The role of smooth muscle cells in calcification of atherosclerotic plaques

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THE ROLE OF SMOOTH MUSCLE CELLS IN CALCIFICATION OF ATHEROSCLEROTIC PLAQUES

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Cover illustration: Immunofluorescent analysis of an advanced human carotid lesion revealed a sleepy baby dragon.

THE ROLE OF SMOOTH MUSCLE CELLS IN CALCIFICATION OF ATHEROSCLEROTIC PLAQUES THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Jede wirkliche Geschichte ist eine unendliche Geschichte.”

“Every real story is a never ending story.”

— Michael Ende, Die unendliche Geschichte

“C’est le temps que tu as perdu pour ta rose qui fait ta rose si importante.”

“It is the time you have wasted for your rose that makes your rose so important.”

— Antoine de Saint-Exupéry, *Le Petit Prince*

ABSTRACT

Calcification (CALC) is a predominant feature of late-stage cardiovascular disease (CVD) but responsible mechanisms and its contribution to the risk of clinical events remain unclear. Formation of highly mineralized extracellular matrix (ECM) leads to progressive aortic valve stenosis (AVS) and has been identified as a surrogate marker for atherosclerotic disease burden. However, we previously found enrichment of genes associated with CALC in atherosclerotic carotid lesions from asymptomatic patients and in patients on statin therapy. This thesis aimed to investigate how CALC correlates with gene expression profiles in human specimens of carotid plaques as well as AVS and functionally characterize the underlying mechanisms associated to osteogenic phenotypic transformation of structural cells.

Study I explored gene expression profiles and biological pathways related to macro-CALC in human carotid lesions, estimated by computed tomography (CT). Microarray profiling, bioinformatic analysis and histological validation based on high- vs low-CALC plaques revealed upregulation of smooth muscle cell (SMC) markers in high-CALC plaques, whereas macrophage markers were downregulated. The most enriched processes in high-CALC plaques were related to SMC differentiation and ECM organization, while inflammation, lipid transport and chemokine signaling were repressed. Proteoglycan 4 (PRG4) was identified as the most upregulated gene in association with plaque CALC and found in the ECM overlapping with SMA, CD68 and tartrate-resistant acid phosphatase (TRAP) positive cells.

Study II characterized PRG4 in the context of AVS and aortic valve CALC. Transcriptomic, histological and immunohistochemical (IHC) analysis of human aortic valves from patients undergoing aortic valve replacement showed significant upregulation of PRG4 in thickened and CALC regions of aortic valves compared with healthy regions. In addition, *PRG4* positively associated with mRNA expression of proteins involved in cardiovascular CALC. Treatment of human valve interstitial cells (VICs) with recombinant human PRG4 (rhPRG4) enhanced phosphate (Pi) induced CALC and increased bone morphogenetic protein 2 (*BMP2*) expression.

Study III analyzed the role of PRG4 in vascular remodeling and intimal CALC. PRG4, detected by IHC, localized to SMCs in early human intimal thickening, while in advanced lesions it was found in the ECM, surrounding macro-CALC. *In vivo* mouse and rat models showed increased *Prg4* expression in SMCs during intimal hyperplasia, correlating with osteogenic markers and transforming growth factor β (*Tgf β*). Moreover, PRG4 was enriched around intimal plaque CALC in apolipoprotein E deficient mice (ApoE^{-/-}) on warfarin. *In vitro*, *PRG4* was induced in primary human vascular SMCs by TGF β and calcifying conditions, while SMC markers were repressed. Silencing experiments showed that *PRG4* expression was driven by transcription factors mothers against decapentaplegic homolog 3 (SMAD3) and SRY-box transcription factor 9 (SOX9). The addition of rhPRG4 increased ectopic SMC calcification, while arresting cell migration, proliferation and osteogenic transformation.

Study IV assessed the influence of biomechanical forces, related to atherosclerotic carotid macro-CALC, on SMC phenotype and plaque stability. Finite element modeling (FEM), based on preoperative CT images, identified that biomechanical stretch was significantly reduced in close proximity to macro-CALC, while pathologically increased levels were observed within distant soft tissues. *In vitro* modeling of these conditions revealed upregulation of markers for SMC differentiation and contractility under low stretch but also impeded SMC alignment and increased osteogenic transdifferentiation. In contrast, high-stretch in combination with calcifying conditions induced rapid SMC apoptosis, suggesting a contribution to atherosclerotic plaque stabilization by the load-bearing capacities of macro-CALC.

Overall, this thesis demonstrates that vascular disease pathology related to CALC can be comprehensively described by linking clinical diagnostics and underlying biological processes *via in silico* analysis, thereby contributing to the basic understanding of disease progression and patient specific phenotypic variability.

LIST OF SCIENTIFIC PAPERS

- I. Karlöf E, **Seime T**, Dias N, Lengquist M, Witasp A, Almqvist H, Kronqvist M, Gådin JR, Odeberg J, Maegdefessel L, Stenvinkel P, Matic LP, Hedin U. Correlation of computed tomography with carotid plaque transcriptomes associates calcification with lesion-stabilization. *Atherosclerosis*. 2019 Sep;288:175-185.
- II. Artiach G*, Carracedo M*, **Seime T**, Plunde O, Laguna-Fernandez A, Matic L, Franco-Cereceda A, Bäck M. Proteoglycan 4 is Increased in Human Calcified Aortic Valves and Enhances Valvular Interstitial Cell Calcification. *Cells*. 2020 Mar 11;9(3):684.
- III. **Seime T**, Akbulut AC, Liljeqvist ML, Siika A, Jin H, Winski G, van Gorp RH, Karlöf E, Lengquist M, Buckler AJ, Kronqvist M, Waring OJ, Lindeman JHN, Biessen EAL, Maegdefessel L, Razuvaev A, Schurgers LJ, Hedin U, Matic L. Proteoglycan 4 Modulates Osteogenic Smooth Muscle Cell Differentiation during Vascular Remodeling and Intimal Calcification. *Cells*. 2021 May 21;10(6):1276.
- IV. **Seime T**, van Wanrooji M, Karlöf E, Johansson S, Matic L, Gasser T.C, Hedin U. Biomechanical assessment of macro-calcification in human carotid atherosclerosis and its impact on smooth muscle cell phenotype. Manuscript.

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OTHER RELATED PUBLICATIONS

- I. Röhl S, Suur BE, Lengquist M, **Seime T**, Caidahl K, Hedin U, Arner A, Matic L, Razuvaev A. Lack of PCSK6 Increases Flow-Mediated Outward Arterial Remodeling in Mice. *Cells*. 2020 Apr 18;9(4):1009.
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LIST OF ABBREVIATIONS

ACAN	Aggrecan
ACTA2	Actin
ApoE	Apolipoprotein E
ApoE ^{-/-}	Apolipoprotein E deficient mouse
AVA	Aortic valve area
AVS	Aortic valve stenosis
BAV	Bicuspid aortic valve
BiKE	Biobank of Karolinska endarterectomies
BMP	Bone morphogenic protein
BMPR2	Bone morphogenic protein receptor type 2
Ca	Calcium
CaPi	Calcium-phosphate
CAC	Coronary artery calcification
CALC	Calcification
CASP3	Caspase 3
CEA	Carotid endarterectomy
CKD	Chronic kidney disease
CNN	Calponin
CT	Computed tomography
Ct	Cycle threshold
CTA	Computed tomography angiography
CVD	Cardiovascular disease
DMP1	Dentin matrix acidic phosphoprotein 1
EC	Endothelial cell
ECM	Extracellular matrix
ECST	European carotid surgery trial
EV	Extracellular matrix vesicle
FEM	Finite element modeling
FGF	Fibroblast growth factor
FH	Familial hypercholesterolemia
GAG	Glycosaminoglycan
GH	Growth hormone
GWAS	Genome wide association studies
HDL	High-density lipoprotein
HPRT	Hypoxanthine phosphoribosyltransferase 1

HU	Hounsfield unit
IBSP	Integrin binding sialoprotein
ICAM-1	Intracellular cell adhesion molecule 1
IF	Immunofluorescence
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IHH	Indian hedgehog
HSPG2	Perlecan
IL	Interleukin
IPH	Intraplaque hemorrhage
ITGAM	Integrin subunit alpha M
LDL	Low-density lipoprotein
Ldlr ^{-/-}	Low-density lipoprotein receptor deficient mouse
LMOD1	Leiomodin 1
LRNC	Lipid rich necrotic core
MATX	Matrix component
MGP	Matrix gla protein
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MYH11	Myosin heavy chain 11
MYOCD	Myocardin
NASCET	North American symptomatic carotid endarterectomy trial
OPG	Osteoprotegrin
OPN	Osteopontin
OSX	Osterix
oxLDL	Oxidized low-density lipoprotein
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDLIM7	PDZ and LIM domain protein 7
PG	Proteoglycan
Pi	Phosphate
PTH	Parathyroid hormone
qPCR	Quantitative real-time polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa B ligand
rhPRG4	Full length recombinant human PRG4

ROS	Reactive oxygen species
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
RUNX2	Runt-related transcription factor 2
SM22 α	Smooth muscle cell protein 22 α
SMA	Smooth muscle alpha actin
SMAD3	Mothers against decapentaplegic homolog 3
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SMTN	Smoothelin
SOX9	SRY-box transcription factor 9
T3	Tri-iodothyronine
TGF β	Transforming growth factor β
TIA	Transitory ischemic attacks
TLR	Toll-like receptor
TNF α	Tumor necrosis factor α
TRAP / ACP5	Tartrate-resistant acid phosphatase
US	Ultrasound
VC	Vascular calcification
VCAM-1	Vascular cell adhesion molecule 1
VEC	Valvular endothelial cell
VIC	Valvular interstitial cell
WT	Wild-type

LIST OF ILLUSTRATIONS

Contractile SMC		Cytokines
Activated SMC		Calcium / Phosphate
Quiescent VIC		Cholesterol Crystal
Activated VIC		Micro-Calcification
Quiescent VEC		Macro-Calcification
Activated VEC		LDL Particle
Activated Macrophage		Oxidized LDL Particle
Foamcell		Turbulent Flow / Hemodynamic Stress
Apoptotic Cell		Biomechanical Stress
Myofibroblast-like Phenotype		Neo-vascularization
Osteogenic Phenotype		Collagen
Osteoclast-like Phenotype		Glycosaminoglycan
		Elastin
		Fibrous ECM

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE – BIGGEST KILLER WORLDWIDE

The term CVD describes a variety of conditions affecting the heart and blood vessels, such as rheumatic and congenital heart disease, coronary and peripheral artery disease, cerebrovascular disease (e.g. carotid artery stenosis), AVS and aortic aneurysm (Figure 1A). However, population-based studies could attribute many of these medical conditions to one major underlying pathology, atherosclerosis².

Atherosclerosis is a systemic disease of large and medium sized arteries that manifests itself in the form of vessel wall thickening (stenosis) with increasing accumulation of lipids and fibrous tissue³ (Figure 1B). Maladaptation of the immune system response, normally required to reestablish vascular homeostasis, generates chronic cardiovascular inflammation further contributing to disease progression⁴. Early atherosclerotic lesions have been discovered to form already during teenage years⁵ but often remain asymptomatic for a long time making complications caused by occlusion, plaque rupture and consequential thrombus formation increasingly prevalent among the elderly⁶⁻⁸.

Figure 1: Systemic impact of CVD. (A) CVD pathologies affect the whole human body and are a major cause of multiple fatal complications, such as stroke, heart failure and aneurysm rupture. (B) Two common manifestations are atherosclerotic carotid artery stenosis and aortic valve stenosis.

1.1.1 Epidemiology and risk factors

To date, CVD poses the foremost cause of global mortality accounting for more than 30% of all deaths worldwide, 4 million in Europe alone every year ⁹⁻¹². Symptomatic carotid artery stenosis causes an estimated 8-20% of all strokes ¹⁰, affecting approximately 75% men and 62% women at 65 years and older ⁶. Up to 7% of the same age group suffer from AVS, reaching a one-year mortality rate above 50% due to cardiac outflow obstruction in severe cases ^{7,13}.

Although knowledge of major risk factors like high blood pressure, cholesterol levels, smoking, physical inactivity, overweight, genetic predisposition and congenital disorders is increasing ^{9,14-16}, lethal complications such as heart failure, stroke and aortic aneurysm rupture remain high. Moreover, prevalence of CVD is rising in developing countries due to population growth and increasing life span ¹⁷.

An important factor inducing vascular remodeling and increasing risk of disease initiation are detrimental fluid mechanics in certain areas of the cardiovascular system. Narrow curvatures or branch points, e.g. at the coronary arteries, carotid bifurcations or aortic valve leaflets, create turbulent flow conditions causing constant biomechanical stress on the vessel wall over time ^{18,19}. While increased biomechanical stress is compensated by vascular remodeling in young healthy individuals, it can lead to pathological hyperplasia when amplified by high blood pressure, physical inactivity and other risk factors.

In addition, a fatty “western-type” diet is considered to be one of the most common risk factors for CVD. Apolipoproteins (ApoA, ApoB, ApoC, ApoE) facilitate the formation of plasma lipoprotein particles within the bloodstream to transport lipids ²⁰. Many lipoprotein particles rich in ApoB (VLDL, IDL, LDL) are main drivers of CVD progression. Especially cholesterol-rich low-density lipoprotein particles (LDL) are associated with decreased nitric-oxide-synthase activity and consecutive endothelial dysfunction, preceding their entry and accumulation within the vessel wall ²¹. However, lipids do not always exhibit the same atherogenic effects. High-density lipoproteins (HDL) mainly containing ApoA, have previously been attributed anti-oxidative and cholesterol-clearing properties, which lead to overall athero-protective effects ^{22,23}.

Hereditary genetic disorders can cause major predisposition to develop CVD at young age. Extensive studies on patients with familial hypercholesterolemia (FH) constitute the basis for our present understanding of the cholesterol metabolism as well as congenital malfunctions in LDL handling. Michael S. Brown and Joseph L. Goldstein received the Nobel prize in 1985 for their analysis of FH as a monogenic dominant trait. They found individuals who carried a homozygote mutation to have severely increased cholesterol levels (up to five times), while patients with a heterozygote genetic defect still showed elevated cholesterol levels (two to three times higher) compared to the normal population ²⁴. As a consequence, FH can cause severe atherosclerosis and coronary infarction already in adolescence. They further described that these clinical impacts were induced on a molecular level by a lack of functional LDL-receptors, severely impairing metabolism of circulating lipids ²⁵⁻²⁸. The recent emergence of genome-wide association studies (GWAS) allowed for a more detailed comparison of genetic variants and revealed that single nucleotide polymorphisms (SNPs) and other mutations account for 30 to 60% of CVD susceptibility variation ^{29,30}. To date the majority of variants are implicated in lipid metabolism and inflammation, but several have been identified to also affect phenotypic changes of structural cells, like SMCs, regarding their contractility, migration and proliferation ³¹⁻³³. These findings raised the hypothesis that SMCs together with disrupted lipid metabolism and chronic inflammation are causal drivers of CVD initiation.

1.1.2 Carotid artery stenosis

1.1.2.1 The arterial wall under physiological conditions

Healthy arteries are composed of three main layers: i) *tunica intima* directly exposed to the blood flow, ii) *tunica media* and iii) *tunica adventitia* ³⁴ (Figure 2). The luminal lining of the vessel wall (intima) is formed by a mono-layer of endothelial cells (ECs) adhering to a

basement membrane mainly composed of collagen type IV, laminin and perlecan (HSPG2)³⁵. ECs together with the basement membrane composition have been shown to support homeostasis by secreting factors inhibiting excessive SMC proliferation in the *media*, inflammatory infiltration and coagulation^{36,37}. Mechanosensing ion channels, integrins and extensive glycocalyx make ECs highly flow responsive. Under laminar conditions they adapt an organized elongated pattern in flow direction, forming a tight non-thrombogenic surface to the blood circulation, only selectively permeable to cells and macro-molecules³⁸. In contrast, turbulent flow, oscillatory shear stress and local hypoxia lead to poor “cobble-stone”-like alignment and secretion of pro-inflammatory and pro-migratory factors like vascular cell adhesion molecule 1 (VCAM-1), interleukin (IL) 8, platelet derived growth factor B (PDGF), TGF β and other cytokines inducing vascular remodeling^{39,40}. This activation of endothelial cells and further signaling, driving the vascular adaptation to changed shear conditions, is fundamentally controlled by nitric oxide (NO) generation^{41,42}, previously known as endothelium-derived relaxing factor (EDRF). Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were jointly awarded the Nobel Prize in 1998 for the description of this pathway in vasodilation. In addition to their role in regulating vascular tone, ECs have been suggested to contribute to *inner elastic lamina* formation by elastin secretion⁴³. The *inner elastic lamina* separates *intima* from *media*, the dominating layer of muscular arteries like the *common carotid*. This layer is mainly formed by a circumferentially orientated, tightly linked network of contractile SMCs. Contractile SMCs display a differentiated state, expressing typical cytoskeletal markers such as smooth muscle alpha actin (SMA), myosin heavy chain 11 (MYH11) and smoothelin (SMTN) to actively distribute strain within the arterial wall and maintain the contractile tone. They establish a basement membrane network of similar composition and function as the intimal counterpart³⁵. Nevertheless, SMCs retain the capacity of de-differentiating to a “synthetic” phenotype, characterized by proliferation, migration and expression of structural proteins and matrix degrading enzymes, relevant for ECM remodeling⁴⁴. This feature is crucial to adapt vessel compliance and regain homeostasis after vascular injury. Equally important for maintaining elasticity and providing general tissue strength is the medial ECM. However, while certain key components like collagens and proteoglycans show a higher turnover rate, being remodeled by SMCs and inflammatory cells, organized elastin fibers are generally considered to only be formed during development⁴⁵. The *outer elastic lamina* marks the border between *tunica media* and *adventitia*. This collagen-rich surrounding layer mainly provides tensile strength to prevent overstretching of the vessel wall. However, large numbers of residing progenitor cells^{46,47}, fibroblasts and immune cells as well as connection to the lymphatic and nervous system allow response to insults by fibrosis and inflammation⁴⁸. In large vessels or cases of severe or chronic remodeling the *adventitia* can develop a substantial *vasa vasorum*⁴⁹.

1.1.2.2 Initiation of carotid artery stenosis

Despite rigorous scientific efforts to define key events causing pathological remodeling of the carotid artery, the sequence of disease initiation remains heavily debated. Nevertheless, three main factors were identified to interact, causing vessel wall destabilization and activation of other players involved during disease progression: hemodynamic shear stress, lipids and genetic predisposition. An interplay of EC activation by turbulent flow conditions, subsequent permeability for lipids and minerals as well as resulting proliferation and migration of SMCs causes adaptive intimal thickening⁵⁰, one of the earliest indicators of atherosclerotic lesion formation^{41,51}. Clinically, this vascular adaptation is measured by B mode ultrasound and widely adopted as a surrogate for the rate of CVD progression, called carotid intimal/medial thickness. Perhaps the most important limitation of this assessment arises from the general measurement acquisition in the *common carotid* artery, whereas advanced atherosclerotic disease occurs predominantly within the *internal carotid*. Moreover, the primary drivers of intimal/medial thickening appear to be of biomechanical nature, defined by age and hypertension, which do not necessarily reflect the full atherosclerotic process⁵². In atherosclerosis, prevailing disruption of the endothelial homeostasis causes permeability to LDL particles, that aggregate to intimal proteoglycans⁵³⁻⁵⁵. LDL particles trapped within the

subintimal ECM have been shown to be oxidized by resident vascular cells⁵⁶ and induce leukocyte adhesion on the endothelium as well as macrophage activation within the sub-intima⁵⁷. Oxidized LDL (oxLDL) particle overload leads to an accumulation of lipid-filled macrophages or so-called foam cells forming early manifestations of a lipid-rich core. These lesions are commonly referred to as *xanthomas*^{3,58,59} (Figure 2). Pro-inflammatory cytokine secretion and release of reactive oxygen species (ROS) by activated macrophages promote further LDL retention and activation of matrix metalloproteinases (MMPs), weakening the vessel wall matrix^{60,61}. Studies on familial hypercholesterolemia confirmed the adverse effects of sustained high LDL plasma levels, provoking atherosclerotic lesion formation in form of lipid depositions within the subendothelial layers already at a young age⁶².

Figure 2: Schematic illustration of the healthy artery wall and atherosclerotic lesion progression. Early intimal thickening is mainly characterized by infiltration of the subendothelial layer by lipid particles and inflammatory cells, leading to SMC proliferation and migration. During later stages of plaque development, pro-fibrotic and osteogenic mechanisms lead to ECM remodeling and formation of calcification.

1.1.2.3 Atherosclerotic carotid lesion progression

While processes leading up to disease initiation remain contested, the overall biological pathways driving lesion progression seem to be more clear. Although early *xanthomas* formed via LDL infiltration and aggregation as well as immune activation might still be completely reversible⁵, chronic influx of lipids and inflammatory cells will lead to excessive SMC activation and loss of their contractile features⁶³. This dedifferentiation is orchestrated by a rapidly changing extracellular milieu including various atherogenic molecules like pro-inflammatory cytokines (e.g. IL 1 β and 6, tumor necrosis factor α (TNF α) and interferon γ (IFN γ)), growth factors (e.g. PDGFB and TGF β) as well as inorganic calcium (Ca) and Pi⁶⁴⁻⁶⁸.

Synthetic SMCs proceed to migrate towards luminal parts of the lesion, contributing to intimal hyperplasia by extensive proliferation and ECM secretion³⁷. This stage of lesion development is called pathological intimal thickening. Interestingly, SMCs have also been reported to show lipid clearing capacities, forming CD68 positive foam-like cells in the process⁶⁹. Together with macrophage activation this may aid initial lipid clearance from the vessel wall but in turn results in enhanced foam-cell accumulation. The strongly pro-inflammatory and pro-apoptotic plaque environment finally leads to expansive caspase 3 (CASP3) -dependent apoptosis and necrosis of macrophages and SMCs⁷⁰⁻⁷². Furthermore, impaired phagocytic clearance results in deposition of apoptotic debris and inorganic cholesterol crystals underneath a layer of foam-cells, forming a continuous lipid-rich necrotic core^{73,74}. This irreversible disruption of the intima is referred to as *fibroatheroma* (Figure 2).

Pathological examination of human atherosclerotic plaques often reveals very advanced lesions that remain asymptomatic for decades due to a protective fibrous cap established by migrating SMCs and subsequent fibrous ECM formation, separating the lesion from the lumen^{75,76}. Although clinical research is starting to recognize the importance of patient specific plaque heterogeneity, the majority of advanced lesions are considered to be characterized by: i) extensive intimal thickening, ii) structural disorganization of the vessel wall and iii) significant aggregation of lipids and fibrotic ECM⁷⁷. Perpetual apoptosis and failing response mechanisms to clear the accumulating debris lead to secondary necrosis and necrotic core stiffening⁷². This biomechanical wall stiffening as well as the narrowing lumen are currently considered to be the major risk factors for a potential plaque rupture. While SMCs are considered to strengthen the fibrous cap integrity by secreting ECM components such as collagen and elastin⁷⁸, inflammatory cells (macrophages and T-lymphocytes) within the same area release MMPs and cytokines that induce ECM degradation⁷⁹. Therefore late stage plaque stability is partially dependent on a fragile balance between SMCs and active inflammation^{4,80}. Insufficient tissue oxygenation within the necrotic core and hypoxic macrophages trigger neo-angiogenesis originating from the *vasa vasorum*⁸¹⁻⁸³. While actively enhancing inflammatory cell recruitment and affecting ECM remodeling via oxygenation⁸⁴, these immature capillaries lack supporting pericytes, which causes their leakiness and fragility⁸⁵. Hence, intraplaque hemorrhages (IPH) are common sources of increased inflammation and destabilization in advanced atherosclerotic plaques⁸⁶⁻⁸⁸. Intimal macro-CALC (> 2 mm) is mostly a feature of late-stage atherosclerotic lesions³. However, micro-CALC (< 15 µm) can already be present in early intimal thickening and the necrotic core⁸⁹. Detailed mechanisms of CALC formation and its impact on plaque stability are subject of section 1.3.

1.1.3 Aortic valve stenosis

1.1.3.1 The aortic valve under physiological conditions

The aortic valve is an avascular tissue surrounding the orifice of the *aorta* with the crucial function of preventing blood-backflow into the left ventricle under diastole. It is formed from three leaflets or cusps which attach to the aortic root forming the *sinus of Valsalva*, origin of the *coronary arteries*³⁴. The valve closes under diastole due to the aortic backpressure on the cusps and opens under systole when this pressure is equalized by the blood flow from the heart. This constant movement is maintained by a refined composition of valvular endothelial cells (VECs), valvular interstitial cells (VICs) and ECM components⁹⁰. Similar to ECs forming the luminal layer of arteries, VECs line the outer leaflet-layer in direct contact to the blood stream, capable of sensing and adapting to hemodynamic changes. In contrast to ECs, VECs align perpendicular to shear inflicted by blood flow⁹¹. Below the VEC monolayer, three distinct layers of VICs and different matrix components form the structural part of each leaflet. On the aortic side, tightly connected VICs and collagen fibers form the *fibrosa*, providing necessary load bearing capacities⁹². The middle layer, called *spongiosa*, is composed of VICs and glycosaminoglycans (GAGs) with inherent cushioning and shear absorbing properties⁹³. The final layer on the ventricular side is called *ventricularis* and consists of elastin fibers and VICs, generating tissue compliance and elasticity⁹⁴ (Figure 3).

1.1.3.2 Initiation of AVS

Similar to arterial pathologies, it is not possible to define a single factor responsible for the initiation of leaflet inflammation, thickening, fibrosis and CALC, finally leading to disturbed functionality and clinical symptoms. However, it is likely that low-shear and high tensile biomechanical stresses, mostly exhibited on the aortic side, induce local VEC dysfunction making the endothelial layer permeable for lipoproteins^{95,96}. Additionally, activated VECs express increased levels of adhesion molecules (VCAM-1 and intracellular cell adhesion molecule 1 (ICAM-1)) triggering inflammatory cell infiltration into the subendothelial layers^{97,98}. Another common factor for AVS initiation and CVD in general is increased oxidative stress, causing oxLDL formation, tissue inflammation, VIC apoptosis and early CALC⁹⁹⁻¹⁰¹. In accordance to *xanthoma* formation, accumulation of lipid-filled activated macrophages enhances secretion of pro-inflammatory cytokines and ECM degradation, finally resulting in valve thickening and early mechanical deficiencies¹⁰².

Figure 3: Schematic illustration of the healthy aortic valve composition vs tissue disruption during progressive AVS. AVS progression is mainly driven by a combination of lipid and mineral infiltration, inflammatory ECM degradation and subsequent fibrosis. Calcification is a final feature of this process commonly found within the fibrosa on the aortic side of the valve.

1.1.3.3 AVS progression

Continuous ECM degradation and chronic local inflammation generate nucleation sites for dystrophic CALC, formed by inorganic Ca and Pi crystals, additionally driven by the release of pro-calcific extracellular vesicles from macrophages and VICs^{103,104}. This marks the onset of progressive pathological cusp stiffening, further activating VIC transformation towards myofibroblastic and osteogenic phenotypes¹⁰⁵ (Figure 3). Transformed VICs start to express increasing levels of fibrotic ECM, contributing to LDL retention¹⁰⁶. Moreover, together with activated macrophages and other immune cells, activated VICs release high levels of MMPs and cysteine endoproteases, disrupting the complex collagen and elastin network. This excessive degradation of functional ECM and replacement by inflamed fibrotic tissue forms a scaffold for further CALC, leading to valve failure. In the final stages of AVS, activated VICs adapt osteogenic expression machineries, forming heterotopic bone-like CALC towards the aortic leaflet side¹⁰⁷.

1.1.4 Current clinical praxis

Characteristic clinical signs of an unstable carotid stenosis with atheroembolism are transitory ischemic attacks (TIA, symptoms resolve within less than 24 hours), sudden vision impairment

(*amaurosis fugax*), minor (symptoms can last for more than 24 hours, not disabling for activities of daily living) and complete strokes (persistent neurological damage, disabling for activities of daily living). Patients diagnosed with one of these conditions carry a cumulative risk at two years of 26% to suffer any ipsilateral stroke and 13.1% to sustain a major or fatal event, despite medical care¹⁰⁸. Nevertheless, many pathological occlusions of the carotid artery remain asymptomatic and are often diagnosed coincidentally during examinations of the neck area. Importantly, these patients still carry a remaining annual risk of 2% to suffer from stroke or myocardial infarction¹⁰⁹.

Clinical signs of AVS, caused by significantly obstructed blood flow, include heart murmur, rapidly fluttering heartbeat, chest pain and dizziness or early fatigue with activity. However, these symptoms can also be connected to other CVDs that often coincide with severe AVS¹¹⁰. Therefore, more detailed diagnostic tools need to be applied in order to validate patient condition.

1.1.4.1 Diagnostic tools

In order to establish a standardized decision tree for clinical stratification of patients with symptomatic carotid stenosis at high risk for stroke as well as providing recommendations for active surgical intervention, two multi-center randomized controlled trials (European Carotid Surgery Trial (ECST)¹¹¹, North American Symptomatic Carotid Endarterectomy Trial (NASCET)¹⁰⁸) were conducted in the 1990's. The aim was to elucidate the benefit of CEA on the risk of severe future events (stroke or death) in comparison to pharmacological treatment alone, in symptomatic patients. In NASCET, a benefit of surgical intervention was found for a stenosis degree of 50-69%, with subsequent risk reduction of 29% in 5 years. For patients presenting with a stenosis of more than 70% the cumulative risk of fatal events was reduced from 13.1% to 2.5% in two years, prompting the assignment for surgery of all patients included in this study group. ECST indicated that a stenosis degree of over 80% was required to motivate CEA. However, this discrepancy was due to differing assessment methodologies resulting in overestimation of the stenosis degree in patients enrolled by ECST¹¹². These studies provided the foundation for international guidelines from the Society for Vascular Surgery and European Society for Vascular Surgery, which clinicians at present use to evaluate symptomatic carotid stenosis.

While traditional risk evaluation of carotid lesions solely relies on stenosis degree assessed *via* invasive angiography or ultrasound (US), modern imaging modalities facilitate a direct visualization of the vessel wall morphology and thereby more direct diagnosis of plaque vulnerability¹¹³. Routine diagnostics to date are mainly based on imaging by ultrasound (US) and computed tomography angiography (CTA), in rare cases magnetic resonance imaging (MRI) is applied as a third imaging modality. Due to its flexibility, low-cost and low-risk, US has become the gold standard for assessment of stenosis degree based on blood flow velocity monitoring. Furthermore, it allows an approximation of plaque morphology based on the greyscale median, separating echo-lucent and echo-dense tissues¹¹⁴. While echo-lucency indicates lipid-rich necrotic cores, inflammatory cell accumulation and intra-plaque hemorrhage, echo-density suggests higher contents of stabilizing fibrous tissue, SMC-rich areas or CALC¹¹⁵. However, US remains subject to high operator variability and physical properties of the patient, obesity or short muscular necks can cause problems during image acquisition¹¹⁶. Most of these pitfalls can be overcome by the implementation of CTA as a diagnostic tool with high availability, providing a rapid high-resolution imaging of the carotid wall and lumen. Recent advances in software development allow for improved reconstruction and radiometric analysis of plaque structures and components based on CT images¹¹⁷. In contrast to US, CTA is outstanding in visualizing dense tissues, like CALC. Tissue density is described in Hounsfield Units (HU) per pixel. Typical thresholds have been proposed *via* histological validation for lipid rich areas < 60 HU and CALCs > 130 HU^{118,119}. Coronary artery CALC measured by CT is an established marker for CVD risk prediction, included in several scoring systems (Agatston, ACCF/AHA guideline)¹²⁰⁻¹²². However, association of CALC score with higher risk in general is controversial as statin treatment, that exerts beneficial effects by decreasing

systemic inflammation as well as serum LDL- cholesterol levels, has also been shown to rather increase vascular calcification (VC) ¹²³. Assessment of CALC patterns by intra-vascular US also revealed that large plaques with low CALC were at higher risk of future events ¹²⁴. While luminal areas are clearly depicted in CTA, assessment of the fibrous cap and IPH remains challenging due to their variable intermediate tissue densities ¹²⁵. The fibrous cap/lipid rich plaque ratio can be assessed *via* CT image analysis software ¹¹⁷ and used as an indirect identifier of plaque phenotype ¹²⁶. Additional knowledge of pro-calcific components and defined molecular targets could further aid improvements of CT imaging modalities. The main disadvantage of CTA lies in the patient risk caused by increasing radiation dose and intravenous contrast agents, especially during longitudinal studies.

Echocardiography is the current routine diagnostic tool to evaluate aortic valve function based on hemodynamics (transaortic peak velocity) as well as mechanical properties such as aortic valve area (AVA) and left ventricular functionality ¹²⁷. The average cusp orifice opening, termed AVA, spans 3 to 4 cm² in healthy individuals, allowing for a transaortic peak velocity below 1 m/s. According to the European Society of Cardiology guidelines severe AVS is defined by an AVA below 1 cm² and a resulting transaortic peak velocity above 4 m/s ¹²⁸. In addition, patients are stratified according to disease progression, accounted for by an annual increase in blood peak velocity above or below to 0.3 m/s. However, resting echocardiographic assessment can lead to ambiguous or uncertain medical readouts especially in elderly patients due to its susceptibility to patient specific hemodynamic differences ¹²⁹. Therefore, CT was recognized as an established method in atherosclerosis to directly assess CALC grade, one of the dominant determinants for AVS severity and valvular dysfunction. Correspondingly, several studies confirmed Ca quantification by CT imaging *via* evaluation of Ca concentrations in explanted aortic valves ^{130,131}, leading to its recommendation as a complementary tool for diagnosis of AVS severity ¹²⁸. A remaining blind spot in current clinical AVS diagnostics is the lack of imaging modalities for non-calcific leaflet thickening. Especially in women, pro-fibrotic transformation has been shown to be a defining factor for AVS progression and pathology ¹³².

1.1.4.2 Specific biomarkers for carotid lesion vulnerability and AVS severity

Since general risk stratification of atherosclerotic carotid lesions has transpired to be widely inefficient, greater emphasis was put on the discovery of biomarkers for the prediction of patient risk. The concept of plaque “vulnerability” refers to the identification of lesions posing a high risk of thrombosis and association with future adverse cardio- and cerebrovascular events ^{133,134}. Plaque rupture is most often connected to erosion of the endothelial layer and fibrous cap, exposing the highly thrombogenic necrotic core to the blood stream. In this regard, IPH caused by neo-angiogenesis or previous ruptures is an attractant for inflammatory infiltration and tissue disruption that recently has been identified as a prime source of circulatory biomarkers for plaque instability in a large human multi-omics study ¹³⁵. While there are no readily available biomarkers for SMC content, decreasing levels of collagen, elastin and proteoglycans, expressed by migrating SMCs, have recently been directly linked to adverse disease progression and may generate potential circulatory biomarkers in future ^{86,136-138}. CALC, as a hallmark of late stage atherosclerosis and AVS, has always been an important determinant of CVD progression ⁵⁹. However, since clinical routine imaging is usually unable to detect the presence of early micro-CALC, monitoring of circulating CALC inhibitors like MGP and fetuin-A ^{139,140} or drivers of CALC formation such as osteocalcin and osteomodulin ¹⁴¹, in addition to plasma mineral imbalance ¹⁴², could be beneficial. As previously mentioned, contradictory effects of CALC on the clinical outcome have become evident in recent years ¹⁴³, therefore it is relevant to evaluate novel circulatory biomarkers linked to CALC, as well as to investigate their role in atherosclerosis pathophysiology.

1.1.4.3 Prevention and treatment

The current clinical practice for prevention of CVD related events, such as ischemic stroke caused by carotid plaque rupture or AVS related heart failure, mainly includes changes of life

style such as: healthy low-cholesterol diet, physical exercise, abstinence from smoking as well as blood glucose and blood pressure surveillance.

Pharmacological therapies

Additional prescription of antiplatelet therapies and HMG-CoA reductase-inhibitors (statins) has become increasingly common as a stabilizing treatment for atherosclerotic patients. In contrast to carotid lesion formation randomized clinical trials could not confirm positive effects of LDL reduction by statins on echocardiographic parameters or CALC in patients suffering from AVS¹⁴⁴⁻¹⁴⁶. While this may surprise at first due to the evident association between LDL levels and AVS progression¹⁵, other lipoproteins implicated in the initiation of AVS were found to be upregulated in patients treated with rosuvastatin, potentially abolishing any positive effects *via* LDL reduction¹⁴⁷. Moreover, statins have been shown to generally increase CALC formation in atherosclerotic pathologies^{123,148}. However, while macro-CALC has been related to stabilizing properties in late-stage atherosclerotic plaques^{136,149}, it is inherently detrimental to cusp flexibility and therefore aortic valve function, suggesting a given discrepancy for an efficient statin treatment. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors recently emerged as a novel LDL lowering therapy *via* upregulation of LDL receptors, interestingly also showing the capacity to downregulate other atherogenic lipoproteins¹⁵⁰. Furthermore, direct targeting of inflammation to reduce CVD risk by IL 1 β targeting antibodies or colchicine has been investigated as a potential clinical treatment in patients with a history of myocardial infarction or chronic coronary disease^{151,152}.

Since CVD commonly presents as systemic pathology affecting various vascular beds, combined therapeutical strategies on a patient specific level may pose a future best clinical praxis, warranting further molecular evaluation¹⁵³.

Surgical treatment

As current pharmacological treatments can be used to delay or stabilize CVD progression but are unable to cure or halt pathologies like carotid stenosis or AVS, surgical intervention is necessary for symptomatic patients or those at high risk.

Figure 4: Schematic explanation of CEA and transcatheter aortic valve replacement. In CEA, the intimal plaque is scooped from the surgically opened vessel in order to restore blood flow and reduce risk of future rupture. Transcatheter aortic valve replacement is a less invasive method to replace a dysfunctional aortic valve with an artificial substitute.

Following ECST and NASCET, it was advised that symptomatic patients with an indicated carotid stenosis of more than 70% to 80% should undergo CEA to remove the intimal plaque from the vessel (Figure 4). Present clinical guidelines are still based on these studies even though they already showed that 9 to 15 surgical interventions had to be performed in order to prevent one stroke, revealing a major gap in diagnostic precision. A benefit of prophylactic surgical intervention on asymptomatic patients based

on stenosis degree alone strongly depends on the expertise of the leading surgeon and subsequent minimalization of procedural morbidity. With increasing advancements in medical therapies, equal treatment efficiency of asymptomatic carotid stenosis by pharmacological stabilization is no longer a subject of controversy^{154,155}. Therefore, the field is moving towards an integrative concept to grade rupture risk by rendering location and extent of the plaque, visualization of plaque composition, combined with assessment of blood parameters and

symptoms. This way, a patient can be considered for surgery or a more intensive lipid-lowering therapy despite a rather mild stenosis or *vice versa* ¹¹³.

In severe cases of AVS surgical replacement of the aortic valve with an artificial equivalent remains inevitable. Nevertheless, application of transcatheter aortic valve implantation, initially developed for patients not suitable for traditional surgery due to co-morbidities, is expanding to patients at moderate and low risk ¹⁵⁶ (Figure 4).

1.2 VSMC AND VIC PLASTICITY – VASCULAR CHAMAELEONS

While vascular SMCs arise from diverse embryonic origins where post-developmental boundaries can only be approximated (e.g. SMCs of the *thoracic aorta* arise from *neural crest* cells while *abdominal aortic* SMCs originates from *splanchnic mesoderm*) ¹⁵⁷, VICs are derived from a mesenchymal origin. Nevertheless, both cell types are major contributors to the structural integrity of our vascular system and possess the capacity to adapt and modify ECM components to maintain tissue functionality. Studies of human specimens representing the full spectrum of atherosclerosis identified early reactions of SMCs and VICs to external stimuli ^{2,158-160}, leaving no doubt that these cells play a major role throughout all stages of cardiovascular pathologies.

The *tunica media* is traditionally considered the “muscular” layer of the vessel wall, home to the majority of differentiated vascular SMCs. Nevertheless, early vascular adaptation to extreme flow conditions leads to “intimal thickening” containing significant numbers of SMCs and proteoglycan rich matrix within the intimal layer ⁴⁴. Compared to SMCs residing in the medial layer, these intimal SMCs show distinct secretory capacities and phenotypic variability ¹⁶¹, leading to the hypothesis of phenotypic plasticity ¹⁶². Similarly, quiescent VICs found in healthy mature aortic valves can adopt a proliferative phenotype in response to VEC activation, lipid infiltration, cytokine release and high Pi, forming early aortic valve leaflet thickening ^{163,164}.

Contractile SMCs represent highly specialized cells characterized by a low rate of proliferation, poor synthetic capacity, quiescence and expression of a unique set of proteins enabling them to control vascular tone. Myocardin (MYOCD) acts as a master transcriptional regulator of the SMC lineage, inducing gene-expression of cytoskeletal components such as myosin heavy chain 11 (*MYH11*), calponin (*CNN*) and actins (*ACTA2*), while inhibiting cell proliferation ¹⁶⁵. Nevertheless, SMCs retain remarkable phenotypic plasticity in response to environmental stimuli. The general term “synthetic phenotype” describes SMCs showing significantly lower expression of cytoskeleton-related proteins, alterations in Ca handling and substantial loss of myofilaments ¹⁶⁶. Additionally, they are highly proliferative, migratory and able to secrete chondrogenic ECM components ^{167,168}, altogether resembling mesenchymal progenitor-like or myofibroblast-like cells ¹⁶⁹ (Figure 5). In reverse, VIC activation is marked by an increase in expression of SMA as well as MMPs and TGFβ allowing them to proliferate, migrate and release fibrotic ECM, similar to synthetic SMCs ¹⁷⁰. However, latest studies strongly suggest that SMC and VIC phenotypic plasticity exceeds the simplified model of quiescent vs. synthetic cells. Various transitory SMC subphenotypes may coexist in atherosclerotic lesions that can be characterized using panels of sensitive markers for cytoskeletal integrity (e.g. SMTN, leiomodulin 1 (LMOD1), PDZ and LIM domain protein 7 (PDLIM7)), contractile function (MYH11), ECM secretion (e.g. collagen 1 and 2, aggrecan (ACAN)), cell interaction and adhesion (e.g. CD34, CD44, integrin subunit alpha M (ITGAM)), surface receptors, Ca channels or vesicle transport ^{44,139,166,169,171,172}. Nevertheless, SMC identification can become challenging when their phenotypic switch results in dedifferentiation and loss of typical markers. *In vivo* fate-tracing models in mice showed that 30-70% of cells within atherosclerotic plaques belong to the SMC lineage but lack sensitive contractile markers, becoming almost indistinguishable from macrophages, mesenchymal stem cells and myofibroblasts ^{173,174}.

During intimal and valvular CALC, SMCs and VICs have been shown to acquire phenotypes similar to chondrocytes and osteoblasts, composing calcified ECM ¹⁷⁵⁻¹⁷⁷ (Figure 5). Initial clues were collected in studies on medial CALC showing mineralized matrix in direct proximity to SMCs expressing alkaline phosphatase (AP) and collagen II ¹⁷⁸. These findings

were confirmed in atherosclerotic intimal lesions showing SMCs positive for osteoblastic (Runt-related transcription factor 2 (RUNX2)) and chondrocytic markers (BMP2, osterix (OSX), SRY-box transcription factor 9 (SOX9))¹⁷⁹⁻¹⁸¹. Moreover, SMCs executed an osteogenic phenotype change, characterized by the loss of SMC markers and gain of RUNX2, osteopontin (OPN), osteocalcin (OCN) and AP, upon high Pi stimulation *in vitro*¹⁷². Similar changes could be triggered *in vivo*, where increased expression of TGFβ1 in the arterial endothelium caused cartilaginous metaplasia in the underlying media of rats¹⁸². Importantly, *in vitro* and *in vivo* studies also identified MYOCD, RUNX2 and SOX9 to exhibit counteractive roles during SMC differentiation, confirming that SMCs maintain basic lineage identity^{183,184}. Such transitional cells, termed “myochondrocytes”, showing both SMC and chondrocyte properties, have been morphologically identified by electron microscopy in human and mouse atherosclerotic lesions¹⁸⁰. Accordingly, VICs involved in active regulation of ectopic CALC in severe AVS have been identified to express high levels of RUNX2 and BMP2, activating subsequent CALC-related signaling pathways¹⁷⁶. Especially bone morphogenic protein receptor type 2 (BMPR2) signaling has been shown to accelerate osteogenic transformation by phosphorylation and activation of several SMAD intracellular transducer proteins^{185,186}. Recent development of single cell sequencing technologies allowed even more detailed tracking of the differential contribution and multitude of SMC phenotypes during vascular pathologies. Novel transition states, not defined by a specific functional role, were either identified or predicted based on central transcription factors such as krüppel-like factor 4 (KLF4)^{169,174,187}.

Figure 5: Graphical summary of SMC and VIC phenotypic transformation related to calcification. Both vascular SMCs and VICs actively contribute to fibrotic and osteogenic ECM remodeling in atherosclerotic disease and AVS. They can acquire a multitude of transdifferentiated states that are difficult to classify as the transition phases are seamless. Interestingly, during the activation process both cell types generate a phenotype not unlike mesenchymal progenitors.

Finally, pathogenic stress (in particular hyperphosphatemia and hypercalcemia) can lead to VIC and vascular SMC apoptosis, connected to the release of pro-calcific extracellular matrix vesicles (EVs), creating nucleation *nidi* for dystrophic CALC^{139,188} (Figure 5). Autophagy, a highly conserved mechanism to clear damaged or aging cells¹⁸⁹, has recently been identified as an important process to mitigate VC¹⁹⁰. It was shown to both inhibit osteogenic transdifferentiation and EV release as well as rescuing overall cell viability within the tissue^{191,192}.

1.3 VASCULAR CALCIFICATION – BONES IN ARTERIES AND VALVES

CALC of the cardiovascular system is a systemic pathology that has been shown to afflict humans for millennia. Both the ice mummy “Ötzi” (who died approximately 5300 years ago) as well as ancient Egyptians have been diagnosed with significant CALC of their arteries^{193,194}. Histological manifestations of VC as well as their respective clinical impact have been found to be as diverse as the numerous inductors leading to cellular ossification (e.g. biomechanical forces, oxidative stress, inflammation or Ca / Pi and lipid overload). VCs, affecting the *tunica media* and *intima* as well as arterial valve tissues, can be defined as micro-CALC (from 0.5 µm, “speckled calcification” up to 2 mm) or macro-CALC (“fragmented calcification” 2-5 mm, “diffuse calcification” > 5 mm), the latter forming sheet-like continuous structures of calcified tissue. This classification mostly relies on histopathological assessment as radiology is only able to discriminate macro-calcified structures^{75,119,195,196}.

Progressive plaque CALC is classically considered to be a marker for increasing atherosclerotic disease burden^{77,197}. Micro-CALC as well as early stages of speckled and fragmented VC, aggregating within the necrotic core, fibrous cap and medial tissue matrix¹⁹⁸, have been related to adverse clinical outcome. This effect is most likely caused by the inherently unfavorable geometry and tissue property of these types of VC, creating tissue stress concentrations, activating further inflammatory response mechanisms and ultimately leading to plaque rupture or thrombus formation¹⁹⁹. In studies of sudden coronary death, assessment by CT identified exclusive presence of speckled CALC in 65% of acute plaque ruptures and either an absence or speckled CALC in over 50% of vulnerable thin-cap fibroatheroma²⁰⁰. In contrast, macro-CALC has rather been reported to be a predominant feature of clinically stable “asymptomatic” lesions^{136,149,201,202}, while it has been suggested that non-calcified plaques contain more destabilizing inflamed and lipid rich regions²⁰³⁻²⁰⁵. In support of these observations, carotid plaques from asymptomatic patients have shown enrichment of biological processes associated with CALC together with markers of SMC differentiation¹⁴⁹. Moreover, gene expression analyses of lesions stratified by pre-operative CTA associated macro-CALC with molecular signatures typically linked to plaque stability and in particular genes related to a differentiated SMC phenotype¹³⁶. Estimation of structural plaque stresses *via* computational *in silico* modeling has indicated that wide-spread macro-CALC can lower overall tissue stress due to greater stiffness and load bearing capacity²⁰⁶ and might result in advantageous stress redistribution²⁰⁷. The diagnostic discrepancy between densely formed continuous CALC and loosely arranged early aggregations is directly highlighted by clinical CAC evaluation. While estimation of CAC volume alone was found to be positively associated with CVD events, the addition of CAC density was identified as a negative predictor across all levels of CAC volume as well as other risk factors in the general population²⁰⁸.

Therefore, a better understanding of molecular mechanisms driving different forms of VC formation as well as their impact on tissue remodeling is crucial to improve patient diagnostics and risk prediction.

1.3.1 Molecular mechanisms of vascular calcification

The continuous process of pathological VC is driven by the interconnection of passive physiochemical and active biological events leading to tissue transformation. While the temporal sequence and clinical consequences remain disputed, consensus exists that VC is generally initiated by a loss of CALC inhibitors and phenotypic changes of structural cells.

Circulating inhibitors such as matrix Gla proteins (MGP) and fetuin-A exhibit protective functions in healthy arteries¹⁴⁰. However, in response to inflammatory priming (characteristic for atherosclerosis and AVS), mineral imbalance (typical for chronic kidney disease (CKD)) and other cell-damaging stimuli, SMCs and VICs can begin to release matrix vesicles loaded with high concentrations of calcium-phosphate (CaPi) or hydroxyapatite^{139,209}. In addition, multiple different resident and circulating cells have been shown to be subject to similar processes, including endothelial progenitor cells, mesenchymal stem cells and macrophages^{181,210}. These microparticles and uncleared debris of apoptotic cells act as the first *nidus* for further mineralization due to their surface-bound Ca binding complexes^{188,211}. Subsequently, micro-CALCs develop *via* transformation of amorphous CaPi into mature crystalline hydroxyapatite particles of spherical or needle like structure, with a diameter of typically up to 50 μm ^{198,212,213}. Extensive degradation of organized ECM structures and replacement by fibrotic tissue, containing large amounts of scattered collagen filaments, forms a scaffold prone for the aggregation of micro-CALC particles¹⁹⁸. This initial stage of mineralization is accompanied by an osteo-chondrogenic phenotypic transition of SMCs and VICs marked by upregulation of transcription factors typically known as master regulators of chondrocyte and osteoblast development and maturation (SOX9 and RUNX2)^{172,183,214}. *In vitro* experiments have shown a direct connection between the degradation of elastin and accelerated Pi-provoked CALC of SMCs²¹⁵. The acquired transcriptional program induces other factors such as OSX, AP, OPN and OCN, which actively regulate extensive formation of sheet-like ossified matrix^{210,216,217}, partially replicating physiological mineralization processes like endochondral ossification²¹⁸⁻²²¹. As a consequence, expression of tissue-nonspecific AP will promote the conversion of CALC inhibiting pyrophosphate to Pi, further contributing to mineral imbalance²²².

Pathological examination of human tissues spanning all age groups identified CALC formation as a dynamic process, characterized by co-existence of micro- and macro-CALC throughout plaque progression. Very advanced CALC formations detected in end-stage CVD can resemble mature calcified matrix with a lamellar structure, including osteoclastic remodeling processes²²³ and even bone marrow²¹⁹.

1.3.2 Lessons learned from bone biology

VC in late stages of CVD pathologies is often compared to skeletal bone formation. While this comparison usually stems from morphological similarities observed by radiography or histology, it has become increasingly evident that bone is an endocrine organ capable of influencing systemic vascular health²²⁴. Osteotropic hormones, such as parathyroid hormone (PTH), exhibit systemic regulatory functions both in skeletal and vascular mineralization. In particular osteocyte-derived fibroblast growth factor 23 (FGF23), a hormone that enhances Pi secretion by the kidney, has been shown to be an important connector between bone-forming cells and VC²²⁵. Elevations in FGF23 might serve as a PTH mediated defense mechanism to attenuate toxicities of early hyperphosphatemia. However, common CVD co-morbidities and risk factors including diabetes, uremia and hyperlipidemia, further disrupt the bone-vascular signaling axis due to reduced Klotho co-factor expression, leading to vascular CALC and endothelial dysfunction²²⁶⁻²²⁸. A potent PTH responsive inhibitor of matrix mineralization is phosphorylated OPN, secreted by skeletal osteoblasts. Due to its stability to proteolysis it serves as a systemic defense to VC when released into circulation²²⁹⁻²³¹. The role of bone-derived OCN appears more complex as it has the capacity to support adiponectin production by adipocytes, thereby suppressing TNF stimulated VC, on the one hand^{232,233}. On the other hand, it may also directly promote osteogenic SMC transformation²³⁴.

With an increasing understanding of the co-regulatory pathways connecting the bone-vascular axis it becomes evident to directly compare the active processes mediating metabolic effects of skeletal and vascular CALC. Both CVD related VC and bone formation are multistage processes following controlled temporal and spatial sequences. Complex molecular mechanisms, involving intra- and extracellular molecular signaling, regulate mineralized tissue formation and have been described so far in more details in bone biology. Commonly, a variety

of local and systemic regulatory factors, including growth respectively differentiation factors, hormones, cytokines and ECM, interact with cell types like bone and cartilage forming cells or even circulating mesenchymal and inflammatory cells ²³⁵.

Similar to early mineralization initiation in atherosclerotic lesions, pro-inflammatory cytokines IL 1, IL 6 and TNF α are mainly known to play an important role in initiating the repair cascade after bone tissue disruption ²³⁶. Therefore peak expression is found within the first 24 hours following fracture, but again later during fracture remodeling. Their main function is the induction of further downstream responses to injury by enhancing ECM synthesis, stimulating angiogenesis and recruiting endogenous fibrotic cells to the injury site. The second peak expression occurs due to involvement of all above mentioned cytokines in tissue remodeling. Especially TNF α promotes endochondral ossification by mesenchymal progenitor cell recruitment ²³⁷, induction of cell death in hypertrophic chondrocytes and stimulation of osteoclastic function by upregulation of the TNF-related cytokine RANKL ²³⁸. Atherosclerosis is commonly accepted to be linked to chronic inflammatory processes, driving disease progression. Cytokines like ILs, IFNs and TGFs play a crucial role in tissue remodeling and initiation of pro-calcific processes ²³⁹. However, apart from its key role in mediating inflammatory disease TNF α also controls VC *via* TNF-related apoptosis inducing ligand (TRAIL), an OPG resembling ligand ²⁴⁰. Due to the striking similarities with TNF α regulation in bone-homeostasis, this system has also been described to mediate bone turnover in the vasculature ²⁴¹.

Two universal transcription factors affecting chondrogenic and osteogenic fate are SOX9 and RUNX2. SOX9 is expressed by proliferative but not hypertrophic chondrocytes, inducing secretion of proteoglycan rich ECM including collagen II ²⁴² and ACAN ²⁴³. It is crucial for normal chondrocyte proliferation rates and delay of apoptosis. In opposition to SOX9, RUNX2 activates the Indian hedgehog (IHH) promoter and thereby maintains a balance of proliferation and hypertrophy of chondrocytes as well as osteoblast differentiation during bone development. Recently, another transcription factor, myocyte enhancer factor-2 (MEF2C), known to regulate muscle and cardiovascular development, has been shown to act upstream to RUNX2 ²⁴⁴. *In vitro* and *in vivo* studies on vascular SMCs were able to causally link RUNX2 and SOX9 expression to osteogenic transformation and concurrent SMC CALC, as discussed in more detail in section 1.2.

Differentiating chondrocytes express OSX, an essential transcription factor for osteoblast differentiation, just like SMCs undergoing an osteogenic phenotypic transition. Acting downstream of RUNX2, null mutations of OSX result in a complete absence of bone formation ²⁴⁵. In bone homeostasis, OSX is chiefly expressed by osteoblasts, but skeletal deformity in the chondrocyte-specific OSX conditional knockout mice proves that OSX expression by chondrocyte lineage cells is essential for bone development ²⁴⁶. Just like intimal CALC, new bone material is composed of mineralized collagen matrix also containing OCN, OPN, BMPs, and inorganic hydroxyapatite. Specific matrix proteins such as OCN and OPN are secreted by mature osteoblasts and regulate the mineralization of the surrounding matrix ²⁴⁷. In the past several years, Wnt signaling was revealed to not only serve as a master controller of bone formation, as it occurs downstream of additional signaling cascades, but also to regulate osteoclast formation and hence bone resorption ²⁴⁸. Wnt/ β -catenin signaling not only promotes the differentiation of mesenchymal progenitors into osteoblasts but also the generation of cardiovascular progenitor cells from mesenchymal stromal cells ²⁴⁹. During bone formation this signaling pathway positively regulates expression of OPG, thereby suppressing osteoclastogenesis and enhancing mineralized matrix formation ²⁴⁸. Similarly, several studies on hypercholesterolemic mice and SMC cultures were able to replicate the regulatory function of Wnt-signaling, inducing an osteogenic phenotypic switch by upregulation of RUNX2, BMPs and ALP ^{250,251}.

Independent hormonal mediators of cartilage and bone growth are insulin-like growth factors (IGFs) and thyroid hormone. During bone growth IGF-I and IGF-II are mainly derived from the bone matrix, endothelial cells, osteoblasts and chondrocytes ^{235,252}. The serum concentration of IGF-I is regulated by growth hormone (GH), therefore it is considered the local mediator of GH's systemic effects ²⁴². Both IGF-I and IGF-II promote cartilage matrix formation in form of

collagen I and non-collagenous matrix proteins and have been implicated during atherosclerotic plaque formation²⁵³ as well as intimal hyperplasia²⁵⁴. Thyroid hormone and tri-iodothyronine (T3) are systemic factors stimulating hypertrophy and death of chondrocytes as well as hypertrophic markers including collagen X and AP²⁵⁵. *In vitro* studies on vascular SMCs from rats were able to translate the regulatory function of T3 to VC, revealing capacities of T3 to downregulate RUNX2, ALP, OCN, OPN and OSX expression under calcifying conditions²⁵⁶. A large group of important growth and differentiation factors like BMP's, activins and inhibins is included in the TGF β superfamily. Most of these molecules act on serine/threonine kinase membrane receptors, affecting nuclear gene expression and thereby ultimately promote various stages of intramembranous and endochondral ossification¹⁸⁶. BMP inhibition in LDL receptor deficient mice (Ldlr^{-/-}) was able to inhibit atheroma development and associated VC or osteogenic activity²⁵⁷. BMP antagonistic FGFs are expressed upon thyroid hormone induction and suppress chondrocyte proliferation in growth cartilage independently of IHH. FGFs are additionally synthesized by monocytes, macrophages, mesenchymal cells and osteoblasts, playing a critical role in early angiogenesis and mesenchymal cell mitogenesis²⁵⁸. Interestingly, *in vivo* experiments on ApoE^{-/-} mice constitutively overexpressing endothelial FGF showed increased EC apoptosis, endothelial to mesenchymal transition and SMC proliferation²⁵⁹. This effect together with concurrent TGF β activation implies an overall pro-atherogenic outcome of high FGF levels²⁶⁰, even though they have been clinically applied to treat ischemic diseases²⁶¹.

In summary, the combination of knowledge gathered in preclinical and clinical studies related to skeletal and cardiovascular health highlights the presence of a bone-vascular regulatory axis. The balance between positive signaling molecules and their inhibitors is a critical determinant of osteogenesis and thus development of mineralized matrix in different tissues. Systemic dysmetabolic states, commonly found in connection to CVD, perturb this sensitive balance, giving rise to severe calciotropic dysfunctions.

1.3.3 Proteoglycan 4 and other important proteoglycans

The group of proteoglycans (PGs) encompasses 43 distinct genes and great variance of different proteins due to alternative splicing. Proteoglycans are often considered as a subclass of glycoproteins with the key difference of repeating units of long unbranched disaccharide chains *vs* short highly branched, non-repeating glycan chains.

Finding a comprehensive classification of the different subgroups has turned out to be a challenging task. However, current literature proposes a simplified categorization according to three criteria: i) cellular and subcellular location, ii) overall gene/protein homology and iii) the presence of specific protein modules within their respective protein cores²⁶². According to this classification, PGs are found intracellularly (within secretory granules), on the cell surface (transmembrane spanning or glycosylphosphatidylinositol (GPI) -anchored), in the pericellular and basement membrane zone or extracellularly (e.g. hyalectans and small leucine-rich PGs). While there is only one intracellular PG, serglycin, mainly responsible for granule integrity in mast cells²⁶³, a big group of heparan sulfate PGs function as major biological modifiers of growth factors at the cell surface, pericellular matrix and basement membrane zone²⁶⁴. Due to their ability to interact and bind with each other as well as to key components of the basement membrane, they achieve optimal growth factor-receptor presentation, as well as maintenance of morphogen gradients^{265,266}. Chondroitin and dermatan sulfate containing PGs additionally function as structural constituents of complex ECM, providing fiber organization, viscoelastic properties and retain water²⁶⁷. Small leucine-rich PGs are the most numerous class, functioning both as structural part and signaling molecules within the ECM. By acting on receptor tyrosine kinases and toll-like receptors (TLRs) ECM PGs are able to modulate fundamental processes involved in development and pathology, such as migration, proliferation, apoptosis, inflammation and autophagy²⁶⁸⁻²⁷³.

Proteoglycans secreted by vascular SMCs exhibit important regulatory functions within the vascular environment. HSPG2, a modular heparan sulfate PG and essential component of the basement membrane in healthy arterial walls, has been shown to maintain endothelial barrier function and inhibit SMC proliferation in the context of intimal hyperplasia^{37,54,271}. However,

in conjunction with its wide expression within other vascular and avascular tissues, HSPG2 has also been shown to modulate cell adhesion²⁷⁴, lipid metabolism²⁷⁵ as well as vascular and skeletal biomechanics^{276,277}. Increased vascular expression of ACAN during intimal hyperplasia formation and atherosclerotic lesion development has been related to SMC activation, phenotypic transformation and apoptosis²⁷⁸. Nevertheless, ACAN is known to form large supramolecular complexes (> 200 MDa) generating a densely packed gel to support the load-bearing capacities of cartilage tissues²⁷⁹. In addition to its mechanical stabilization, ACAN has the physical properties to counteract oxidative stress by scavenging redox-active cations²⁸⁰. These cation-scavenging capabilities are mainly generated by a large amount of negatively charged carbohydrate chains, a general feature of most PGs. In connection to the lipid retention hypothesis, one of the most accepted theorems to explain initiation of atherosclerotic lesion formation²⁸¹, PGs have been shown to particularly bind positively charged regions of apoB-containing lipoproteins⁵⁵, thereby contributing to lipid accumulation within the media and neo-intima. Genetic modification of the heparan sulfate side-chain formation in the HSPG2 protein of ApoE^{-/-} mice has been shown to result in significantly reduced lipid retention during the early phases of disease development⁵⁴. This example highlights the conflicting roles of PG's inherent physical properties within the vasculature, on the one hand providing vital structural support to maintain vessel wall homeostasis, but becoming a contributor to pathological processes typical for atherosclerosis on the other.

We identified lubricin/PRG4 as the most significantly upregulated transcript in calcified plaques, correlated to bone metabolism and inhibition of inflammatory pathways. PRG4 is a large molecule (~ 345 kDa) that consists of approximately equal proportions protein and

Figure 6: Illustration of the PRG4 protein structure. (A) Predicted 3D structure using AlphaFold¹. (B) Simplified schematic of the main domains.

oligosaccharides, either referred to as proteoglycan or glycoprotein due to harboring structural features of both classes (Figure 6A). Besides heparin-binding domains, multiple mucin-like repeats and an aggregation domain, it contains more than 168 glycosylation sites, making it highly hydrophilic²⁸², while the non-glycosylated regions can bind to collagens and other cartilage proteins^{283,284} (Figure 6B). Mainly produced by synovial fibroblasts and superficial zone chondrocytes²⁸⁵, PRG4 has been shown to be crucial for joint homeostasis *via* surface lubrication, suppression of inflammatory responses and exhibiting other cytoprotective functions^{286,287}. Its biological functions are mediated by direct binding to a number of receptors including CD44 as well as TLR 2 and 4. PRG4-CD44 interaction inhibits nuclear factor kappa B (NF- κ B) translocation in human synovial fibroblasts attenuating IL 1 β -induced synoviocyte proliferation and expression of

matrix-degrading enzymes²⁸⁸⁻²⁹². PRG4 has also been shown to affect synovial fibroblast to myofibroblast transition by reducing fibrotic marker expression (SMA and collagen I)²⁹³ and to regulate accumulation and polarization of macrophages in the synovium²⁹⁴.

However, PRG4 expression is not limited to joints and cartilage as it has also been detected in other tissues like tendon, lung, liver, heart, bone, ligament, muscle, and skin^{285,295,296}. Moreover, it poses neutrophil binding capacities within human plasma via L-selectin ligand activity²⁹⁷.

Therapeutically, recombinant forms of PRG4 have been shown to exhibit disease-mitigating effects in pre-clinical osteoarthritis (OA) models^{288,298}. In this thesis, we investigated whether PRG4 has similar roles in late stages of atherosclerosis and osteogenic SMC transition.

2 RESEARCH AIMS

This PhD project is part of the EU Marie Curie International Training Network program INTRICARE that has a focus on various aspects of VC and its role in vascular disease.

The aims of this program were to:

- Identify and functionally characterize novel sensitive markers of calcification.
- Understand the underlying mechanisms associated to cellular phenotypic switching in atherosclerotic ossification.
- Address the outstanding clinical need for improved diagnostic accuracy, to reliably predict future strokes and other lethal CVD related events, by highlighting a potential translational pipeline between clinical imaging, *in silico* analysis and basic cell biology.

More specifically, the objectives of this thesis were to:

- I. Relate calcification grade, assessed by patient diagnostic CT imaging, to the overall gene expression profiles in human plaques. (*Study I*)
- II. Investigate the role of PRG4 in atherosclerotic disease progression, intimal and valvular calcification. (*Study II and III*)
- III. Define the influence of macro-calcification on local plaque biomechanics and SMC phenotype in human carotid lesions. (*Study IV*)

3 MATERIALS AND METHODS

In an effort to connect clinical diagnostics with a deepened understanding of the underlying basic biology, all studies presented in this thesis originate from human biobank data, subsequently investigated and validated by *in silico*, *in vivo* and *in vitro* methodologies.

3.1 HUMAN BIOBANKS

In order to facilitate translatability of our studies into clinical settings, discovery and initial characterization was conducted utilizing several human biobanks.

The Biobank of Karolinska Endarterectomies (**BiKE**), prospectively enrolls patients undergoing surgery for (>50% NASCET ²⁹⁹) carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Stockholm, Sweden. Symptoms are defined as TIA, minor stroke and *amaurosis fugax*. Patients not showing symptoms within 3 months prior to surgery are categorized as asymptomatic. The biobank was established in 2002 and currently consists of more than 1500 patients comprising i) a BioBank with plaque tissues, plasma, PBMCs, DNA and RNA including a material register, ii) an ImageBank with quantified diagnostic images by CT/US and iii) a DataBank of 100 clinical variables (i.e. age, gender, comorbidities, therapy and blood measurements), microarrays from plaques and peripheral blood monocytes, proteomic analysis of lesions by mass spectrometry and plasma by Luminex. Additionally, biomaterial for further analysis is deposited as cDNA for qPCR and paraffin-embedded plaque tissues for histology.

Study I and *III* were based on a selected cohort of BiKE patients (n=40), stratified according to the presence of macro-CALC visualized by preoperative CTA. The cohort included 20 highly calcified carotid plaques and 20 lesions diagnosed with low CALC grade, collected from 2008 to 2013.

In *study I*, findings related to CALC grade were investigated and validated using a subcohort (n=61) from an independent biobank called **KärITx**, enrolling CKD patients undergoing living donor kidney transplantation surgery since 2009 at the Department of Transplantation Surgery, Karolinska University Hospital, Stockholm, Sweden. This biobank is designed to deepen the understanding of bone turnover and VC in CKD patients by collection of prospective and retrospective blood and urine samples as well as arterial (*epigastric artery*), fat and muscle biopsies obtained during transplant surgery. These samples facilitate a wide range of analyses, including discovery of biomarkers for disrupted inflammatory, and metabolic processes as well as tissue characterization by IHC, DNA and RNA assays. Moreover, arterial biopsies are subjected to histopathological examination assessing the medial CALC grade, also called Mönckeberg calcification, occurring systemically in the arterial *media* of patients with advanced chronic kidney disease, diabetes mellitus or other chronic inflammatory conditions. Of note, while the median age of these patients was only 45 years, 10% already suffered from CVD comorbidities.

To investigate mechanisms related to human disease progression, *study III* utilized a rare collection of peri-renal aortic biopsies representing atherosclerotic pathology within all age groups between 5 and 76 years ⁷⁶. Samples included in this biobank called **SOKRATES** are collected during organ transplantation and classified according to the adapted American Heart Association classification ³ as proposed by Virmani et al ³⁰⁰.

Extending our investigations of PRG4 in intimal CALC to **AVS** in *study II*, we benefitted from a third biobank at Karolinska University Hospital collecting tricuspid aortic valves from AVS patients undergoing aortic valve replacement. After surgery, macroscopic evaluation of the aortic valve leaflets is conducted to visually identify and dissect non-calcified from calcified regions, thereby establishing a model of disease progression within each individual valve. Observed pathology ranges from healthy or fibrotic (non-calcified) to advanced stages, characterized by the presence of macro-calcified nodules. Subsequently, the separate tissues can be used for proteomic, transcriptomic and histological analyses as well as VIC isolation to characterize and compare expression profiles and cell phenotypes in relation to progressive

AVS. In *study II*, 64 valves were used for transcriptomic analyses, 4 for immunohistochemistry and 20 for cell isolation, respectively.

3.2 IN SILICO CTA IMAGE ANALYSIS AND BIOMECHANICAL MODELING

In silico analysis of carotid intimal lesions, included in *study I, III* and *IV*, was conducted on CTA exams routinely performed in preoperative health care at hospitals in Stockholm area. Image acquisition with a kilovoltage peak of 100 or 120 was performed in *caudo-cranial* direction from *aortic arch* to *vertex* after intravenous contrast injection. Axial images were constructed with a common slice thickness of 0.625 mm.

Image analysis

The initial vessel segmentation is a semi-automated process requiring digital placement of the vessel centerline and selection of the plaque area within the respective software workstation for vascular CT-scans (Figure 7). Outer and inner borders of the artery are then automatically recognized, with an option for manual adjustment, to calculate wall and lumen volumes. Following these initializing steps, specialized software can be used for morphological tissue analysis.

Study I utilized **TeraRecon** (iNtuition, TeraRecon, Foster City, CA, USA, 2015), a modular software that can be optimized for many applications due to the free adjustment of HU thresholds according to the examination objectives. We used settings specifically developed for plaque analysis, with a threshold of >400 HU to mark intimal CALC volume (Figure 7). The CALC degree was then calculated in relation to the total wall volume in the same region.

Figure 7: Representative images of a classic CTA view in comparison with TeraRecon and vascuCAP analysis software.

the initial vessel segmentation and manual adjustment, this software allows for automated tissue composition analysis including many additional components (lipid rich necrotic core (LRNC), IPH and tissue matrix (MATX)) (Figure 7) and structural measurands (plaque burden and cap thickness). To avoid limitations of fixed thresholds, accuracy is achieved by algorithms that account for distributions of tissue constituents, for which overlapping densities were classified by expert-annotated histology, rather than assuming constant material density ranges. The implementation of these algorithms not only enhances separation of complex tissue compositions but also reduces inter-observer variability by increased automation.

Biomechanical modeling

As previously introduced, the arterial wall is constantly subjected to complex biomechanical forces induced by the pulsatile intraluminal blood flow. Changes of these forces due to turbulent flow or pathological hypertension have been proposed as one of the earliest causal drivers of atherosclerotic disease initiation. In short, mechanical load on solid materials can be quantified as stress, measured in pascal (Pa). Forces applied in parallel to the matrix surface are defined as shear stress, while forces acting perpendicular to the matrix are called normal stress.

Study III and *IV* upgraded this simple annotation by use of **vascuCAP** (Elucid Bioimaging, Wenham, MA, USA), a histology-validated tool for computer-aided phenotyping of atherosclerotic lesions based on diagnostic CTA images¹¹⁷. After

Due to the multidirectional transmission of forces within a tissue matrix a numerical terminology becomes very complex, therefore conversion to von Mises stress allows the expression of a general multiaxial stress state by a single number. The physical deformation of a matrix in response to stress is measured as unitless strain or stretch in %, representing the relative change of angle or length. The degree of tissue strain is determined by the specific tissue properties and quantified as stiffness. Healthy non-calcified vascular tissue has non-linear stress-strain properties with increasing stiffness over strain due to elastin and collagen fiber elongation. Calcified tissue adopts linear non-elastic stress-strain properties due to the greatly increased ECM stiffness. A material that preserves its volume during deformation is defined as incompressible, in biological tissues this is a rather idealized characteristic but often necessary to design computationally feasible modeling tasks. In order to generate a constitutive biomechanical model, these and other mechanical properties would have to be consistently formulated. To bypass the complexity of such a comprehensive model, continuum mechanics define a macro-material by a continuum of representative elements, thereby modeling a structure based on one known sub-module. Using this approach, successful modeling can be conducted while heterogeneity is gradually increased.

Evaluation of structural plaque stresses requires computational FEM due to the complex geometries and non-linear material properties. In *study IV*, FEM analysis of four selected patients was performed using COMSOL Multiphysics 5.6 (COMSOL AB, Stockholm, Sweden) by creating a converted matrix mesh based on segmentations obtained through vascuCAP³⁰¹. Slight manual surface smoothing was applied to avoid local mesh irregularities due to the limited resolution of routine clinical CTA imaging. The generated surface mesh was then converted into a 3D solid domain of the vessel wall and represented by approximately 20k quadratic tetrahedral finite elements. A spatial interpolation function was used to specify the local tissue characteristics of LRNC, CALC and MATX. The mechanics of each of these tissues were modeled using material properties based on previous experimental research³⁰² and enforced tissue incompressibility. Diastolic and respective systolic blood pressure were applied to the wall and the tissue stretch assessed at three cross-sections distal to the bifurcation to cover different regions around the maximal-stenotic part of the lesion.

3.3 IN VIVO MODELS OF OSTEOGENIC TRANSFORMATION AND CALCIFICATION

Since wild-type (WT) rodents are widely resistant to the development of atherosclerotic disease burden or CVD in general, most *in vivo* models to study mechanisms of disease progression in a complete organism have to be induced by either genetic, surgical or pharmacological manipulation.

Commonly applied genetical modifications to induce susceptibility for atherosclerosis in mice are $Ldlr^{-/-}$ ³⁰³ and $ApoE^{-/-}$ ³⁰⁴. Both modifications target the clearance of lipids from the circulation which is mainly controlled by binding of the LDL receptor to the ApoE and ApoB-100 components of LDL particles. The importance of functional LDL receptor binding had already been shown in studies on familial hyperlipidemia in humans. Accordingly, $Ldlr^{-/-}$ as well as $ApoE^{-/-}$ mice suffer from chronically elevated circulating cholesterol levels, initiating atherosclerotic plaque formation³⁰⁵. Old $ApoE^{-/-}$ mice will develop atherosclerotic lesions even on normal chow, however plaque formation is significantly more severe when combined with a western-type diet. Despite noticeably different lipid profiles compared to human patients diagnosed with severe atherosclerosis, lesions in $ApoE^{-/-}$ mice resemble human plaque progression in many morphological features, such as lipid accumulation, inflammatory cell infiltration, SMC proliferation and migration, fibrous cap as well as late-stage necrotic core and even CALC formation³⁰⁶. Therefore, $ApoE^{-/-}$ mice were used in *study III* to model different stages of carotid plaque formation and CALC.

Osteogenic transformation in intimal hyperplasia

Intimal thickening, triggered by endothelial activation is one of the first signs of atherosclerotic disease initiation. It is known that structural cells like SMCs and VICs are able to respond to

injury and changes in biomechanical stress by proliferation, migration and ECM remodeling. However, it remains unclear to what extent osteogenic phenotypic transformation is involved during these early steps of vascular remodeling.

Two surgical methods have been established to reliably induce intimal hyperplasia in rodents. One triggers endothelial activation and neo-intima formation by disturbing the blood flow. This model is commonly generated *via* ligation of the left carotid artery in proximity of the bifurcation and has been presented as an efficient method to study SMC phenotypic changes during vessel remodeling³⁰⁷. Complete ligation induces obstruction of the blood flow and subsequent thrombus formation. Significant intimal thickening can already be observed 2 to 4 weeks post-surgery, characterized by inflammatory cell infiltration and vascular SMC proliferation leading to complete lumen occlusion distal to the ligation. While this model is generally easily reproducible, omitting complex surgical manipulations, it lacks more complex disease characteristics that are provoked by disturbed blood flow. Alternatively, more experienced animal surgeons perform partial ligations in order to create turbulent flow conditions, even more enhanced by the addition of a rigid cuff surrounding a short area of the vessel. These techniques are able to replicate human lesion formation to a larger extent and in combination with genetic manipulation even clinical complications³⁰⁸. Therefore, to unravel the contribution of osteogenic SMC transformation and PRG4 expression during early lesion formation in *study III*, transcriptomic analysis and IHC was conducted on ApoE^{-/-} mice

undergoing partial carotid ligation on a western-type diet (Figure 8A).

Another surgical method inflicts endothelial denudation by mechanical injury to the vessel wall. This technique is either performed by repeatedly moving a spring wire through the carotid artery of mice or utilizing a balloon catheter in larger rodents like rats³⁰⁹. The endothelial damage causes platelet aggregation to the injury site as well as inflammatory infiltration, subsequently activating vascular SMCs. Neo-intima formation is commonly observed within 2 weeks after surgery. This method consistently replicates complex biological processes following endothelial injury in order to regain vascular homeostasis and has been used for detailed description of cellular adaptation and ECM remodeling⁴⁴. However, in rodents the vasculature is usually able to regain homeostasis while in human pathology remodeling processes would

Figure 8: Schematic illustration of the three animal models utilized in this thesis to study SMC phenotypic transition throughout intimal hyperplasia and atherosclerotic lesion calcification.

lead to chronic intimal hyperplasia and luminal stenosis. Hence, *study III* applied the carotid balloon injury model in Sprague-Dawley rats (Figure 8B) as an additional *in vivo* study of intimal hyperplasia with a focus on SMC contribution to vascular healing *via* osteogenic phenotypic changes, ECM remodeling and PRG4 expression.

Atherosclerotic plaque calcification

ApoE^{-/-} mice are a widely accepted model to achieve atherosclerotic lesion formation in rodents. However, only very old mice have been shown to sporadically develop late-stage disease features, like macro-CALC, as found in human tissues.

Different additional methods have been developed to accelerate vascular mineralization processes. Genetic deletion of systemic CALC inhibitors (MGP, Fetuin-A and Klotho) has been shown to cause extensive VC, however in many cases with the drawback of associated disruption of skeletal homeostasis and symptoms of premature aging^{227,310,311}. Surgical removal of 5/6th of the kidney has been shown to mimic CALC processes similar to severe CKD^{312,313}. A pharmacological treatment to induce rapid hyperphosphatemia and medial CALC utilizes high-dose subcutaneous injections of vitamin D3³¹⁴, while low levels of vitamin D3 in connection to uremic conditions have also been shown to induce VC similar to pathologies found in CKD, causing somewhat ambiguous interpretations in relation to general CVD related VC³¹⁵. Warfarin, an anticoagulant in clinical use (including CVD patients), has been shown to cause VC by inhibiting the carboxylation of Gla proteins via blocking of vitamin-K recycling. Although requiring longer treatment in mice, this model provides a reliable approach for CVD related VC with clinical impact. A previous study validated the accelerated atherosclerotic lesion progression and CALC³¹⁶ which was utilized in *study III* to achieve conditions similar to fibro-calcific plaques (Figure 8C), thereby identifying the temporal involvement of PRG4 throughout atherosclerotic plaque CALC. In brief, ApoE^{-/-} mice received either a western-type diet + vitamin K1 or a western-type diet + vitamin K1 + warfarin. Additional vitamin K1 supplementation is necessary to control toxic effects on the liver and prevent bleeding, while introducing vitamin K-deficiency in the vasculature.

3.4 IN VITRO MODELS OF OSTEOGENIC TRANSFORMATION AND CALCIFICATION

Primary human vascular SMCs and VICs are a resource for mechanistic studies of CALC processes as well as high throughput screening of pharmacological interventions without deviating too far from a natural setting. While these cells, when used at low passage numbers, express typical markers and show functional features of differentiated structural cells, some of the more sensitive markers may already be downregulated during cell isolation, contributing to the onset of phenotypic modulation. Moreover, cells isolated from different donors might show inherently distinct predispositions to CALC and transdifferentiation. In *study III* and *IV*, we conducted exploratory studies on commercially available primary ascending aortic SMCs from donors not diagnosed with any vascular comorbidities or risk factors for CVD. For *study III*, we extended our analysis to SMCs isolated from biopsies classified as normal aortic wall tissue, in patients undergoing thoracic aneurysm surgery. Primary human VICs used in *study II* were isolated from aortic valves directly after valve-replacement surgery and phenotypically characterized. Nevertheless, it cannot be excluded that results are affected by interpatient variability.

In vitro CALC is commonly achieved by supplementation of either inorganic Pi and Ca or organic β -glycerophosphate. β -glycerophosphate supplementation relies on the active excision of glycerol by SMC or VIC derived tissue non-specific AP, to increase free phosphate levels in the cell culture media. Additional supplementation with ascorbic acid and dexamethasone promotes collagen production, leading to calcified nodules containing collagenous inner cores^{317,318}. Inorganic Pi and Ca overload has been shown to exhibit direct effects on SMC and VIC osteogenic transformation. On the one hand, Pi can act as a direct signaling molecule, promoting osteoblastic transformation³¹⁹. On the other hand, inorganic Pi and Ca will form CaPi-crystal precipitations in a concentration-dependent manner (at approximately 2 mM) if

not actively inhibited by released CALC inhibitors. Moreover, in the presence of live cells these CaPi-crystals are embedded in an organic amorphous matrix, resembling the structure of hydroxyapatite crystals ³²⁰. High Ca treatment has also been connected to active release of calcifying EVs, similar to early CALC processes *in vivo* ¹³⁹. SMCs in early passages have been reported to react to these ossifying stimuli by phenotypic modulation in a similar fashion as observed *in vivo*, showing increased expression levels of osteogenic markers such as BMP2, SOX9 and RUNX2 ^{66,183,184,209,230,320}. We applied this methodology in studies II and III to test different factors typical for the atherosclerotic plaque milieu and assess their effect on SMC CALC and phenotypic changes.

To induce CALC, medium was supplemented with 3.6 mM Ca or 2.6 mM Pi for up to 12 days and refreshed every 3 days. In order to address the specific impact of typical cytokines previously implicated in atherosclerotic disease progression and particularly osteogenic transformation, primary SMCs were stimulated with 20 ng/ml of human IGF1, IGF2, TGFβ, TNFα or IFNγ, or 50 ng/ml of human PDGFB, respectively. VICs were treated with 10 ng/ml TGFβ or 20 ng/ml BMP2. In a functional assay investigating the impact of extracellular PRG4 on SMC and VIC CALC, we combined Ca and Pi mediated CALC with full length rhPRG4 (Lubris BioPharma, USA) supplementation at concentrations of 10 and 100 µg/ml.

3.5 IN VITRO MODELS OF TISSUE STRETCH

Nevertheless, VC does not only have direct effects on the phenotype of structural cells. Especially sheet-like macro-CALC leads to a significant increase in tissue stiffness, reaching an average Young's modulus of up to 25 GPa ³²¹, approximately 10k-fold higher than soft or only partially calcified fibrous tissue. This causes changes in load-bearing and stress-redistribution within the tissue. Vascular SMCs are known for their phenotypic adaptation in order to maintain vessel function under stretch ^{322,323} but pathologically increased tissue stress, typical for chronic hypertension and CVD, has been shown to induce apoptosis of differentiated SMCs ³²⁴. Different *in vitro* models of cyclic stretch have been established to delineate SMC phenotypic changes according to frequency and level of applied stress ³²⁵. These models mostly utilize stretchable membranes instead of rigid culture plates as culture surface. While matrix gels further enhance tissue-resemblance, experimental set-up and analysis becomes increasingly complicated. In *study IV*, we applied a 2D system to investigate the impact of specific stretch-parameters, acquired within highly calcified human carotid plaques by *in silico* FEM, on vascular SMC morphology, phenotype and survival. In addition to stretchable silicone membranes, used to model the soft tissue environment, we included standard rigid culture plates as the closest biomechanical replication of a calcified plaque matrix.

3.6 GENE EXPRESSION ANALYSIS

Analytical methods to detect and quantify changes in gene and protein expression due to disease processes, phenotypic adaptations or pharmacological interventions are crucial to understand underlying molecular mechanisms resulting in pathological tissue modification and clinical symptomatology. Scientists have developed many methods mostly applicable to tissues and cell culture bulk samples, allowing for simultaneous assessment of gene-sets or specific detection of few targets.

Gene expression analysis by microarray

In order to characterize differences in gene expression from human carotid lesions (*Study I*), human aortic valves (*Study II*) and *in vivo* models of intimal hyperplasia (*Study III*), we conducted microarray profiling. The basic principle of this technique is to utilize specific DNA probes, bound to a solid surface, to simultaneously study the relative expression of a large number of genes. Sample preparation includes reverse transcription of high quality RNA samples into cDNA which hybridizes to the complementary probes on the microarray and is subsequently labeled by a fluorescent dye. Signal intensity is then quantified by a fluorescence scanner. Commonly, several probes of slightly different sequence but targeting the same transcript are randomly distributed across the chip, referred to as a probe set. However, the raw

readout cannot be linearly correlated with the absolute RNA concentration but needs to be assessed relative to other samples within the same batch. Additional data post-processing steps are performed in order to summarize probe sets, perform data normalization (e.g. guanine cytosine count normalization to adjust probe affinity variations) and transformation (e.g. background correction and log transformation) for clear graphical presentation. Microarray analysis has become a fast and increasingly affordable method with the ability to quantify a myriad of coding and non-coding transcripts. However, relative quantification always requires direct comparison of treatment conditions within the same batch and chip design can introduce a bias towards already known and expected transcripts.

Gene expression analysis by qPCR

Quantitative real-time polymerase chain reaction (qPCR) is a technique to monitor amplification of target DNA molecules during a PCR process. Similar to microarrays, a reverse transcription step is required to generate cDNA from RNA samples. Common methods for the detection of PCR products include non-specific fluorescent dyes (e.g. SYBR green) that intercalate with any double-stranded DNA and sequence-specific DNA probes (e.g. TaqMan) consisting of oligonucleotides that are labelled with a fluorescent reporter, ensuring detection only after hybridization of the probe with its complementary sequence. Generally, the PCR process requires a series of temperature cycles that are sequentially repeated 25 to 40 times. Each cycle normally consist of three stages: i) the denaturation (around 95 °C) enforces separation of the nucleic acid double chain, ii) probe and primer to DNA hybridization is facilitated at a temperature of around 50 to 60 °C and iii) strand elongation and polymerization by DNA polymerase is executed between 68 to 72 °C. Optimized polymerase enzymes and short target sequences allow to skip the last step as DNA replication is completed already during the change between alignment/hybridization and the next denaturing stage. In addition, fluorescence intensity is quantified during short phases at the end of each cycle, with a temperature of around 80 °C to reduce background caused by the presence of primer dimers in case of non-specific dyes. The specific temperatures and timings depend on a wide variety of parameters such as the polymerase enzyme, divalent ions and deoxyribonucleotide triphosphate (dNTPs) concentration and primer design. The fluorescent readout-data of qPCR assays is represented for each sample by the cycle threshold (Ct) value, the number of amplification cycles required to generate a fluorescence signal significantly above the background fluorescence. Therefore, Ct values are inversely proportional to the original relative RNA expression level. Similar to microarray data, Ct values have to be compared to samples run within the same batch and are commonly transformed *via* the $\Delta\Delta C_t$ method to present the fold change of a target gene relative to a reference or “housekeeping” gene. Good reference genes have to show stable expression under all treatment conditions, hence it can be an advantage to test several reference genes for optimization. Apart from the necessity for good reference genes, qPCR data strongly depends on high quality RNA extraction and storage. DNA contaminations within the original samples could otherwise cause positive signal detection independently of the existence of transcribed RNA.

In studies II / III and IV we utilized qPCR analysis based on TaqMan assays to quantify genes of interest in samples from *in vitro* experiments. Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) was used as reference gene in SMC samples and hypoxanthine phosphoribosyltransferase 1 (HPRT) in samples extracted from VICs.

3.7 HISTOLOGICAL PROTEIN ANALYSIS

Transcription products assessed in gene expression assays are often subjected to multiple post-transcriptional modifications (e.g. alternative splicing, 5'capping and 3'polyadenylation) affecting temporal and quantitative parameters of protein translation as well as creating several protein variations from one transcript. For that reason, it is necessary to validate the relationship between transcriptomic dysregulation and protein presence, localization and interaction within morphological distinct tissue features.

Tissue characterization by histology

Histology (new Latin from Greek histos = tissue and logia = study, first mentioned using this terminology in 1919 by August F.J.K. Mayer) studies the microscopic anatomy, combining assessment of organs, tissues and cells. Histopathology is the delineated clinical application to identify dysfunctional tissues, often required for accurate diagnosis of cancer or other diseases.

General sample preparation includes fixation of cell and tissue structures (e.g. by 4% formaldehyde creating methylene bridges, 2,5% glutaraldehyde creating pentene bridges or snap freezing), dehydration and embedding within protective substrates preserving antigens and tissue integrity, such as paraffin wax, epoxy resin or cryo embedding matrix (e.g. OCT) for hydrated samples. For staining and microscopy, the samples are sectioned utilizing a microtome or cryostat, with common section thickness between 4 to 12 μm .

Sections can be stained with a multitude of different histological stains derived from natural sources or chemical reactions, developed to generate tissue contrast for morphological assessment as well as to highlight specific cellular features. In the studies presented in this thesis we utilized:

- 1) Alizarin red, a dihydroxyanthraquinone isomer forming precipitates with free Ca ions and generating bright red staining of tissue components containing Ca. This procedure works best under acidic conditions, slightly degrading the mineralized matrix to expose contained Ca ions.
- 2) Weigert's elastin, a combination of stains often including orcein (natural extract from lichen) or a combination of resorcinol (an isomeric benzenediol) and fuchsin (a chemical magenta dye), staining elastin fibers dark purple. Cell nuclei were counterstained using hematoxylin (a dark, almost black natural stain extracted from the logwood tree).
- 3) Masson's trichrome, combining hematoxylin, acid fuchsin, xylydine ponceau (a red azo dye), phosphomolybdic acid and methyl blue (chemical blue dye) to create dark red stain of muscle fibers, dark blue collagen matrix, light red cytoplasm and dark nuclei.
- 4) Movat's pentachrome, a very complex stain originally developed by Henry Z. Movat in 1955 to highlight the various constituents of connective tissue, especially cardiovascular tissue, by five colors in a single stained slide³²⁶. This stain was subsequently modified multiple times and optimized protocols differ greatly between laboratories. General components usually include alcian blue (a polyvalent basic dye), Verhoeff hematoxylin, crocein scarlet (a bright red azo dye) combined with acidic fuchsin and saffron. This stain allows identification of elastic fibers (black or blue), nuclei (black or blue), collagen and reticular fibers (yellow), mucin and proteoglycans (bright blue), fibrin (bright red) and muscle (dark red).

Specific protein detection by IHC and IF

IHC and immunofluorescence (IF) are sensitive and specific methods to detect and visualize the presence of proteins in tissues by use of antibodies. Based on similar sample preparation as general histology, this detection procedure involves deparaffinization and antigen retrieval to expose proteins of interest (e.g. by heating, citrate buffers or proteases), however these steps are usually not necessary for cryopreserved specimens. Subsequently, unspecific antibody binding sites are blocked and a primary antibody targeting the protein of interest added. A detectable signal is commonly generated by a tagged secondary antibody specific for the species of the primary antibody, thereby attaching either an enzyme based colorimetric (e.g. horseradish peroxidase or alkaline phosphatase) or fluorescent assay. This method heavily relies on the primary antibody specificity and blocking of unspecific binding, whereas assay sensitivity can be enhanced by optimized secondary antibodies and improved tags.

While IHC and IF are excellent qualitative methods for specific protein detection and localization, they have inherent weaknesses when it comes to high throughput or stringent quantitative requirements. Nevertheless, optimized staining protocols and good microscopic set-ups allow for semi-quantitative analysis by counting of positive signal in relation to total area.

We applied both IHC (*Study I, II, III*) and IF (*Study I, III, IV*) in order to validate and characterize changes in protein expression on tissue and cellular level in comparison to detected transcriptomic dysregulations. The specific protocols are described within each manuscript's methods section.

3.8 ETHICAL CONSIDERATIONS

This thesis aims to not only understand the basic disease mechanism of atherosclerosis but to also generate knowledge about potential new targets which could be applied to directly improve diagnostics and patient health. These are two very important motivations for all experiments conducted.

The starting point of our studies was human tissue collected during carotid endarterectomy or kidney transplantation surgery. Each patient signed an informed consent upon being introduced to the procedure and project by the leading surgeon. This material was then analyzed with various molecular-biological and histological methods and correlated to important clinical risk factors. Patient ID's are pseudonymized and only accessible to selected staff over secure hospital computers. Human tissues and plasma samples are also coded and stored in monitored freezers to ensure the security of the respective biobank. The use of human material within this project was granted by Swedish ethical permits following the guidelines of the declaration of Helsinki or in agreement with the guidelines of the Dutch medical and ethical committee and the code of conduct of the Dutch federation of biomedical scientific societies. Sample extraction didn't confer additional risk to the patient as the surgery was conducted in exactly the same procedure. Patients without symptoms were only assigned for CEA in cases of severe stenosis, after being informed about the risks by a surgeon.

To understand the complex mechanisms of age related diseases animal models are unfortunately still irreplaceable. Since zebrafish, mice and rats never develop atherosclerotic lesions in their wildtype forms, either genetically modified or surgically induced models have to be used. This drawback is most probably caused by the much shorter lifespan and higher healing capacity of animals. To induce different kinds of plaque phenotypes and test possible treatments, genetically modified animals can be injected with compounds that would also be used in clinical practice. One example is the injection of warfarin, a commonly used anticoagulant, which induces a more calcified plaque phenotype. When using genetically modified mice it is of highest importance to ensure that the animals are not subjected to prolonged suffering or effects that severely decrease their quality of life. Apart of developing early to medium stages of CVD, ApoE^{-/-} mice showed normal behavior and no signs of distress. We also used rats to create surgical models of vascular injury and follow the healing and remodeling process. All animals lived under monitored conditions in a professional animal facility. They were anesthetized prior to any surgery and received pain relieve afterwards. In case of complications and for tissue harvesting animals were sacrificed using a quick and painless method. All animal care and experimental procedures were performed in accordance with the guidelines for use of experimental animals and were approved by the local animal experimentation ethics committee.

In order to track functional mechanisms and molecular targets, cell culture models have to be applied. We used primary vascular SMCs either sourced from commercial providers or isolated from surplus tissue fragments extracted during aneurysm surgery, in agreement with the Dutch code for proper secondary use of human tissue. These cells can only be kept for a limited amount of time until they die or lose their typical phenotype due to complex transdifferentiation processes. Therefore these cultures are very valuable and have to be used in a responsible manner. If cells have to be moved between universities special agreements have to be signed to ensure secure handling.

With this considerate approach we try to build an ethical fundament that allows us to justify our experiments with the gain of knowledge and the prospect of improving life span as well as life quality of future patients.

4 RESULTS AND DISCUSSION

Combined, the studies presented in this thesis aspired to unravel the intricacies of SMC and VIC phenotypic changes, leading to osteogenic ECM remodeling and VC. Moreover, we aimed to link molecular biology and clinical routine diagnostics by *in silico* modeling techniques in order to contribute to a better understanding of risk prediction based on atherosclerotic plaque morphology.

In short, we characterized PRG4 as a novel marker for early osteogenic SMC and VIC transformation, playing a key role in subsequent ECM remodeling and VC. With respect to clinical diagnostics, we demonstrated that macro-CALC in human carotid atherosclerosis assessed by routine CTA was associated to general biological processes of plaque stabilization and contributed to favorable tissue biomechanics.

4.1 MACRO-CALCIFICATION RELATES TO STABILIZING BIOLOGICAL PATHWAYS IN LATE-STAGE CAROTID LESIONS

In *study I*, we investigated the general genetic signatures of human carotid lesions stratified by the morphological feature of high vs low CALC grade. Patients ($n = 40$) enrolled in this study were matched for symptomatology, comorbidities, age and sex. Since the level of CALC was assessed by routine clinical CTA, only macro-CALC could be accounted for. Per definition micro-CALC falls below the spatial resolution of this technique.

Global gene expression analysis revealed a majority of upregulated genes in highly calcified plaques to be connected to cytoskeletal formation and contractility, typically expressed by differentiated SMCs, but to some extent also by myofibroblasts (e.g. *MYOCD*, *CNN1*, *ACTA2*). Moreover, genes that have been characterized to sensitively indicate SMC differentiation, such as *MYH11*, *LMOD1* and *PDLIM7*, showed a corresponding increase together with genes involved in ECM and basement membrane integrity (e.g. elastin, collagens and *HSPG2*). Overall, this transcriptomic data indicated activation of multiple pathways in high CALC plaques commonly connected to reacquisition of vessel homeostasis, previously only known from rodent *in vivo* models but not human pathology. These findings were confirmed on protein level by IHC and histological tissue analysis. In contrast, genes related to inflammatory cells and their recruitment (e.g. IL 8, CD68, CD36), lipid metabolism and ECM degradation (MMPs and cathepsin) were downregulated.

Figure 9: Graphical representation of major upregulated biological pathways in highly calcified plaques. Slice size indicates gene set enrichment.

SMC contractility, ECM integrity and fibrous cap formation are commonly considered to be major stabilizing attributes of advanced atherosclerotic lesions⁷⁵, while increased inflammation and MMP activity leading to ECM fiber degradation has been linked to adverse clinical outcome^{80,327-329}. Therefore, these results are a first indication for a more stable expression profile of highly calcified lesions in late-stage atherosclerosis. Gene set enrichment analysis, estimating the impact of dysregulated genes on biological pathways, supported our initial findings (upregulation of SMC differentiation, muscle contraction, ECM organization), but additionally revealed induction

of processes involved in both SMC fate and osteogenic transformation, such as SMAD and TGF β signaling^{186,330,331} (Figure 9). Repressed biological pathways confirmed mitigation of inflammation, ECM degradation, cholesterol metabolism and cytokine responses, with TNF, IL 1 β , IFN γ mediated signaling among the most significantly affected. When conducting the same analysis on highly calcified plaques from symptomatic patients only, upregulated pathways of ECM organization and elastic fiber formation were accompanied by processes most likely related to IPH, such as platelet degranulation, fibrin clot formation and iron uptake. Nevertheless, ECM degradation and inflammation remained repressed compared to low calcified plaques from symptomatic patients, suggesting that macro-CALC can be a feature of lesion stabilization even after partial rupture. Extraction of such “healed” lesions is not uncommon since patients are often referred for CEA after a sequence of minor symptoms.

Importantly, this profiling revealed a distinct genetic signature for highly calcified lesions that allowed for complete separation of high vs low calcified plaques by predictive modeling using a panel of only 20 genes. We had previously utilized a similar model including 30 genes to separate symptomatic from asymptomatic carotid lesions with a precision of 78%¹⁴⁹, suggesting that the inclusion of high CALC grade as phenotypic determinant could improve clinical risk prediction for patients diagnosed with advanced carotid stenosis. On a cellular level, detection of sensitive markers for differentiated SMCs within intimal plaques in close proximity to macro-CALC indicated that this plaque phenotype could facilitate SMC redifferentiation, a novelty in late-stage human CVD.

4.2 FORMATION OF INTIMAL AND VALVULAR MACRO-CALCIFICATION IS MARKED BY PRG4 EXPRESSION

In *study I*, *PRG4* emerged as the top significantly upregulated gene in highly calcified advanced carotid lesions, interestingly correlating with markers for differentiated SMCs (*SMTN*, *MYH11*, *ACTA2*) but also several genes typically strongly involved in matrix mineralization and remodeling (*BMP2*, *SOX9*, *RUNX2*, *TRAP/ACP5*)^{186,242,332,333}. More detailed characterization of histological sections found a significant overlap of extracellular PRG4 with areas also showing strong signal for OPN and OCN expression. Moreover, IF identified direct overlap with SMA and TRAP positive cells surrounding macro-calcified nodules. Together these findings suggest a connection of PRG4 expression to intimal CALC formation, likely involving osteogenic SMC transformation. The presence of PRG4 in plaques classified as low-calcified can be explained by the limited resolution of clinical CTA, making micro-CALC undetectable. This was supported by speckled Alizarin-red positive areas in low-calcified lesions, a histological technique specifically staining tissue Ca. The ambivalent relationship to both mineralized matrix anabolism (*BMP2*, *RUNX2*, *OCN*) as well as catabolism (*TRAP/ACP5*, *OPN*) could reflect their temporal overlap throughout atherosclerotic lesion progression²⁵⁶. PRG4 appeared to have effects on both processes as a major part of the plaque ECM.

Biological pathway analysis associated PRG4 mainly to repression of SMC migration and hematopoietic progenitor differentiation, nevertheless PRG4 had previously been shown to directly mitigate inflammation in joint tissue^{289,294} as well as atherosclerotic disease in mice^{137,334}. This biological function may explain the positive correlation of *PRG4* gene expression with *CD36*, *TNF* and *IL 1 β* as well as overlapping IF signals between PRG4 and CD68 positive cells in human carotid plaques from our study. Of note, PRG4 protein was only found sporadically within highly calcified medial biopsies from CKD patients which often lack the severe inflammatory response typically driving atherosclerotic intimal lesion progression. While PGs have generally been proposed as molecular components of the ECM contributing to lipid retention when overexpressed within the media and neo-intima⁵⁵, a more detailed morphological analysis of late-stage carotid plaques, performed in *study III*, rather found a negative relationship between PRG4 and LRNC plaque volume proportion. However, multiple linear regression analysis indicated that this effect was mainly caused by increased levels of CALC.

Another CVD pathology with strong inflammatory contribution and severe late-stage CALC formation is AVS. In *study II*, we analyzed *PRG4* expression in a cohort of 64 patients

undergoing aortic valve replacement. While the analyzed valves are mainly representative of end-stage disease, similar to carotid plaques removed by CEA, leaflet dissection according to severity of pathological changes in tissue morphology (healthy, thickened and calcified regions) allowed for an estimation of disease progression areas within the same patient. *PRG4* expression levels were elevated in both thickened and calcified tissues compared to morphologically healthy regions but there was no further increase between thickened and calcified areas. Overall, *PRG4* positively correlated to genes facilitating matrix mineralization (*BMP2*, *RUNX2*, dentin matrix acidic phosphoprotein 1 (*DMPI*) and integrin binding sialoprotein (*IBSP*)) and inflammation (*IL 6*). Interestingly, the significance of these correlations diminished with increasing grade of CALC, suggesting that *PRG4* expression is part of the early osteogenic transformation, likely triggered by chronic tissue inflammation, but not directly involved in genetic processes driving accumulation and remodeling of macro-CALC. Another valid explanation is the increasing heterogeneity of the thickened and calcified cusp tissue, making a strict separation difficult. Accordingly, *PRG4* protein deposition was detected by IHC within the ECM of thickened leaflet regions surrounding macro-calcified nodules. These areas were histologically characterized by high proteoglycan and collagen content and partially overlapped with *RUNX2* and *BMP2* positive cells in close proximity to calcified matrix.

In order to track *PRG4* contribution to human atherosclerotic lesion formation in a more stringent manner, we employed a rare cohort of aortic biopsies representing the full disease spectrum, classified from AHA stages I to V (SOKRATES biobank, *Study III*). By IHC, intracellular *PRG4*, together with *SOX9* positive cells, was detected within the intima and media already during early intimal thickening and xanthoma formation. In later stages, *PRG4* localization shifted towards the ECM, surrounding necrotic core regions and overlapping with *SOX9* and *RUNX2* positive cells. These areas also contained neo-vascularization, likely facilitating tissue oxygenation, remodeling and bone-like macro-CALC processes.

Overall, our analysis of human tissues representing different vascular beds and stages of CVD progression indicates that elevated *PRG4* expression marks osteogenic tissue transformation, likely triggered by chronic inflammatory processes and other atherogenic stimuli, preceding the formation of severe macro-CALC.

4.3 PRG4 EXPRESSION IS DEPENDANT ON SMC AND VIC OSTEOGENIC PHENOTYPIC MODULATION

Our findings from human tissues were supported by three rodent *in vivo* models (*Study III*), showing that elevated *PRG4* levels were involved in early medial activation and intimal hyperplasia, accumulating in areas later undergoing severe mineralization. Additionally, these models suggested a possible molecular pathway consisting of *Tgf β* mediated induction of *Prp4* expression, coupled to *Sox9* and other osteogenic factors (*Bmp2*, *Runx2*), during the acute response to vascular injury and atherosclerotic disease initiation. Moreover, initial *PRG4* induction was connected to SMC activation marked by significant negative correlation to *Myocd*, *Myh11* and *Smtn*. Nevertheless, in rat carotid intimal hyperplasia induced by balloon injury *PRG4* protein persisted within the extracellular matrix throughout the late resolution phase while its gene expression slowly returned to baseline, suggesting a functional contribution of the extracellular protein to the reacquisition of vascular homeostasis and SMC differentiation.

Considering the central contribution of structural cells like SMCs and VICs to the formation of intimal hyperplasia and valvular thickening we investigated their connection to *PRG4* expression in more detail using primary human cells (*Study II* and *III*, Figure 10). Indeed, our experiments showed a strong induction of *PRG4* by TGF β signaling in carotid and aortic SMCs isolated from patients not diagnosed with CVD as well as aortic SMCs and VICs isolated from patients undergoing surgery due to CVD related pathologies. In SMCs *PRG4* activation was related to a strong initial upregulation of *SOX9*, while VICs showed a spontaneous *SOX9* upregulation over time *in vitro*. This effect might be explained by the isolation from diseased aortic valves and concomitant predisposition for osteogenic phenotypic transition as SMCs

isolated from CVD patients also showed varying levels of osteogenic susceptibility. Moreover, siRNA knock-down experiments on healthy aortic SMCs confirmed a signaling axis *via* *SMAD3* and *SOX9* to be an upstream regulator of *PRG4* expression, similar to the genetic machinery controlling PG expression in chondrocytes³³⁵. Both VICs and vascular SMCs showed a strong upregulation of *PRG4* when stimulated with high inorganic Pi, a factor known

Figure 10: The role of *PRG4* in atherosclerotic lesion progression and its influence on SMC phenotypic modulation. *PRG4* expression is upregulated during SMC activation by *TGFβ*, mineral imbalance or changed tissue biomechanics. It marks the onset of osteogenic phenotypic transformation and increases ectopic calcification formation as a key component of the remodeled ECM. In a negative feedback mechanism, extracellular *PRG4* has the capacity to inhibit SMC proliferation, migration and osteogenic transformation.

to induce osteogenic phenotypic modulation. Accordingly, high Ca treatment resulted in a similar transition of SMCs, indicating mineral imbalance to be a general driver of *PRG4* activation. However, while *PRG4* expression positively correlated with *SMAD3* and *SOX9* in both conditions, we observed some striking differences in the effect on typical SMC markers. Under high Ca, *PRG4* levels correlated to a strong loss of all typical SMC markers, even affecting *MYOCD*, *ACTA2* and *CNN1*, opposed to Pi treatment where *PRG4* upregulation positively correlated to *MYOCD* and *ACTA2* levels. These findings may reflect different phenotypic modulations of vascular SMCs in response to mineral overload as Pi has been shown to directly activate osteogenic transformation⁶⁶, while Ca overload increased EV release and SMC apoptosis¹³⁹. In both scenarios, strong expression of *PRG4* appeared to be a cellular response mechanism, probably *via* capturing the positively charged ions and mineralizing particles within the dense proteoglycan matrix, thereby releasing structural cells from osteogenic pressure. This hypothesis is complemented by an increased formation of ectopic CALC when calcifying conditions were combined with extracellular rhPRG4 supplementation.

4.4 EXTRACELLULAR PRG4 COUNTERACTS SMC MIGRATION AND DEDIFFERENTIATION

The initial identification of macro-CALC as a morphological feature of a more stable lesion phenotype in late-stage carotid atherosclerosis seemed ambivalent considering the clear implication of CALC as a marker of increased disease burden. Nevertheless, high levels of extracellular *PRG4* may present one possible mediator responsible for its positive molecular and cellular impact. In *study III* we could show that addition of rhPRG4 to the culture medium of vascular SMCs significantly reduced proliferation and migration even under *TGFβ*, *PDGFB* and high serum treatments, all known to induce SMC activation¹⁶⁶. Moreover, it was able to

reduce endogenous *PRG4*, *SOX9* and *SMAD3* expression under calcifying conditions and prevented further loss of general markers for SMC differentiation (*MYOCD*, *CNN1*) (Figure 10). However, it appeared unable to prevent increased *RUNX2* and *SOX9* expression over time in VICs and even induced *BMP2* without additional osteogenic stimuli (*Study II*), indicating inherently different cellular effects of a PRG4-rich ECM on osteogenic transformation in different vascular beds.

4.5 MACRO-CALCIFICATION EXERTS LOAD-BEARING CAPACITIES IN HUMAN CAROTID PLAQUES, SUPPORTING SMC SURVIVAL AND DIFFERENTIATION

A passive effect of continuous sheet-like macro-CALC is the significant increase of tissue stiffness. Purely hypothetical *in silico* models had previously suggested that such CALC-related stress redistribution may exhibit advantageous load-bearing capacities in atherosclerotic lesions, depending on position and geometry within the tissue^{199,204}. In *study I*, we showed that extensive macro-CALC was associated with a reacquisition of more differentiated properties of plaque SMCs and ECM formation in late-stage carotid atherosclerosis. To evaluate whether

Figure 11: Graphical summary of the analysis pipeline utilized in study IV to connect routine clinical diagnostics, in this case CTA, with laboratory analytics via *in silico* and *in vitro* modeling techniques.

macro-CALC related biomechanical forces can influence SMC phenotype and contribute to plaque stabilization, we generated an analysis pipeline connecting routine clinical CTA with *in vitro* experiments via *in silico* modeling (Figure 11).

FEM analysis based on *in silico* tissue morphology annotation confirmed a significant reduction of tissue stretch within and in close proximity to macro-CALC areas, while other soft tissue components of the carotid lesion were subjected to stretch values commonly found in pathological hypertension. When replicating these conditions in a 2D *in vitro* cyclic stretch model we found that neither condition was physiologically optimal, as some stretch is required to stimulate SMC alignment and functional contractility requiring *MYH11* expression³³⁶.

Nevertheless, the general gene expression profile of SMCs growing on unstretched soft membranes (modeling soft tissue surrounding CALC) and standard rigid culture plates (most similar to the CALC surface) was characterized by increased expression of cytoskeletal markers including genes previously shown to be lost early during SMC dedifferentiation (*ACTA2*, *CNN1*, *LMOD1*, *PDLIM7*)¹⁶⁶. Additionally, higher *HSPG2* expression under unstretched conditions may indicate reacquisition of a basement membrane composition favoring SMC differentiation. However, complete depletion of cyclic stretch also led to an increase in *RUNX2*, suggesting an increased potential of these SMCs for continued matrix mineralization. In late-stage atherosclerotic disease this may still pose the lesser of two evils as SMCs were able to adapt to high levels of Ca and Pi without stretch, but in combination with elevated stretch committed to rapid cleaved CASP3 driven apoptosis. Interestingly, high amounts of PRG4 were detected on transcriptomic and proteomic level in SMCs on both stretched and unstretched silicone membranes, while it was widely absent on rigid plates. Considering the effects on SMC differentiation, proliferation and migration described in *study III*, this may be another factor for increased osteogenic potential under rigid conditions but on the other hand inhibit SMCs to efficiently adapt to pathological stretch. This hypothesis is supported by increased SMC apoptosis under stretch in combination with rhPRG4 supplementation, warranting further functional investigation.

5 CONCLUSIONS

The results presented in this thesis demonstrate that macro-CALC in late-stage human atherosclerosis, can be connected to stabilizing biological processes, in particular SMC differentiation and survival. Moreover, routine clinical CTA in combination with *in silico* analysis can be utilized to assess the impact of patient specific plaque morphology on tissue biomechanics and SMC phenotype.

PRG4 emerged as a specific target marking the onset of osteogenic transformation and matrix mineralization in intimal carotid atherosclerosis as well as AVS, while being a key component of the ECM with the capacity to modulate SMC differentiation. Together, these findings contribute to a better understanding of atherosclerotic lesion remodeling and vulnerability, consequently with the potential to improve diagnostic accuracy and risk prediction for the individual patient, warranting further clinical evaluations.

- I. Our analysis uncovered that highly calcified carotid lesions exhibit a distinct phenotype related to SMC differentiation and ECM organization as well as reduction of inflammatory activity. As macro-calcification is a morphological plaque feature that is rather easily detectable utilizing diagnostic tools already in routine clinical use, position and geometry of densely calcified nodules should be investigated as surrogate marker in clinical risk prediction (*Study I*).
- II. Analysis of pathological samples, representing different vascular beds and stages of human CVD progression, identified elevated *PRG4* expression as a marker for osteogenic tissue transformation, likely triggered by chronic atherogenic stimuli, preceding the formation of severe macro-calcification (*Study I, II, III*).
- III. *In vivo* and *in vitro* models identified early upregulation of *PRG4* in VICs and vascular SMCs by osteogenic phenotypic transformation in response to TGF β signaling and calcifying conditions. We showed that *PRG4* expression is part of the vascular remodeling response, preceding and enhancing the formation of ectopic CALC (*Study II, III*).
- IV. While endogenous *PRG4* expression is connected to osteogenic phenotypic transformation *via* SMAD3 and SOX9, extracellular *PRG4* mediates positive effects on SMC differentiation and quiescence, indicating a feedback mechanism (*Study III*).
- V. We were able to generate a potential pipeline to connect plaque composition, biomechanics and molecular processes on a patient specific level. Our data confirms, based on clinical CTA imaging and *in vitro* studies, that macro-calcifications can support SMC survival and differentiation under atherosclerotic conditions by generating low stretch zones within advance lesions.

6 SOCIETAL IMPACT AND RESEARCH PERSPECTIVE

CVD treatment has been a focus of clinical research for many years, being the major cause of death worldwide but the World Health Organization (WHO) predicts these numbers to still rise towards claiming a staggering annual 22 million lives by the year 2030³³⁷. Coupled to an aging population, the rising prevalence of CVD is putting pressure and substantial additional costs on healthcare systems and businesses worldwide. In particular, the treatment of heart attacks and strokes not only requires intensive clinical care but also substantial recovery time, adding to the total cost. In Europe, the annual expenditure for CVD is estimated at 210 billion Euro per year, with about 53% (111 billion) due to health care costs and 26% (54 billion) due to productivity losses. The considerable remaining part of approximately 21% (€45 billion) mostly affects the family of patients and other informal care-givers, who invest crucial time and effort in order to restore quality of life³³⁸. While clinical procedures improve, major risk can still be attributed to failing recovery from cardiovascular episodes, leading to premature death and decreased productivity. Innovative medicine has the responsibility to reduce hospitalizations and expensive surgical procedures by improving routine diagnostics as well as patient specific medical care.

To date, most patients with advanced carotid stenosis are classified by the degree of luminal stenosis and clinical symptomatology. In most centers the indication for CEA is still depending on a stenosis degree above 50-70% (NASCET) and the occurrence of neurological symptoms, unprecise tools for the assessment of lesion morphology, disease biology and future risk prediction. However, interest in the assessment of plaque morphology has increased over the past years, especially after new recommendations were included within the 2017 ESVS guidelines. In *study I* and *IV* of this thesis, we demonstrate the translational potential of utilizing detailed *in silico* analysis of clinical CTA images to generate a more accurate representation of patient specific disease morphology. Moreover, we were able to link this modeling data to specific disease modifying biological pathways and cellular responses. Macro-CALC of carotid atherosclerotic lesions stood out as a positive modifier of biological pathways related to plaque stabilization. Biomechanical analysis in combination with *in vitro* experiments confirmed a positive effect on SMC differentiation and survival in proximity to macro-CALC compared to other regions of the same plaque. Expanding this analysis to a wider range of biomechanical conditions as well as comprehensive transcriptomic and proteomic analysis would generate valuable data to make one step forward towards predictive modeling of disease progression on a patient specific level. Ideally, future patients suffering from vulnerable atherosclerotic lesions would be detected prior to developing severe symptoms. Combined screening methods, including detection of circulating blood biomarkers, CT imaging of the neck and heart vasculature and subsequent *in silico* analysis, could form a valuable foundation to predict disease progression, detect patients at high risk and suggest personalized treatments.

On a molecular level PRG4, a novel proteoglycan in the context of CVD, emerged as a key factor within the ECM matrix of atherosclerotic lesions, marking osteogenic transformation. Matrix bound proteins can be utilized as biomarkers following their release into circulation by tissue erosion or injury, commonly occurring in late-stage atherosclerotic lesions. Moreover, the specific biological functions of extracellular PRG4 could be translated towards clinical application. Pre-clinical studies have already confirmed disease mediating capacities of recombinant PRG4 in osteoarthritis *via* reconstitution of joint lubrication and protection from articular cartilage degeneration²⁹⁸. Additionally, it has been shown to modulate cellular responses by direct binding to TLR 2 and 4 as well as CD44 receptors²⁹² and is a substrate for proprotein convertase subtilisin/kexin type 6 (PCSK6)³³⁹, a protease more recently identified as key mediator of SMC migration, vascular and cardiac remodeling³⁴⁰⁻³⁴². It is likely that the modulating effects of rhPRG4 on SMC proliferation, migration and gene expression (described in *study III*) are driven by direct interaction, possibly manipulated by post-translational modification, warranting more detailed investigation of mechanistic pathways *in vitro*. Moreover, *in vivo* disease intervention models on conditional PRG4 gene-trap mice should

investigate the potential of PRG4 to prevent vascular pathologies, improve vascular healing and reduce risk of complications after interventions like stenting or aneurysm repair. As rhPRG4 treatment of dry eye disease is already subject of a phase 2 clinical trial (LubrisBiopharma), positive pre-clinical findings can be expected to find clinical translation more rapidly in future.

Importantly, the studies presented in this thesis are available to the public in peer-reviewed journals and the results have been largely disseminated and shared with the scientific community through posters and oral presentations at international conferences, symposiums, and other meetings. In addition to outlining several important directions towards improved CVD diagnostics, risk prediction and novel pharmacological approaches, this thesis may stimulate a deeper understanding and interdisciplinary discussions regarding VC formation and its clinical implications.

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To me, research is more than a hobby or even a profession, it is a lifestyle. Choosing to embark on a PhD gives the curious mind carte blanche to question traditional concepts and contribute new perspectives. While pointing at scientific holes may still look like an easy game for restless minds, getting to the bottom of them and emerge with answers requires determination, resilience and remarkable people backing your journey. This is to everyone whom I had the honor to meet, be inspired, supported and encouraged by during these last four years of asking questions and searching for answers.

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Illustrations created using BioRender

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9 CURRICULUM VITAE

About the author:

Till Seime was born on January 30th, 1989 in Saalfeld (Saale), Germany. He grew up in Jena, Germany, where he completed his secondary education at the Carl-Zeiss-Gymnasium, a high school specialized on natural sciences, following his childhood dream to become a researcher. After an internship at the Max-Planck-Institute of Biogeochemistry, he moved to Innsbruck, Austria, to study Engineering-, Environmental- & Biotechnology at the Management Center Innsbruck (MCI), with a focus on the utilization of algae for molecular farming. Till completed his Bachelor's degree including an exchange semester at the Indian Institute of Technology in Madras (IITM), Chennai, India, in 2012 and proceeded at MCI with the Master's program in biotechnology. Instead of a career plan in pharmaceutical industry, he decided to focus on medical and regenerative research and completed his master's degree 2014 at the Orthopedic Research and Biotechnology Unit, Children's Hospital Westmead, Sydney, Australia, specializing in bone regeneration and fracture repair under the supervision of Assoc. Prof. Aaron Schindeler. Thereafter, he returned to Innsbruck to work for two years as a biomedical scientist in molecular oncology at the Daniel Swarovski Research Laboratory, Department of Visceral-, Transplantation- and Thoracic Surgery, supervised by Prof. Jakob Troppmair. In 2017, he was accepted for PhD studies at Karolinska Institutet, Stockholm, Sweden, as part of the EU Horizon 2020 *International Network for Training on Risks of Vascular Intimal Calcification and roads to Regression of Cardiovascular Disease* (INTRICARE) Marie-Curie program. His research has been focused on the role of smooth muscle cells in calcification of atherosclerotic plaques utilizing human bio-banks, primary human cell cultures, rodent *in vivo* models and *in silico* analysis. The PhD project was performed under the supervision of Prof. Ulf Hedin and Assoc. Prof. Ljubica Perisic Matic at Karolinska Institutet, Department of Molecular Medicine and Surgery, Division of Vascular Surgery as well as Prof. Leon J. Schurgers and Prof. Chris Reutelingsperger at Maastricht University, Department of Biochemistry, Cardiovascular Research Institute Maastricht, The Netherlands. The scientific output of this double degree project between Karolinska Institutet and Maastricht University is summarized in the presented thesis entitled "The role of smooth muscle cells in calcification of atherosclerotic plaques".

Publications:

Seime T, Akbulut AC, Liljeqvist ML, Siika A, Jin H, Winski G, van Gorp RH, Karlöf E, Lengquist M, Buckler AJ, Kronqvist M, Waring OJ, Lindeman JHN, Biessen EAL, Maegdefessel L, Razuvaev A, Schurgers LJ, Hedin U, Matic L. Proteoglycan 4 Modulates Osteogenic Smooth Muscle Cell Differentiation during Vascular Remodeling and Intimal Calcification. *Cells*. 2021 May 21;10(6):1276.

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Other manuscripts:

Seime T, van Wanrooji M, Karlöf E, Johansson S, Matic L, Gasser TC, Hedin U. Biomechanical assessment of macro-calcification in human carotid atherosclerosis and its impact on smooth muscle cell phenotype. *Manuscript*

Skenteris NT, **Seime T**, Witas A, Karlöf E, Wasilewski G, Heuschkel M, Jaminon A, Oduor L, Dzhanayev R, Kronqvist M, Lengquist M, Peeters F, Söderberg M, Hultgren R, Roy J, Maegdefessel L, Arnardottir H, Bengtsson E, Goncalves I, Quertermous T, Goettsch C, Stenvinkel P, Schurgers L, Matic L. Osteomodulin attenuates smooth muscle cell osteogenic transition in vascular calcification. *Clin Transl Med.* *In Revision*

Zhou Z, Collado A, Sun C, Tratsiakovich Y, Mahdi A, Winter H, Chernogubova E, **Seime T**, Narayanan S, Jiao T, Jin H, Alvarsson M, Zheng X, Yang J, Hedin U, Catrina SB, Maegdefessel L, Pernow J. Downregulation of Erythrocyte miR-210 Induces Endothelial Dysfunction in Type 2 Diabetes. *Diabetes.* *In Revision*

Presentations at international scientific conferences:

“Biomechanical assessment of carotid intimal macro-calcification and its impact on smooth muscle cell phenotype” (*Poster, Virtual*) European Atherosclerosis Society Congress (EAS), Helsinki, Finland, May 30th – June 2nd, 2021

“Proteoglycan 4 modulates osteogenic smooth muscle cell differentiation during vascular remodeling and intimal calcification” (*Oral, Virtual*) European Society for Vascular Surgery Translational Meeting (ESVS), Krakow, Poland, September 29th – October 2nd, 2020

“Proteoglycan 4 is a key factor in chondrogenic smooth muscle cell differentiation during vascular remodeling & intimal calcification” (*Poster, Virtual*) International Vascular Biology Meeting (IVBM), Seoul, South Korea, September 9th – 12th, 2020

“Proteoglycan 4 is implicated in osteo-chondrogenic smooth muscle cell differentiation during vascular remodeling and intimal calcification” (*Poster*) Atherosclerosis, Thrombosis and Vascular Biology Meeting (ATVB), Boston, USA, May 14th – 16th 2019

”Proteoglycan 4 is implicated in osteo-chondrogenic smooth muscle cell differentiation during vascular remodeling and intimal calcification” (*Oral*) Vascular Research Initiatives Conference (VRIC), Boston, USA, May 13th, 2019

“Macro-calcification associates to lesion-stabilization in advanced carotid plaques” (*Poster*) International Vascular Biology Meeting (IVBM), Helsinki, Finland, June 3rd – 7th, 2018

Grants:

Svenska Sällskapet för Medicinsk Forskning (SSMF) travel grant, 2020: “Investigation of Proteoglycan 4 as a mediator of inflammation and micro-calcification in atherosclerosis”

Hjärt-Lungfonden (HLF) travel grant, 2019: “Proteoglycan 4 is implicated in osteo-chondrogenic smooth muscle cell differentiation during vascular remodeling and intimal calcification”

Karolinska Institutet (KI) travel grant, 2018: “Proteoglycan 4 is associated to calcification in advanced atherosclerotic plaques”

Hjärt-Lungfonden (HLF) travel grant, 2018: “Macro-calcification associates to lesion-stabilization in advanced carotid plaques”