linagliptin in plasma. However, the increased glucose tolerance and reduced

DPP-4 activity after 4 weeks of treatment indicated its presence. In humans, the half-life of linagliptin is >100h and steady-state, i.e. the equilibrium of intake and elimination, is reached after 4 days of daily administration.[199] In Wistar rats, linagliptin is absorbed following oral administration (10 mg/kg), reaches a maximum plasma concentration after 1 hour and has a half-life of 27,5h.[200] In general, the pharmacologic steady-state of a drug is estimated to occur after 4-5 half-lives of the specific compound. However, this assumption might not be applicable for estimating the time to steady-state of linagliptin in rats since it does not apply in humans. The time to steady-state in Wistar rats remains to be investigated. Therefore, it is possible that our results could have been influenced by an inadequate plasma concentration of linagliptin during the initial injury response since the animals were not treated prior to surgery. Previously, it was shown that pretreatment (2 weeks) and continuous oral administration of linagliptin (3 mg/kg) reduced the IH formation following wire injury in mice.[201] However, it was recently reported that treatment with linagliptin (3mg/kg) reduces IH formation at six weeks after carotid balloon injury in Wistar rats without pretreatment.[202] Investigation of the IH formation process has shown that the major increase in IH thickness occurs during the first 4 weeks after which it slows down and ceases at 8 weeks after injury.[117] Therefore, it is unlikely that an additional two weeks in time from injury to sacrifice would have generated the similar findings in our study.

Previous studies have shown that linagliptin may reduce endothelial dysfunction and decrease the inflammatory response of ECs.[197,203] Sitagliptin, another DPP-4 inhibitor, has been shown to improve re-endothelialization following injury in mice.[204] However, we could not detect any effect of linagliptin on the re-endothelialization process in either normal or diabetic conditions. Indications from a correlation analysis revealed that DPP-4 activity was associated with percent re-endothelialized areas in the GK rats. However, the concentration of linagliptin in plasma was not measured and conclusions regarding the beneficial effects on endothelial regrowth should be made with caution. In addition, these results were not corroborated in the Wistar group.

Linagliptin has been shown to reduce systemic inflammation and presence of circulating inflammatory cytokines (IL-6, tumor necrosis factor-α) in rats and humans.[202,205,206] Since inflammation influences arterial wall healing and the IH formation[57,207], we sought to investigate the influence of linagliptin on the acute inflammatory response. In the short-term experiment, we used a higher concentration of linagliptin (10mg/kg) and pretreated the animals

the day before the surgery. The targets for evaluation of early inflammatory response were selected based on a previous study.[208] We could detect a reduced CD44 expression and a decreased intensity of CD44 in IHC staining. CD44 has previously been associated with SMCs proliferation, IH formation, leukocyte recruitment and contractile inward remodeling.[209–212]

In vitro experiments revealed a GLP-1 independent anti-proliferative effect on SMCs, which are in line with a previously published study.[201] Also, exposure to linagliptin retained expression of contractile genes, which indicates conservation of more differentiated SMC phenotype. Previously, it has been suggested that soluble DPP-4 induces proliferation and upregulation of inflammatory signaling (IL-6, IL-8) by direct activation of protease-activated receptor 2 on SMCs.[198] Whether our results *in vitro* are related to selective inhibition of the protease-activated receptor-2 activation remains to be investigated.

4.3 STUDY III

The carotid balloon injury model is an established animal model used to investigate the biology of the healing arterial wall and in particular the intimal hyperplastic response.[117,119] Advancements in large-scale gene expression analysis and computational biology have revolutionized the understanding of biological processes. Here, we hypothesized that utilization of the carotid balloon injury with large-scale multi-level transcriptomic analysis would bring further insights to the arterial wall healing process. We aimed to create an encyclopedia of the temporal alterations in gene expression of the arterial injury response and provide the research field with a resource for validation and future collaborations. Male SD rats (n=7-10/group) were subjected to left carotid balloon injury and euthanized at different time points (prior to injury, 0h, 2h, 20h, 2 days, 5 days and 2, 6, 12 weeks). At euthanization, multiple tissues were harvested in a systematic manner. Carotid arteries were harvested for histological and microarray analysis and plasma was used for lipid analysis. Microarray profiling with subsequent bioinformatic exploration of the microarray data and histochemical characterization of the injury process was performed. IHC staining was used to validate the bioinformatic analysis.

Global gene expression profiling revealed presence of three dynamic phases: acute injury (0-2h), tissue remodeling (20h-5d) and late homeostasis (2-12w). The initial injury phase was characterized by upregulation of genes and pathways related to inflammation, leukocytes and coagulation and reduced expression of genes related to ECs. Prediction of key transcription factors indicated presence of both cell proliferation and apoptosis. IHC evaluation revealed an

increased presence of adventitia resident lymphocytes and macrophages. The dynamic tissue remodeling phase was marked by an increased expression of genes and pathways related to ECM modulation, SMC migration/proliferation, cytokine signaling and osteoblast differentiation. Analysis of associated transcription factors indicated SMC phenotypic modulation and activation. During late homeostasis phase, a reduced expression of genes related to cell proliferation with a concomitant upregulation of genes associated with cytoskeleton and vasodilation was observed. Also, an upregulation of pathways related to SMC contractility, reduced cell proliferation and maintained chondrocyte differentiation could be detected. The IHC staining confirmed presence of contractile markers in SMCs in this phase. The contralateral artery displayed a dynamic dysregulation of genes related to tissue metabolism. In addition, bioinformatic analysis revealed potential novel pathways in arterial healing.

Acute endothelial denudation with concomitant mechanical trauma to the vessel wall induces platelet aggregation, thrombus formation, inflammation and recruitment of circulating leukocytes.[117,207] As expected, we could detect a downregulation of genes related to ECs and upregulation genes associated with leukocytes and inflammation. A temporal downregulation of Il6 expression was detected, which is in line with previous studies.[208,213] Interestingly, we could not detect the similar initial upregulation as detected by Fedorov A et al.[208] This could be related to the use of different time points, differences in bioinformatic approach or related to differences in parts of the artery used for gene expression analysis. The associated pathways were initially related to coagulation, immune response and apoptosis, which is known to occur in response to mechanical injury[207], and later related to cell adhesion and interferon-gamma production (Figure 13). Interferon-gamma signaling has been associated with SMC proliferation and IH formation.[214,215]

The morphological alterations during tissue remodeling phase have previously been well characterized and are marked by transmigration of SMCs to the intima and subsequent proliferation.[57,216] Our results revealed a gradual separation from the acute phase in global gene expression profile and dysregulation of genes related to ECM, SMC proliferation and cell migration. Similar to Li J et al[213], a significant increase in C1qtnf3 expression was detected, which has been associated to SMC proliferation.[217] Further analysis displayed a dynamic pattern with upregulation of pathways related to the adaptive immune system and later also proliferation and cell migration (Figure 13).

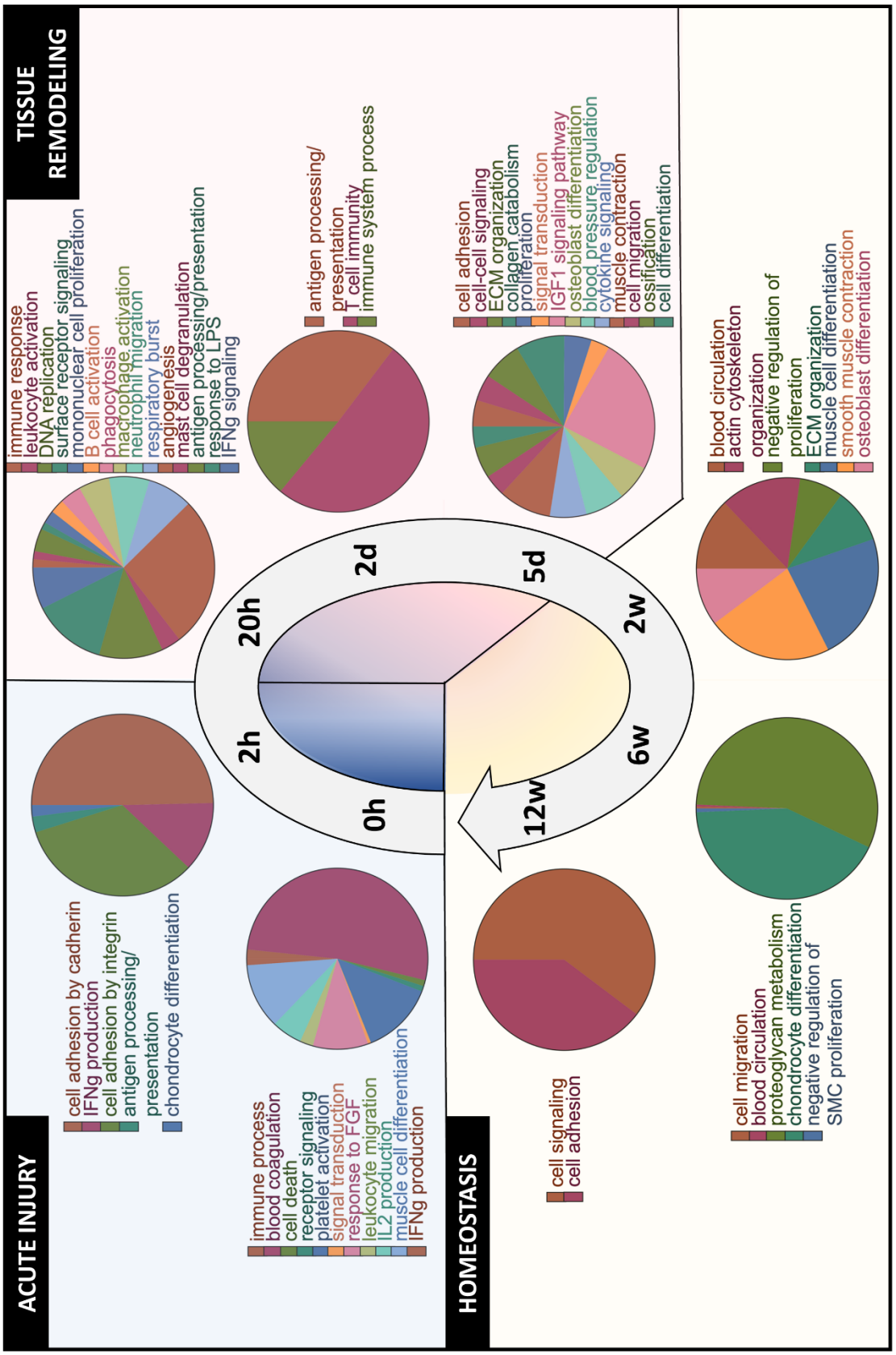

Figure 13. Bioinformatic pathway analyses characterizes three phases in response to injury. Gene set enrichment analyses of significantly upregulated genes at each time-point upon injury *vs.* the previous one. Plots show enrichment of gene ontology categories, only significant processes with p-value<0.05 shown.

Interestingly, during this phase we could detect presence of an osteoblast differentiation pathway. To facilitate transmigration, SMCs undergo a de-differentiation process from quiescent contractile to activated synthetic. The phenotypic transition induces downregulation of contractile genes and upregulation of a variety of genes commonly not related to SMCs.[218] Evaluation with IHC confirmed phenotypic modulation of SMCs and revealed presence of a spatial pattern of de-differentiation, detected as a gradual loss of SMTN from the lumen towards deep within the media.

Late homeostasis displayed a return of the global gene expression profile to the similar levels as seen in the intact artery. This phase was characterized by reduced expression of genes associated with proliferation and ECM degradation and upregulation of genes related to cytoskeleton and vasodilation. Further analysis revealed increased expression of pathways associated with reduced proliferation, SMC contractility and metabolism (Figure 13). Prediction of key transcription factors showed contribution of Tp53 during this phase, which could be explained by its extra apoptotic functions related to cell-cycle arrest and DNA repair.[219]

Analysis of global gene expression profile revealed a homogenous clustering at each time point, which indicates that the injuries were performed in a similar manner. In concordance with previous studies, we could identify dysregulation of specific genes throughout the healing process. These findings indicate a robustness of our methodology and data. Therefore, we performed further exploration of our data, which suggested presence of novel pathways in arterial wall healing, such as immune-priming, clonal expansion and osteo-chondrogenic pathways.

It has previously been shown that SMCs in atherosclerotic plaques may gain characteristics and express markers primarily related to macrophages.[220] Our results indicate that the arterial injury induces an upregulation of the macrophage associated markers Cd11b and Csf1r in SMCs. The expression of these genes is reduced over time but persists even at later time points. These results suggest that SMCs may express monocyte-macrophage markers and possibly contribute to monocyte-related inflammatory responses during the healing process. Furthermore, it has been suggested that the IH is formed from a minor fraction of the SMCs rather than a general uncontrolled proliferative response.[221] Expression of Pou5f1 (Oct4) and Pax3 has been associated with pluripotent stem cells and been implicated to be expressed by cells contributing to healing responses.[222–224] Analysis of gene expression revealed a general

increase in Pou5f1 expression and later upregulation of Pax3 at 12 weeks after injury. Interestingly, we could detect an increased expression of both these markers in the media of the injured artery on a protein level. An increased presence of Pax3 could be detected in the medial SMCs even at later time points. However, the IHC stainings displays a strong signal for Pax3 in the adventitia, which could explain the differences detected in the gene expression analysis. The role and influence of oligoclonal expansion on the arterial wall healing process is currently being further investigated.

The increase in osteo-chondrogenic pathways inspired us to further explore specific targets that are currently being investigated in our research group. We could predict an increased expression of the transcription factor Runx2, which has previously been identified as a key driver of osteo-chondrogenic differentiation but has also been related to phenotypic modulation of SMCs and vascular calcification.[225–229] Runx2 is also a regulator of aggrecan (ACAN), which has been suggested to mediate mechanical protection of SMCs.[230,231] The histological analysis revealed presence of a spatial pattern in the Runx2 and ACAN expression. The expression was reduced in deeper parts of the media with a gradual increase towards the lumen at 2 weeks after injury followed by an absence of ACAN at later time points. Interestingly, the reduced expression of ACAN coincided with *de novo* formation of an elastic membrane, located beneath the most luminal SMCs in the neointima. Previous studies have suggested that loss of endothelium and disruption of the IEL increases the transmural interstitial flow with subsequent exposure of shear stress directly on to the SMCs, which may induce migration and proliferation.[232,233] Therefore, our findings could be related to a protective response to the biomechanical stress exerted on the SMCs by the interstitial flow. As the IH thickness increases, the influence of the transmural interstitial flow on the deeper parts of the arterial wall is reduced resulting in the spatial pattern of ACAN expression seen at 2 weeks after injury. *De novo* formation of the lumen elastic lamina at later time points decreases the interstitial flow resulting in a loss of ACAN expression. These findings warrant further investigation to understand the influence of interstitial flow on osteo-chondrogenic gene expression in SMCs.

Validation of large-scale gene expression analysis is commonly performed using quantitative methods, such as qRT-PCR. This was not performed since our methodological approach to gene expression data was validated in previous studies.[234,235] Here, we used IHC for qualitative evaluation of our gene expression data on a protein level. The gene expression analysis was performed on the proximal and distal parts of the carotid artery whilst the middle part was used

for histological evaluation. Regrowth of the endothelium occurs in the distal and proximal parts of the artery until approximately 6 weeks after injury, leaving the middle third of the artery without endothelial coverage. Presence of ECs influences the healing process and reduces IH formation.[9,51] Therefore, it is possible that our results may have been influenced by the re-endothelialization process. Also, our analysis may have been influenced by the gene expression of adventitia resident cells. The influence of the adventitia in the arterial wall healing process should be further investigated.

4.4 STUDY IV

Vascular remodeling is an important process needed to uphold the arterial homeostasis upon changes in the physiological milieu. Alterations in blood flow or local inflammation induces a structural rearrangement of the ECM, which may result in stiffening of the vascular wall and changes in vessel geometry.[75,90,236] Complications related to a defective remodeling response, such as constrictive remodeling, following invasive vascular interventions are common.[73,237] The vascular remodeling process may also be emphasized in atherosclerosis and is associated to plaque destabilization and rupture.[75,238,239] Our group have previously shown that increased PCSK6 expression is associated with plaque instability in patients with carotid stenosis.[104] In an ongoing study, we could detect indications of an increased outward remodeling process in PCSK6$^{-/-}$ mice. Therefore, we hypothesized that PCSK6 deletion increases flow-mediated outward remodeling. Here, we aimed to investigate the impact of PCSK6 deletion on vascular physiology and morphology in a model of flow-mediated vascular remodeling. Male C57Bl/6J and PCSK6$^{-/-}$ mice were subjected to carotid ligation in order to increase the blood flow in the contralateral artery. The mice were subjected to repeated UBM examinations and wire myography was performed upon euthanization. The experiment was repeated for histological and TEM evaluation of the tissue.

We found that PCSK6 deletion increased the flow-mediated outward remodeling response, detected as an increased lumen circumference over time. Further analysis revealed a gradual reduction of PI and RI over time in the PCSK6$^{-/-}$ mice. In wire myography, the PCSK6 deficient mice displayed a flow-mediated increase in lumen circumference with an absence of effect on active tension at optimal stretch. A remodeling associated increase in elastic lamina content could be detected in the WT mice, which could not be detected in PCSK6$^{-/-}$ mice. IHC revealed a reduced expression of contractile SMC markers (SMA, MYH11, LMOD1) in the PCSK6$^{-/-}$ mice exposed to increased blood flow.

The PCSK6 deficient mice displayed a progressive flow-mediated outward remodeling response, which could not be detected in the WT mice (Figure 14A). Interestingly, a non-significant pattern could be observed in the WT mice with an initial increase followed by a return to baseline. Previous studies have shown differences in regards to the impact of increased flow on the contralateral artery geometry in the carotid ligation model.[125,240–242] The absence of significant alterations in arterial lumen geometry in the WT mice could be related to an adaptive remodeling process. The compensatory arterial remodeling response in C57Bl/6J mice has been thoroughly described by Eberth JF et al in the murine aortic banding model.[130] In this model, the dramatic increase in blood flow induces an initial progressive expansion of the arterial diameter until 2 weeks after surgery and is followed a gradual reduction until stabilization.[130] In comparison, the aortic banding model induces myocardial hypertrophy and an increased arterial wall thickness while the carotid ligation model has been shown to induce alterations in the arterial caliber without affecting the arterial wall thickness.[126,130,240] Due to the differences in surgical procedure and intensity of the physiological response, direct comparisons between the methods should be made with caution. However, the adaptive remodeling as a physiological response to the increased blood flow is a fundamental biological process. Therefore, our result indicates that PCSK6 is involved in the adaptive remodeling response.

Assessment of volume flow rate confirmed an increased flow in the contralateral artery in response to carotid ligation, which is in line with previous studies.[128,148] Investigation of the flow velocities revealed difference in the pattern of PI and RI over time between PCSK6 deficient and WT mice. In the WT, a gradual increase in PI and RI was observed whereas the PCSK6 deficient mice displayed a progressive reduction in PI and RI without significant alterations in end-diastolic velocity. These alterations could be related to local remodeling in the CCA but also to a defective remodeling process in the distal vasculature or changes in the myocardium. However, differences in local adaptive remodeling response could be detected in the wire myography experiment. In the PCSK6 deficient mice, a remodeling associated increase in circumference at optimal stretch was observed. Interestingly, a flow-mediated increase in active tension was seen in the WT mice, which could not be observed in PCSK6$^{-/-}$ mice (Figure 14B). These differences did not influence the media thickness. Also, there was no difference in length-force curves between the PCSK6$^{-/-}$ and WT, which indicates that PCSK6 deficiency does not significantly alter the functional elastic properties of the arterial

wall. These results confirm that PCSK6 deletion induces a defective remodeling response in the CCA.

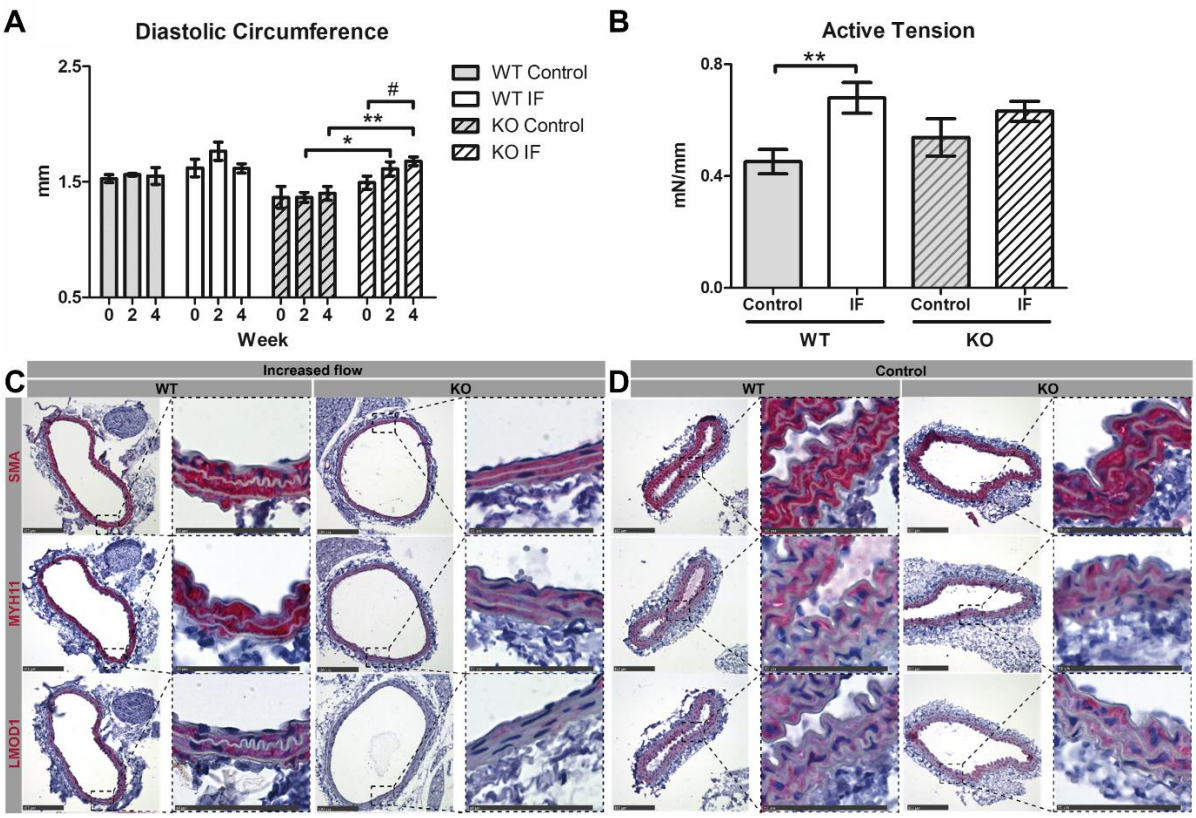

Figure 14. The influence of PCSK6 deficiency on the flow-mediated outward remodeling in the right common carotid artery. Measurements of A) diastolic circumference in ultrasound biomicroscopy and B) active tension at optimal stretch in wire myography Representative images of immunohistochemical staining for Smooth Muscle alpha-actin (SMA), Myosin Heavy Chain 11 (MYH11) and Leiomodin-1 (LMOD1) from WT and KO mice under C) increased flow and D) control conditions. 2-way ANOVA with Bonferroni Multiple Comparison test and Kruskal-Wallis with Dunn's multiple comparison test was used for comparing differences between strain specific ligated and controls at the same time point, *=$p<0.05$ and **=$p<0.01$. Wilcoxon signed rank test was used for comparing data within the same group, #= $p<0.05$. IF= Increased flow, KO= PCSK6$^{-/-}$ mice. WT= C57Bl/6J mice.

Transmission electron microscopy was performed on pressure-fixated arteries to further investigate the structural differences in flow-mediated remodeling. Our analysis revealed an increased elastic lamina content per media area in the WT mice exposed to increased flow, which could not be detected in the PCSK6$^{-/-}$ mice. However, the adaptive remodeling process is known to induce arterial stiffening by increasing the vessel wall collagen content.[130] Hence, the increase in elastic lamina area could be related to a reduced dilation of the remodeled arteries upon pressure-fixation. Furthermore, histochemical analysis revealed a remodeling associated increase in cells per media area in the PCSK6$^{-/-}$ mice. This finding instigated us to

further explore the influence of PCSK6 deficiency on phenotypic modulation of SMCs in the remodeling process. Our results revealed a remodeling associated reduction in expression of contractile SMC makers (SMA, LMOD1, MYH11) in the PCSK6$^{-/-}$ mice (Figure 14C and 14D). These results suggest that the absence of adaptive contractile response in the PCSK6$^{-/-}$ mice could be related to a decreased fraction of contractile SMCs.

PCSK6 has previously been shown to influence the activity of growth factors related to the arterial remodeling process such as PDGF-B and TGF-B_1.[243,244] Interestingly, TGF-B_1 has been shown to be a mediator of arterial stiffening and also related to SMC hypertrophy.[245,246] Therefore, it is possible that PCSK6 deficiency results in reduced TGF-B_1 signaling leading to a hampered adaptive remodeling response. Also, PDGF-B is a potent mitogen but has also been associated with hypertrophy of SMCs *in vitro*.[247] However, the influence of PCSK6 deficiency on TGF-B_1 and PDGF-B activation and its relation to SMCs in vascular remodeling remains to be investigated.

5 CONCLUSIONS

Complications related to an excessive healing process following vascular interventions are a major clinical problem. Current treatment options rely on decreasing disease progression, reducing the risk of thrombosis and inhibition of the intimal hyperplastic response with non-selective anti-proliferative drugs. Hence, there is a need for selective treatments to optimize the healing processes. The purpose of this thesis was to investigate different components of the arterial healing process: re-endothelialization, intimal hyperplasia and vascular remodeling.

5.1 GENE EXPRESSION AND ARTERIAL WALL HEALING

Investigation of the transcriptomic landscape of the arterial wall healing process over time revealed presence of three distinct phases and its associated pathways (**Study III**). We could identify dynamic dysregulation of specific genes, which were in line with previous studies, and confirm our methodology. This descriptive study was conducted in order to create an encyclopedia of the rat carotid balloon injury model to be used for validation and further exploration of possible treatment targets. Tissue samples, organs and plasma from different time-points after injury were collected in order to create a biobank, to be used for hypothesis generation and testing prior to performing an actual experiment, which will improve the accuracy of future experiments and reduce the number of animals needed. Importantly, our analysis also indicated presence of novel pathways, such as clonal expansion, immune-priming and osteo-chondrogenic differentiation of SMCs, which are currently being further investigated.

5.2 RE-ENDOTHELIALIZATION

Regrowth of the endothelial lining following arterial injury is crucial for restoration of the vessel wall homeostasis in order to reduce the risk of restenosis and thrombosis. Further studies are needed to investigate targets for selective improvement of the re-endothelialization process to reduce the risk of complications following invasive vascular interventions. In **Study I**, we revealed that UBM can be used to estimate the re-endothelialization process using intimal hyperplasia formation as a surrogate marker in the rat carotid balloon injury model. This model may serve as a highly relevant tool for non-invasive *in vivo* assessment of the re-endothelialization and IH formation in future pharmacological studies.

Treatment with linagliptin, in normal or diabetic conditions, did not influence the re-endothelialization process (**Study II**). Similar results was observed for the GLP-1 receptor

agonist Exendin-4 in normal conditions.[43] These results indicate that modulation of the incretin system does not influence the re-endothelialization process in rat carotid balloon injury model.

5.3 INTIMAL HYPERPLASIA FORMATION

Despite the effect on SMC proliferation *in vitro*, we did not detect any effect of linagliptin on intimal hyperplastic response in either normal or diabetic conditions (**Study II**). This study was conducted at a time when the influence of DPP-4 inhibitors on vessel wall healing and cardiovascular mortality was largely unknown. Therefore, we had a responsibility towards the research community to report and share our experiences, despite having negative results.

5.4 VASCULAR REMODELING

Remodeling of the vessel wall is a fundamental biological process, which can be emphasized in the arterial healing response and atherosclerosis. In **Study IV**, we reveal that PCSK6 deletion induces outward remodeling, influences the contractility and capacity of SMC to differentiate. The findings from an ongoing study reveal that deletion of PCSK6 reduces IH formation. Combined, these results indicate that inhibition of PCSK6 could be a potential target for reducing the risk of restenosis by promoting outward remodeling and reducing IH formation.

6 IF I WERE TO DO IT AGAIN?

While writing up the thesis, one cannot help to reflect and wonder what could have been done in a better or different way in order to come closer to the truth and improve the contribution of each study to the research community.

Due to the retrospective approach used in **Study I**, we could not use a standardized image acquirement protocol, which probably would have improved our results. We did not perform any direct comparisons of the re-endothelialization border detected in ultrasound with the Evans-blue staining. This could have been performed upon euthanization using placement of a suture at the UBM defined border prior to performing *en face* staining. Advancements of the UBM technology and the image analysis software have made speckle-tracking and 4D ultrasound available, which would have improved the possibility to detect alterations in the movement of and within the arterial wall. These techniques could have added further understanding of the influence of the endothelium on arterial wall healing and possibly revealed differences in the physiological characteristics of the areas with and without intact endothelium.

Study II could have been improved by adjustments in methods and study design. A pilot study with subsequent power calculation would have been of importance for the study design. Pretreatment of the animals with confirmation of DPP-4 activity and glucose tolerance test prior to injury would have confirmed proper uptake of the drug. Also, the concentration of linagliptin should have been measured both prior to injury and upon euthanization. Use of different concentrations of linagliptin, low, medium and high dose, would further have improved this study by determining whether the potential beneficial effects would be concentration dependent. In addition, ELISA should have been performed on serum to elucidate the effect of linagliptin on the systemic inflammatory response.

In **Study III**, we had a potential to include other techniques to further understand the different aspects of the arterial wall healing process. UBM is a fast and non-invasive imaging method, which would have been useful to evaluate the IH formation over time but also for validation of the injury response between the groups. Analysis of the gene expression separately in the different vessel wall layers would have further improved the potential and novelty of this study. Since the flow-conditions vary between the proximal and distal third of the artery, it would have been of interest to analyze these parts of the artery separately. Also, comparison of the

gene expression in the middle un-endothelialized part of the artery to the endothelialized would have further improved our results.

Study IV would have been improved by addition of physiological parameters and evaluation of cardiac function using ultrasound. Measurement of blood pressure would have increased the possibility to characterize the physiology of the arterial wall. Assessment of cardiac physiology with ultrasound would have identified possible cardiac anomalies and differences in myocardial function in response to carotid ligation between WT and knock-out mice. Also, it would have been of interest to evaluate the endothelial function of the remodeled arteries in wire myography. This was not performed since we could detect a negative influence of the length-force experiment on the endothelium-dependent relaxation in our pilot experiment. It would also have been interesting to evaluate the vascular remodeling process in the distal vasculature using magnetic resonance imaging or micro-Computer Tomography.

7 CLINICAL PERSPECTIVE AND FUTURE DIRECTIONS

In this thesis, different aspects of arterial wall healing have been investigated in order to further increase the understanding of the basic mechanisms related to this injury response. However, there is obviously more research needed in order to translate this mechanistic knowledge into better outcomes for patients.

The influence of T2DM on arterial wall healing should be further explored to optimize the surgical management and improve the outcome for these patients. Also, the effect of combination therapies on the healing response should be investigated in experimental models. Analysis of UBM parameters in **Study II** revealed presence of a constrictive remodeling process in the diabetic GK rats treated with placebo following carotid balloon injury (Figure 15). Hence, further investigation of the influence of T2DM on arterial wall healing should be performed by utilizing the similar set up as in **Study III** with GK and Wistar rats. In addition to large-scale transcriptomic analysis and tissue samples, such a study should include a systematic evaluation of temporal changes in arterial wall thickness, lumen geometry and physiology with UBM. The transcriptome of vessel wall samples from patients with T2DM is being investigated in an ongoing study. Therefore, this would allow for comparison of differences and similarities in the gene expression between an experimental injury model and patients with T2DM. Using this approach would bring further insights to the translational value of the diabetic animal model and potentially reveal overlapping molecular pathways to be further investigated.

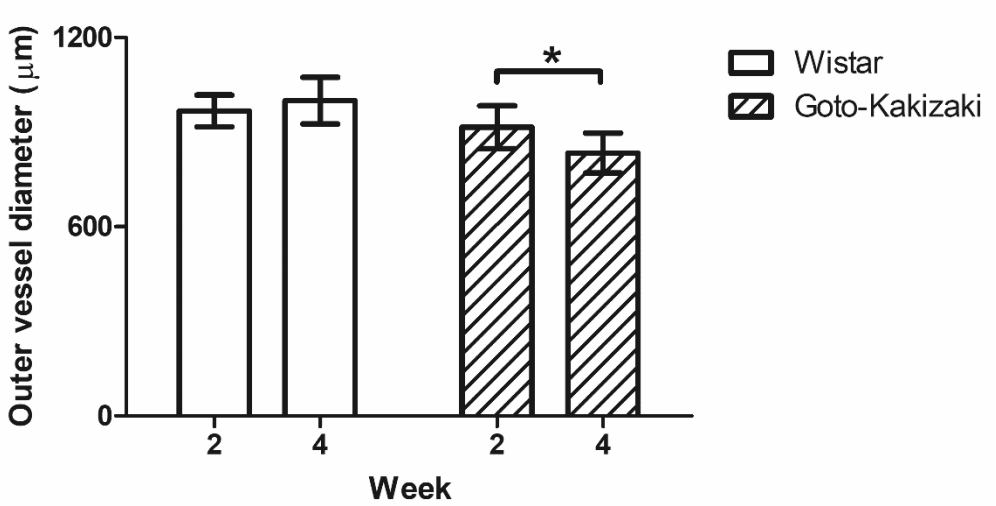

Figure 15. Negative remodeling in diabetic rats following arterial injury. Measurements of outer vessel diameter from animals treated with placebo in ultrasound biomicroscopy following carotid artery balloon injury. *=$p<0.05$.

The role of PCSK6 in arterial wall healing should be further investigated. Evaluation of the IH formation on the ligated side in **Study IV** revealed morphological differences in the intimal hyperplastic response (Figure 16). Interestingly, the SMCs increase in number but seem to be unable to penetrate the IEL and transmigrate to the intima. These results indicate that modulation of PCSK6 activity could be an attractive approach for reducing IH formation by selective inhibition of transmigration of SMCs. The molecular mechanisms related to this finding is being investigated in an ongoing study.

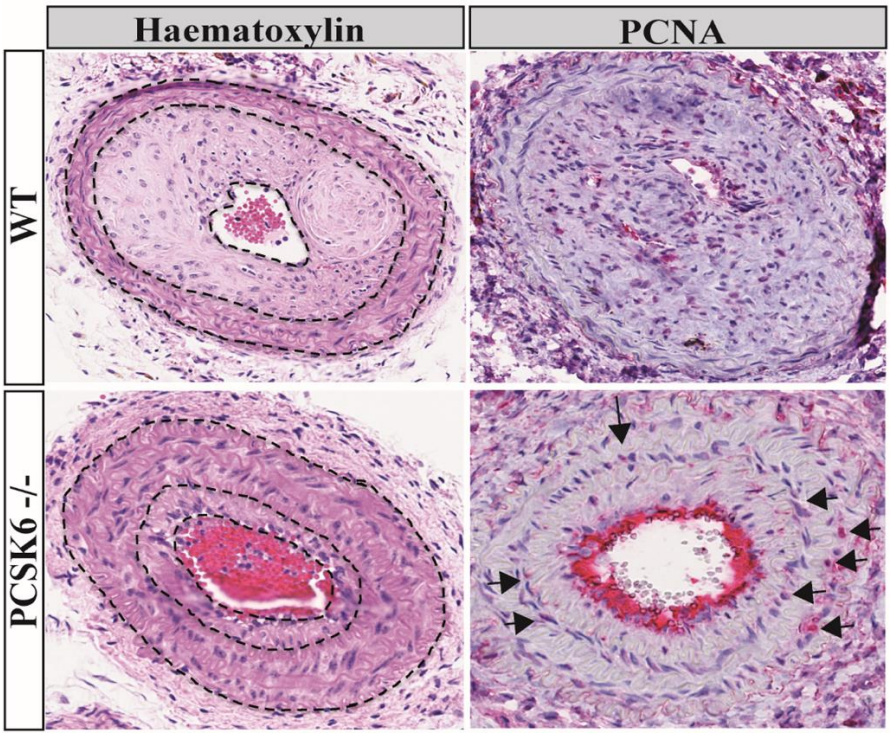

Figure 16. Reduced intimal hyperplasia thickness in response to carotid ligation in PCSK6$^{-/-}$ mice. Histo- and immunohistochemical staining from serial sectioning of intimal hyperplasia formation following ligation of the common carotid artery. PCSK6$^{-/-}$ mice displayed a significant reduction in intimal hyperplasia formation with concomitant increase in PCNA+ cells (red staining in nuclei). Arrows indicate proliferative cells trapped in the *tunica media*. PCNA= Proliferating cell nuclear antigen, WT= Wild-type.

A clinical example of the full impact of vessel remodeling is in arteriovenous dialysis fistulas where the functionality of the fistula is crucial for hemodialysis and patients' life. Complications related to maturation failure are common.[248,249] Hence, more studies are needed in order to further understand the molecular mechanisms involved in maturation failure. The anatomic location of the fistulas allow for the use of ultrasound biomicroscopy and subsequent advanced software analysis, which should be further explored. Since creation of dialysis fistulas is performed with open surgical technique, this would allow for tissue harvest.

Combining large-scale transcriptomic analysis, myography and histological evaluation would be an attractive approach to further understand the influence of biomechanical forces on vascular biology, identify novel molecular mechanisms and possible treatment targets.

There is today a knowledge gap in regards to the differences in arterial wall healing response in different parts of the arterial tree. It is known that the large-arteries have differences in embryological origin and that the flow conditions vary depending on the distance from the heart, which is reflected in the arterial wall structure and ECM content.[8,250] Interestingly, the timeline and influence of patient characteristics on the arterial healing response at different locations of the arterial tree remains largely unknown. Utilization of intravascular imaging techniques, such as optical coherence tomography or intravascular ultrasound, could be a way to further explore these differences.

The field of endovascular treatment is rapidly evolving and number of devices is increasing. Use of these devices, such as DES, have been extensively investigated in regards to coronary artery disease, however, the benefit of using these for peripheral artery disease remains to be fully elucidated. In comparison to coronary interventions, length of the injury inflicted to the vessel wall is increased. Interestingly, the morphology and biology of the atherosclerotic plaques in peripheral artery disease have been shown to differ from coronary artery disease.[251,252] It would therefore be of great interest to investigate how these differences influence the vessel wall healing process.

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