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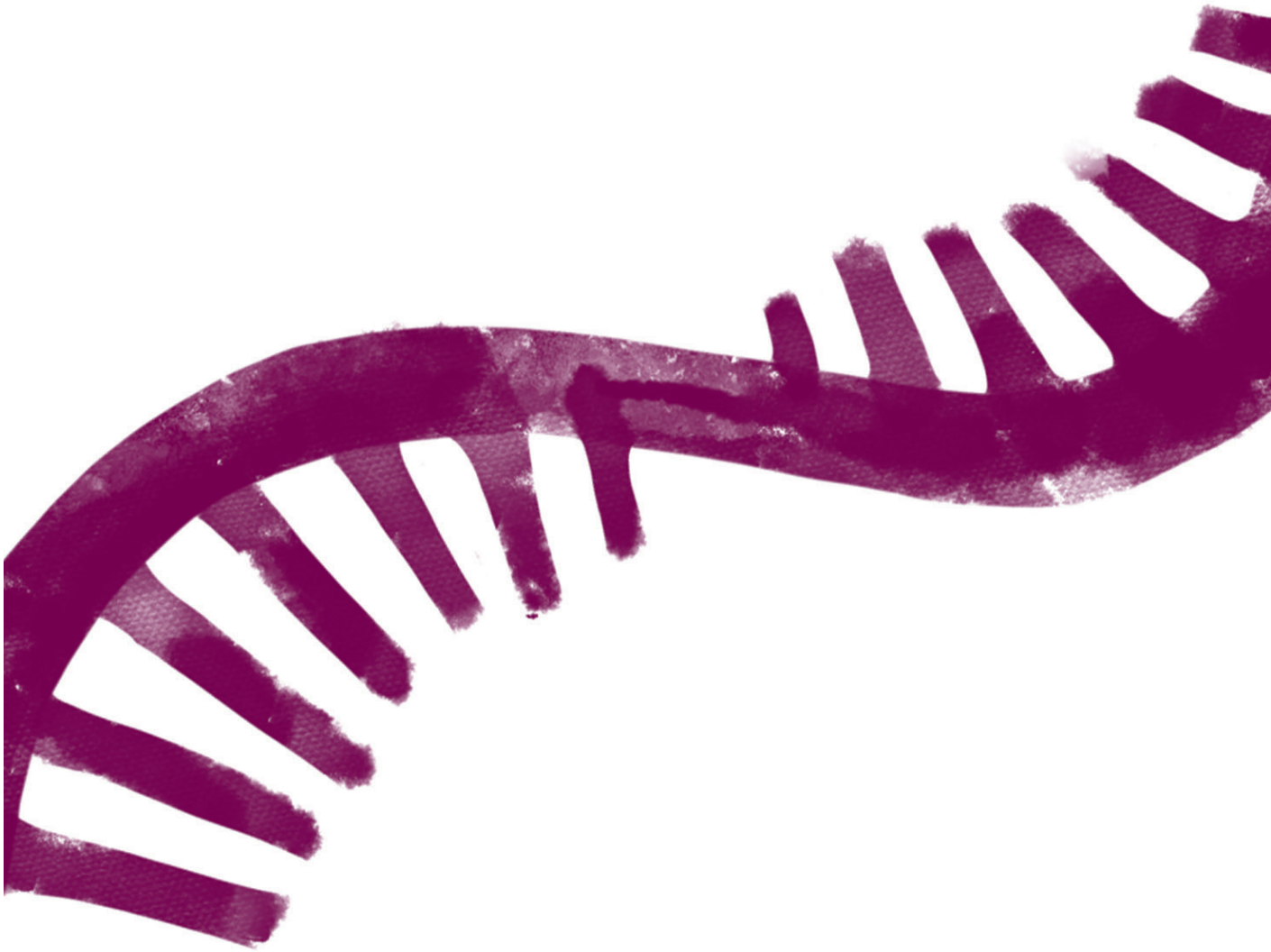
Non-coding RNA-based therapeutics and biomarkers for treatment and detection of vascular disease



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NON-CODING RNA-BASED THERAPEUTICS AND BIOMARKERS FOR TREATMENT AND DETECTION OF VASCULAR DISEASE

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To my grandparents, for their unconditional love
and for inspiring me with their limitless curiosity.

Moim Dziadkom, za ich bezwarunkową miłość
i nieustanne inspirowanie mnie ciekawością ludzi i świata.

ABSTRACT

Cardiovascular disease (CVD), with atherosclerosis as its main underlying pathology, is the most prominent cause of death worldwide. Progression and rupture of atherosclerotic plaques lead to potential adverse pathological events such as myocardial infarction and stroke. Although largely successful, primary and secondary prevention strategies have thus far been insufficient in minimizing the vast consequences of atherosclerotic disease progression on global health. Abdominal aortic aneurysm (AAA) disease shares a similar risk profile with atherosclerosis. A consequence of undiagnosed AAAs can be their subsequent rupture, which up to 90% of patients will not survive.

In both atherosclerosis and AAA, treatment and prevention are complicated by the fact that they progress silently and rarely lead to significant health impacts in their early stages. In addition, different pathological processes are known to be of importance as the diseases progress. These are also affected by patient-specific genetic and environmental risk factors. It would therefore be of benefit to find better ways of stratifying patient-specific disease risk and develop novel treatments. In the past decades, non-coding RNAs have emerged as powerful disease regulators in CVD and have been implicated as disease biomarkers in several research fields. In this thesis, we have sought to: (1) identify novel long non-coding RNAs (lncRNAs) involved in late-stage atherosclerotic disease and AAA, (2) establish techniques of their targeted delivery to affected vasculature, and (3) identify novel microRNA biomarkers of AAA with direct roles in disease development and progression.

In **study I,** we have identified lncRNA *MIAT* as a novel regulator of vascular smooth muscle cell (VSMC) dynamics in carotid atherosclerotic disease, with positive effects on their survival – a beneficial trait in late-stage disease. Its effects on earlier disease stages were however detrimental through regulation of VSMC phenotypic switching into macrophage-like phenotypes and through regulation of macrophage-specific processes. In **study II**, we identified the lncRNA *NUDT6,* the natural antisense transcript of *FGF2*, to be up-regulated in fibrous caps of vulnerable vs stable plaques. *NUDT6* was also up-regulated in AAA vs control aortic tissues. In experimental animal models of atherosclerosis and AAA, *FGF2* de-repression by the way of *NUDT6* inhibition had a beneficial effect on disease phenotypes and was successful in limiting the progression of these diseases. In **studies II and III**, we successfully used drug-eluting balloons to deliver therapeutics to the abdominal aorta of the translational mini-pig model of AAA. In addition, in **study III**, we observed beneficial effects of lenvatinib (VEGF-signaling inhibitor) on experimental AAA disease phenotype through positive effects on VSMC contractility and decreased diameter growth. Finally, in **study IV**, we identified miR-15a-5p as a novel disease biomarker of AAA. We showed miR-15a-5p to be relevant in AAA pathogenesis through its ability to modulate VSMCs into more inflammatory phenotypes, and its inhibition was able to limit experimental murine AAA diameter growth.

In conclusion, our studies not only confirm that non-coding RNAs are promising targets for treatment of CVD, but also underline the translational feasibility of their use.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts:

* = *shared first authorship*

I. **Long non-coding RNA *MIAT* controls advanced atherosclerotic lesion formation and plaque destabilization.**
Fasolo F*, Jin H*, Winski G*, Chernogubova E, Pauli J, Winter H, Li DY, Glukha N, Bauer S, Metschl S, Wu Z, Koschinsky M, Reilly M, Pelisek J, Kempf W, Eckstein HH, Soehnlein O, Matic L, Hedin U, Bäcklund A, Bergmark C, Paloschi V, Maegdefessel L. *Circulation.* 2021;144:1567–1583.

II. **Inhibition of long non-coding RNA *NUDT6* enhances smooth muscle cell survival during vascular disease development.**
Winter H, Winski G, Chernogubova E, Busch A, Bäcklund A, Pauli J, Paloschi V, Rykaczewska U, Soehnlein O, Eckstein HH, Matic L, Hedin U, Boon RA, Jin H, Maegdefessel L. *Manuscript.*

III. **Lenvatinib halts aortic aneurysm growth by restoring smooth muscle cell contractility.**
Busch A, Pauli J, Winski G, Bleichert S, Chernogubova E, Metschl S, Winter H, Trenner M, Wiegering A, Otto C, Fischer J, Reiser J, Werner J, Roy J, Brostjan C, Knappich C, Eckstein HH, Paloschi V, Maegdefessel L. *JCI Insight.* 2021;6(15):e140364.

IV. **microRNA-15a-5p regulates progression of abdominal aortic aneurysms and can be utilized as a circulating biomarker of disease prevalence and phenotype.**
Winski G, Chernogubova E, Busch A, Eken S, Jin H, Lindquist Liljeqvist M, Khan T, Bäcklund A, Paloschi V, Roy J, Hultgren R, Brostjan C, de Boorst GJ, Sachs N, Eckstein HH, Boon RA, Spin JM, Tsao PS, Asselbergs FW, Maegdefessel L. *Manuscript.*

RELATED PUBLICATIONS

Proteoglycan 4 Modulates Osteogenic Smooth Muscle Cell Differentiation during Vascular Remodeling and Intimal Calcification.
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.
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Tsantilas P, Lao S, Wu Z, Eberhard A, Winski G, Vaerst M, Nanda V, Wang Y, Kojima Y, Ye J, Flores A, Jarr KU, Pelisek J, Eckstein HH, Matic L, Hedin U, Tsao PS, Paloschi V, Maegdefessel L, Leeper NJ. *Cardiovasc Res.* 2021 Jan 20; cvab014. Online ahead of print.

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MicroRNA-210 enhances fibrous cap stability in advanced atherosclerotic lesions.
Eken SM, Jin H, Chernogubova E, Li DY, Simon N, Sun C, Winski G, Busch A, Bäcklund A, Österholm C, Razuvaev A, Renné T, Eckstein HH, Pelisek P, Eriksson, González Díez M, Perisic Matic L, Schellinger IN, Raaz U, Leeper NJ, Hansson GK, Paulsson-Berne G, Hedin U, Maegdefessel L. *Circ Res.* 2017;120:633–644.

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LIST OF ABBREVIATIONS

α-SMA	Alpha smooth muscle actin
AAA	Abdominal aortic aneurysm
AngII	Angiotensin II
APOE	Apolipoprotein E
ASO	Antisense oligonucleotide
BAPN	ß-aminopropionitrile
BiKE	Biobank of Karolinska Endarterectomies
CEA	Carotid endarterectomy
CVD	Cardiovascular disease
DEB	Drug-eluting balloon
EC	Endothelial cell
ECM	Extracellular matrix
ECST	European Carotid Surgery Trial (clinical trial)
EVAR	Endovascular aortic repair
FFPE	Formalin-fixed, paraffin-embedded
FISH	Fluorescent in situ hybridization
GSEA	Gene set enrichment analysis
HDL	High-density lipoprotein
ILT	Intraluminal thrombus
ISH	In situ hybridization
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
lncRNA	Long non-coding RNA
MIAT	Myocardial Infarction Associated Transcript
miRNA	MicroRNA
miR-15a	miRNA-15a-5p
MMP	Matrix metalloprotease
MVB	Munich Vascular Biobank

NASCET	North American Symptomatic Carotid Endarterectomy Trial Collaborators (clinical trial)
NAT	Natural antisense transcript
ncRNA	Non-coding RNA
ORA	Overrepresentation analysis
oxLDL	Oxidized low-density lipoprotein
PPE	Porcine pancreatic elastase
RBC	Red blood cell
ROS	Reactive oxygen species
RT	Reverse transcriptase
SMART	Second manifestations of ARTerial disease (cohort)
SMC	Smooth muscle cell
SM-MHC	Smooth muscle myosin heavy chain
StAAAB	Stockholm AAA Biobank
TIMP	Tissue inhibitor of matrix metalloproteinase
UTMD	Ultrasound targeted microbubble destruction
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell

1 LITERATURE REVIEW

1.1 INTRODUCTION TO VASCULAR DISEASE

Cardiovascular disease (CVD) is the most common cause of death in developed countries [2, 3]. CVD-related death amounts to 45% of deaths in Europe and approximately every third death worldwide [3]. CVD risk factors are widely known and include hypertension, hyperlipidemia, smoking, and type II diabetes [4, 5]. During the second half of the 20th century, it became increasingly known that CVD is largely driven by modifiable risk factors, and the focus for the treatment of CVD has shifted towards preventive measures among risk groups [6]. Atherosclerotic disease is responsible for a vast majority of CVD. Among these are coronary artery disease, cerebrovascular disease (e.g. stroke), and other diseases of the arteries (including aortic aneurysms) [7].

1.2 VASCULAR WALL BIOLOGY

The vascular wall consists of three layers ('*tunicas*'): intima, media, and adventitia (**Figure 1**). The intimal layer consists of endothelial cells (ECs) attached to the basal lamina, supported by the internal elastic lamina. The medial layer, rich in smooth muscle cells (SMCs) and elastin, is located between the internal and external elastic lamina. Medial elastin forms sheets (lamellae) between which proteoglycan-rich extracellular matrix (ECM) and SMCs reside. The outermost, adventitial layer, attached to the media through an external elastic lamina, consists mainly of a collagen-rich ECM produced by its inherent fibroblast cells. [8]

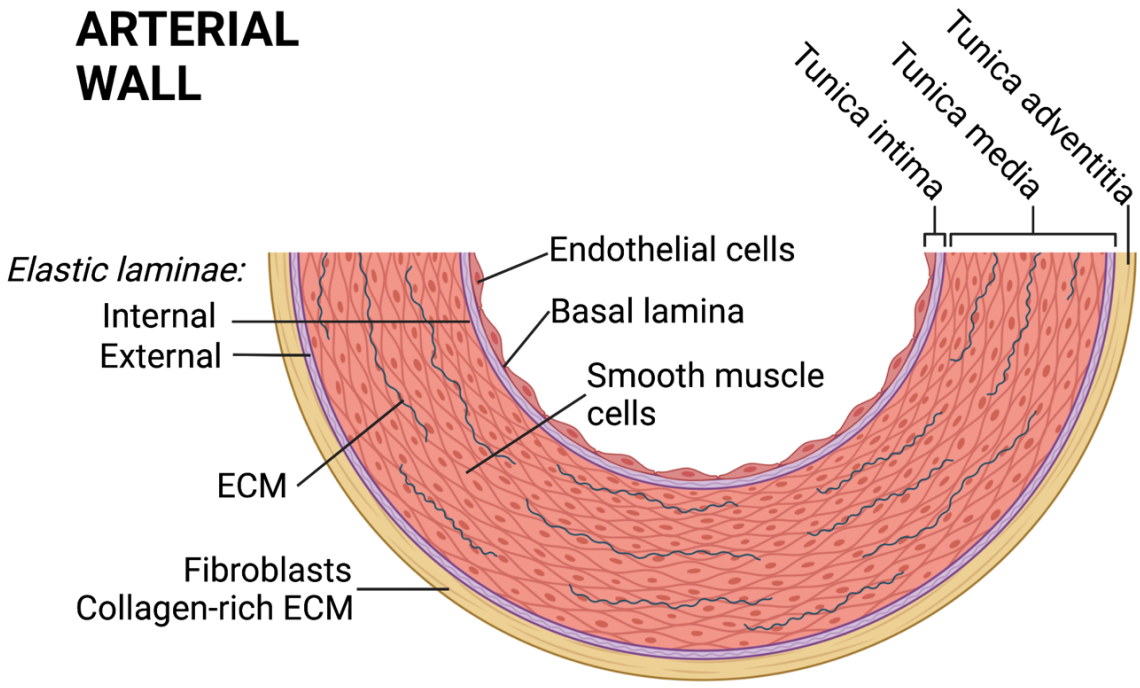

Figure 1. Basic histology of the arterial wall. *Illustration by the author.*

The elastic properties of the human arterial tree have evolved due to the pulsatile nature of human heart function. Without elastic arteries, the strain on the heart in systole would be significantly increased. Meanwhile, blood pressure would fall rapidly in diastole with subsequent flow decrease in distal arterioles. The elastic properties of the ECM enable storage of energy in the aortic wall during systole and later, through elastic recoil, discharge this energy in diastole. This physiological phenomenon ensures a more uniform flow throughout the cardiac cycle. Collagen and elastin, produced and deposited by SMCs, are the main components giving the ECM its mechanical properties. Upon intraluminal pressure increase, elastin allows the arterial wall to expand. At physiological pressure levels, only a small minority of collagen fibers are recruited. The fibers only become active upon further increased wall stretch, gradually reducing the elastic properties, yielding a tensile strength in the arterial wall. Other key components of the ECM are proteoglycans, which attract water and give resistance to compression, and glycoproteins which take part in ECM organization and act as anchors for the attachment of its cells. The ECM also allows for the binding and retention of molecules secreted by its inherent cells or delivered through circulation. These molecules, attracted and bound to proteoglycans, include growth factors, proteases, protease inhibitors, cytokines, and lipoproteins. [8, 9]

1.3 ATHEROSCLEROSIS

The pathogenesis of atherosclerosis consists of lesion formation, their slow progression, and subsequent luminal narrowing [9]. The development of these lesions, referred to as *plaques*, is known to begin early in life and develop slowly over the course of up to 50 years. Risk factors such as hypertension, hyperlipidemia, smoking, obesity, diabetes, and age are associated with increased prevalence and severity of atherosclerotic disease [10]. Atherosclerosis is driven by a progressive process of cholesterol-rich lipid accumulation, their oxidization, modification, and subsequent inflammatory response. While early-stage lesions can sometimes regress, intermediate and advanced lesions are of a chronic, progressive nature [11]. Different stages of atherosclerotic plaque progression are summarized in **Figure 2**.

Development of '*fatty streaks*', precursors of atherosclerotic lesions, starts in the innermost layer of the artery wall, the *intima*, through retention of low-density lipoprotein (LDL) particles from circulating blood. This process begins in childhood and early adolescence [11]. It commonly occurs at sites with disturbed flow ('*atheroprone regions*'), such as branch points [12]. It was long believed that upon oxidative modification due to the presence of reactive oxygen species (ROS) or oxidizing enzymes, these oxidized LDL (oxLDL) particles trigger an inflammatory response. However, recently the scientific consensus has started to move away from oxLDL presence being necessary for atherosclerosis initiation [13], which may as well be mediated through the sole presence of non-modified native LDL or LDL aggregates. Intimal inflammation leads to activation of ECs, through several mechanisms, including upregulated expression of adhesion molecules, whereas SMCs contribute to this process by secreting chemokines and ECM components. Consequently, this leads to infiltration by immune cells – monocytes, lymphocytes, mast cells, and neutrophils. Monocytes transform into macrophages,

which, once activated through scavenger receptors in the presence of oxidized/native LDL, take up cholesterol droplets and form '*foam cells*' [10, 11]. While the majority of foam cells were long thought to be solely of macrophage origin, recently, much focus has been put on the fact that vascular SMCs (VSMCs) can also become lipid-laden [14]. In response to cholesterol uptake, VSMCs can undergo a phenotypic shift, thereby losing their traditional contractile or synthetic phenotypic features and instead assuming the appearance of macrophage-like foam cells [15, 16].

Further, LDL accumulation and chronic inflammation promote cell apoptosis and lead to lesions with histopathologically evident '*necrotic cores*'. These more advanced atherosclerotic lesions, called '*fibro-atheromatous plaques*', contain a subendothelial fibrous cap, composed of SMCs and collagen [17]. Further atherosclerotic stages are characterized by fibrous cap thinning, which makes them increasingly susceptible to rupture [18]. Through almost all disease stages, atherosclerotic plaques remain clinically silent. They only manifest themselves when their severity directly impedes blood flow, or through rupture of the fibrous cap, which leads to thrombus formation and either full or partial flow obstruction [19]. In both situations, the limited blood supply deprives the tissues of oxygen, leading to potential organ damage and dysfunction [11].

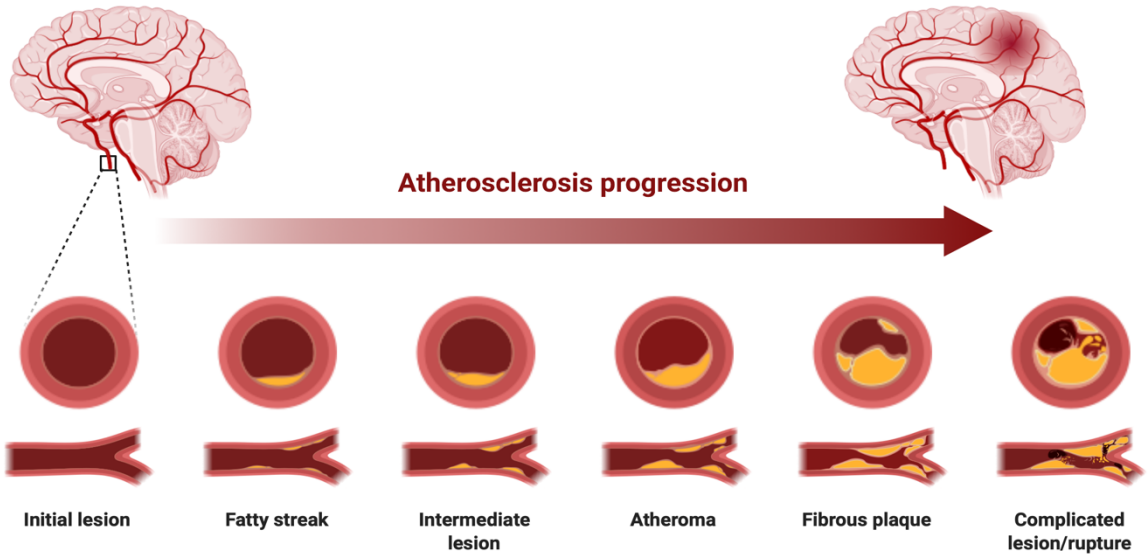

Figure 2. Progression of atherosclerosis and characteristics of different stages of the disease. *Illustration by the author.*

1.3.1 Carotid artery disease and stroke

Stroke affects one in four people over their lifetime. Being the second leading cause of death and the third leading cause of disability in adults [20], it is one of the major diseases of the cardiovascular system with often dire consequences. It accounts for close to 5% of all disability-adjusted life-years lost [21] and 10% of all deaths worldwide [22]. Ischemic stroke is approximately twice as common as hemorrhagic stroke [20], and is defined as *"an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction"* [23]. The leading cause of these infarctions is an occlusion of a cerebral artery [24], which quickly leads to irreversible tissue damage [25]. While other ischemic diseases such as myocardial infarction

are caused by *in situ* plaque rupture leading to thrombosis and artery occlusion, ischemic stroke is most commonly embolic – either cardioembolic (due to atrial fibrillation or valvular heart disease) or atheroembolic (due to atherosclerotic disease in either carotid or vertebral arteries) [26]. Approximately 15 to 20% of ischemic strokes can be attributed to atherosclerotic carotid disease [27, 28].

Like other atherosclerotic plaques, carotid plaques can differ in degree and vulnerability [29]. Carotid stenosis is defined as a narrowing of the vessel lumen due to atherosclerotic plaque build-up, where progressive narrowing has been associated with increased risk of future ischemic stroke [30–33], although mainly in previously '*symptomatic*' patients. Carotid stenosis is considered symptomatic upon simultaneous presentation with either stroke, transient ischemic attack (temporary period of symptoms similar to those of a stroke), retinal emboli, or amaurosis fugax (temporary unilateral vision loss caused by decreased blood flow to the retina) [34].

To limit the risk of future stroke, the plaque can be excised surgically through carotid endarterectomy (CEA) or stented (carotid artery stenting). CEA, where the plaque in the carotid artery is excised, is considered the gold standard treatment [35]. As is true for all surgical decision-making, the risk of the surgery needs to be weighed against the risk of future events, and in this case, the risk of stroke. Surgical treatment alone is not sufficient, and all patients without contraindications are treated with antiplatelet therapy, statins, and are recommended smoking cessation [29, 35]. In the 1990s, two large randomized control trials were performed to understand which patients with symptomatic carotid artery disease would benefit from undergoing CEA – European Carotid Surgery Trial (ECST [36]) and North American Symptomatic Carotid Endarterectomy Trial Collaborators (NASCET [37]). One significant difference between the studies was the method of stenosis quantification – NASCET compared the stenosed diameter to a distal, healthy part of the carotid artery, whereas ECST compared it to the full diameter of the artery at the stenosis site. The studies resulted in recommendations for CEA in patients with >80% (ECST) or >50% (NASCET) stenosis grades (50% NASCET being equivalent to approximately 75% ECST). Current clinical guidelines refer to the NASCET classifications, recommending CEA in patients with ≥70% stenosis and suggesting it at 50-69% [38, 39].

Asymptomatic carotid stenosis can in a limited number of cases be identified during routine examination (through the presence of an audible carotid murmur), but more commonly incidentally through radiologic imaging of the head and neck area [40]. These patients are also recommended medical treatment. However, the role of CEA in treatment of asymptomatic carotid stenosis is controversial, partly because the selection of patients, with expected benefit from surgery larger than the peri-surgical risk, is difficult [41]. Consensus, reflected in current clinical guidelines, is that the degree of carotid stenosis in these patients is not as clearly connected to future risk of stroke as it is in symptomatic patients [42]. However, this question is still lively debated and recent studies show that the surgical benefits in asymptomatic patients with severe carotid stenosis might have been underestimated [42].

1.3.1.1 Plaque vulnerability and rupture

> ***"The major clinical consequences of atherosclerosis such as myocardial infarction or stroke are not a function of gradual narrowing of the lumen, but rather due to thrombotic events associated with acute rupture or erosion of an unstable plaque."*** (Bennett *et al. Circ Res* 2016) [43]

In past decades, vast efforts have been made to better understand which plaques contribute to adverse events such as myocardial infarction or stroke [44]. These events can in most cases be attributed to a thrombotic event due to rupture (65%) or erosion (30%) of an atherosclerotic plaque [43]. Remaining 5% are due to the presence of calcified nodules protruding through the fibrous cap of the lesion [38, 39]. In plaque rupture, a fissure in the fibrous cap exposes the highly thrombogenic plaque core to circulating blood, triggering formation of a thrombus [40]. Plaque erosion pertains to a local desquamation of the endothelial cell layer, triggered by changes in endothelial sheer stress due to changed flow conditions, and subsequent formation of neutrophil extracellular traps and thrombosis [41]. In absence of sufficient collateral circulation, the resulting occlusion or significant reduction of arterial lumen, either *in situ* or downstream through embolization, leads to ischemic tissue damage and often irreversible injury [49, 50]. '*Vulnerable*' or '*unstable*' plaques are defined as *"thrombosis-prone plaques and plaques with a high probability of undergoing rapid progression"* [42]. These plaques are characterized by features such as: thin fibrous cap poor in collagen covering a large necrotic core, fibrous cap rich in macrophages and few SMCs, neovascularization, and presence of intraplaque hemorrhage and spotty calcification [19]. Given that VSMCs have been implicated in most of these processes, they are intriguing candidates for development of novel therapeutics for stabilization of atherosclerotic plaques. Further, development of better risk stratification and optimized medical treatment strategies could provide new alternatives to CEA in certain patient groups.

1.3.2 Novel insights into the molecular landscape of atherosclerosis development and progression

The above-described mechanisms constitute the 'traditional' view of atherosclerosis etiology and development, which over the past decades has been progressively refined and modified to better reflect insights from studies using novel methods and exploring new ideas [13]. While the mechanisms of initial insults leading to the development of atherosclerotic plaques are still lively debated, the subsequent increase in immune cell infiltration within atherosclerotic plaques is considered a key event [49]. It is therefore understandable that for a long time the prevailing view had been that these externally recruited immune cells were the main contributors to the highly inflammatory milieu that characterizes advanced stages of atherosclerotic disease [51, 52]. Similar bias, based on histological images of human plaques, where high SMC content had been associated with more stable plaque phenotypes, drove the conclusion that proliferating SMCs, migrating from the medial layer of the artery into the intima, had solely a stabilizing role on plaques [51]. This unfortunate 'guilt by association' view has at least in part been caused by methodological limitations. As experimental methods

are refined, it allows us to challenge certain conclusions once considered evidence. This is very much the case when it comes to the role that VSMCs play in the development and progression of atherosclerotic plaques.

Differentiated VSMCs of the medial layer of healthy arteries are of a contractile phenotype, characterized by the expression of contractile proteins such as SM-MHC (smooth muscle-myosin heavy chain) and α-SMA (alpha smooth muscle actin) [53]. It has long been known that upon stimulation (in response to stress), for example by PDGF-BB, VSMCs can de-differentiate into a proliferative, '*synthetic*' phenotype, characterized by increased synthesis of ECM-proteins [53]. As VSMCs are characterized by their expression of contractile proteins, immunostaining and subsequent histological examination is the common method of spatially visualizing the location of VSMCs. However, this approach risks losing track of de-differentiated VSMCs that have lost the expression of typical contractile proteins. The majority of VSMCs are localized within the medial layer, but under pathological conditions they can also be found within the intima [54]. Experimental insight into how VSMCs end up in the intimal layer, which involves crossing the internal elastic membrane of the vessel wall, had long been considered elusive [53]. The long-prevailing view, first suggested by Wisler in 1968, was that VSMCs proliferate and migrate from the medial into the intimal layer of the lesion [55].

Recent studies, made possible by the rapid advancement of single-cell sequencing and lineage-tracing technologies, have challenged some of these 'traditional' views on VSMCs, and show that the temporal changes and the de-differentiation process of these cells to be much more complex than originally thought [56]. These studies confirmed that it is indeed almost exclusively local medial VSMCs that give rise to VSMC populations within the plaque [57], and showed that they do so in an oligoclonal fashion. In fact, up to 30-70% [56] of cells in experimental plaques are now believed to be of VSMC origin, and these populations seem to originate from only a small number of differentiated VSMCs [57–59].

1.3.3 VSMC phenotypic switching

As mentioned above, the differentiated VSMCs within the medial layer are of a contractile and non-proliferating phenotype. Differentiation and maintenance of the contractile phenotype is under control of MYOCD, which together with SRF forms a complex able to bind to the '*CArG box*', a DNA sequence expressed in promoters of contractile genes considered key markers for contractile phenotype [60, 61] such as *MYH11* (encoding for SM-MHC), *ACTA2 (*encoding for α-SMA), *CNN1* and *CALD1*. Promoters of contractile genes have also been shown to be under negative control of G/C-rich repressor elements [62, 63], and TGF-β control elements [64]. Phenotypic switching refers to de-differentiation and/or trans-differentiation of VSMCs into non-contractile phenotypes. Contractile VSMCs are responsible for many of the essential auto-regulatory capabilities of muscular arteries and arterioles, and for maintaining their surrounding environment by secretion of ECM components [65]. Different phenotypes that VSMCs have been described to trans-differentiate into are summarized in **Figure 3** and further discussed in the sections below.

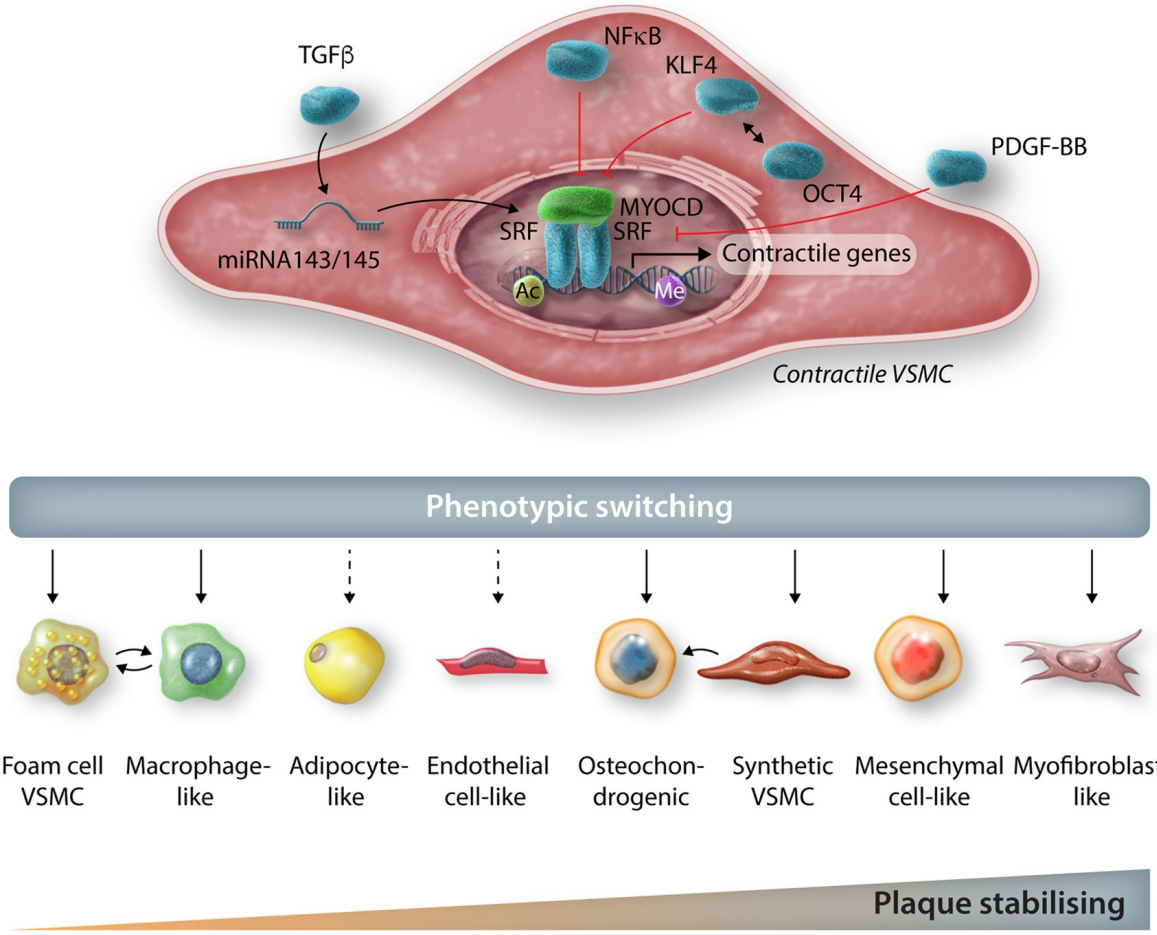

Figure 3. Vascular smooth muscle cell (VSMC) phenotypes and their proposed role in plaque stability. Foam cell and macrophage-like VSMCs have predominantly negative effects. Osteochondrogenic and synthetic VSMCs are context dependent and play both beneficial and detrimental roles. Mesenchymal cell-like and myofibroblast-like VSMCs are mostly considered protective. EC-like and adipocyte-like VSMCs have been described, but evidence on their roles in human atherosclerosis is still insufficient. *Reprinted with permission from Grootaert & Bennett, Cardiovascular Research 2021* [56].

1.3.3.1 Maintenance of VSMC contractile phenotype

Given the key role of MYOCD in regulating contractile gene expression, much of phenotypic regulation has been described to occur via regulation of the aforementioned MYOCD-SRF-CArG mechanism. KLF4, a key mediator of VSMCs phenotypic switching, has been described to interfere with SRFs ability to bind to MYOCD and interfere with the ability of MYOCD-SRF to bind to the CArG-box of contractile genes [66]. In cooperation with p-ELK1, it can bind to the G/C repressor elements and recruit HDAC2, leading to epigenetic silencing via deacetylation of chromatin [67, 68]. p-ELK1, phosphorylation of which is activated by PDGF-BB, is also an independent repressor of contractile phenotype as it competes with MYOCD for the same binding site on SRF [69]. KLF4 is not expressed in healthy differentiated VSMCs, but its expression can be activated by PDGF-BB through activation of Sp1, which possesses multiple binding sites on the KLF4 promoter [70]. Recent studies have revealed a possible equally important role of OCT4 in VSMC phenotypic regulation, with it having strikingly opposite roles to KLF4 in experimental models of atherosclerosis [71, 72]. Other factors described to repress MYOCD are the p65 subunit of NFκB [73], FOXO3a [74], and TCF21 [75].

1.3.3.2 Synthetic and myofibroblast-like VSMCs

Synthetic VSMCs are characterized by the loss of contractile marker expression and an increase in synthetic, migratory, and proliferative capabilities. This phenotypic switch is relevant during all stages of atherosclerosis [76, 77]. The synthetic phenotype switch was early described to be required for the ability of the arterial vessel wall to react to injury and, through increased production of ECM components, either restore its integrity or limit irreparable damage [78, 79]. While re-differentiation of these synthetic VSMCs has been shown in medial VSMCs after resolution of vascular injury [53], the de-differentiated VSMCs that have migrated to the intima seem to retain their synthetic properties on a more permanent basis, likely through continued stimulation due to the largely irreversible nature of atherosclerotic disease progression [56].

Recently, a single-cell RNA sequencing study of modulated VSMCs by Wirka *et al*. characterized a cluster of myofibroblast-like (or '*fibromyocyte*') VSMCs [80]. These were defined by down-regulation of VSMC differentiation markers *Tagln*, *Cnn1* and up-regulation of *FN1*, *Tnfrsf1b* (osteoprotegerin), as well as otherwise fibroblast-specific *Lum*, *Dcn*, *Bgn*. Further, they showed that this phenotypic switch was under the control of *Tcf21*, and its loss inhibited this phenotypic transition. During embryogenesis, TCF21 is known to control the divergence of coronary VSMCs and cardiac fibroblast lineages, and expression of TCF21 seems to be required for fibroblast development [81]. Interestingly, while TCF21 expression levels are low in differentiated VSMCs, lineage-traced VSMCs within experimental lesions start to express *Tcf21* in early stages, to thereafter localize mainly to the fibrous cap in advanced lesions [82]. In addition, *TCF21* expression is increased in fibrous caps of stable lesions compared with ruptured plaques [82]. Thus, it remains to be seen whether the protective role previously assigned to '*synthetic*' VSMCs may in fact be mediated through the presence of these myofibroblast-like VSMCs.

1.3.3.3 Macrophage-like and foam cell-like VSMCs

Under certain conditions, VSMCs can acquire a macrophage-like phenotype which can be found in both experimental and human atherosclerotic plaques [68, 83]. Upon cholesterol loading, cultured VSMCs up-regulate macrophage-specific inflammatory cytokines [68, 84], increase phagocytic behavior [84, 85], and decrease expression of SRF and MYOCD [84], responsible for maintenance of contractile phenotype. This transition is further characterized by loss of contractile markers and gain of macrophage markers such as CD68 and LGALS3 [85]. Further, cholesterol loading of VSMCs has been shown to limit their ability to synthesize ECM-proteins [86], otherwise one of the main plaque-stabilizing effects of stimulated, synthetic VSMCs [87]. The pluripotency factor KLF4 is heavily involved in the regulation of VSMC trans-differentiation into this macrophage-like state [68].

The traditional view of a foam cell is of a macrophage that has undergone phagocytosis of LDL particles. While it was widely accepted that VSMCs exposed to LDL deposits can become lipid-laden, the extent and relevance of this VSMC phenotype was unclear [88, 89]. Lineage-tracing studies have not only confirmed this effect to be of vast importance but also showed

that the majority of foam cells are in fact of VSMC origin [83, 90]. The exact mechanism for this phenomenon is not yet clear but could at least in part be explained by the fact that VSMC-derived foam cells express much lower levels of cholesterol exporter ABCA1 compared to foam cells derived from macrophages [83]. Cholesterol overload can in turn trigger VSMC apoptosis [91] and VSMC-like foam cells have been found to line the necrotic core of experimental plaques [84], originally thought to comprise of mainly lipids and macrophages.

While increasing evidence is pointing towards foam cell VSMCs, due to their limited capacity to clear cholesterol compared to their macrophage-derived counterparts, playing detrimental roles in atherosclerosis, the net impact of macrophage-like VSMCs is not entirely clear [56]. Even though these cells express macrophage-specific markers, recent evidence points towards the fate of these cells not being completely sealed. Firstly, this phenotype can be experimentally reversed *in vitro* through '*lipid unloading*', by providing the cells cholesterol acceptors such as Apolipoprotein A1 and HDL (high-density lipoprotein) [84]. Secondly, even though macrophage-like VSMCs express macrophage-specific markers, their transcriptomes are vastly different from traditional macrophages [84]. Their closer resemblance has been noted to the Mox macrophage subtype [92], characterized by its lower phagocytic capacity compared to M1 and M2 macrophages [93]. Thirdly, expression of *Lgals3* does not necessarily need to signify a functional macrophage-like phenotype [71]. The recent study by Alencar *et al.* [71] shows that LGALS3 might instead be a marker of a '*transitional*' VSMC subtype. They show that the single-cell RNA sequencing cluster of this subtype, capable of subsequent progression into several different VSMC phenotypes, is the first one to acquire *Lgals3* expression along with *Ly6a/Sca1*. The latter has been proposed to be a marker of primed VSMCs in intermediate states ('*mesenchymal cell-like*' VSMCs), which in response to inflammation can give rise to distinct VSMC subtypes present in atherosclerotic plaques [94].

1.3.3.4 Osteochondrogenic VSMCs

Calcification of atherosclerotic plaques has historically been one of the signature characteristics of the disease. It has been used as a surrogate marker of atherosclerosis since the 1940s [95, 96] and coronary artery calcium score is still a relevant clinical aid to help predict the risk of a future atherosclerotic coronary event (i.e. myocardial infarction) [97]. Originally thought to be a passive and degenerative phenomenon, vascular calcification was described by the pathologist Virchow in 1858 [98], who observed due to the presence of bone structure-like features likened it to an "*ossification*". More than a century later, in 1983, Tanimura *et al.* [99] described VSMC-derived "*matrix vesicle-like structures*" to be involved in the calcification of atherosclerotic lesions. Today we know that vascular calcification is indeed a process driven largely by osteochondrogenic VSMCs and lineage-tracing studies have shown that a whole 98% of osteogenic cells in experimental plaques are VSMC-derived [100]. Secretion of membrane-bound matrix vesicles is among the mechanisms proposed for VSMCs-mediated calcification [60]. It should be noted that medial and intimal calcification are considered different diseases, where the former is connected to vascular stiffness, hypertension, and heart failure, and the latter to the atherosclerotic consequences of arterial obstruction and plaque

rupture [60]. VSMCs with osteochondrogenic phenotypes are thought to be detrimental in the pathogenesis of late-stage atherosclerotic plaques [71]. The transformation into osteochondrogenic VSMCs has been described to be under the control of the osteogenic transcription factor RUNX2 [101–103], and KLF4-dependent [104]. However, the exact trigger for this switch has not been entirely elucidated. Seemingly, several signaling pathways seem to be involved, among these Wnt/β-Catenin [105, 106], BMP2 [107, 108], and VEGF [109]. Overall, the process looks to be driven by changes in the surrounding environment during atherosclerotic plaque progression. VSMCs affected by ongoing inflammation, oxidative stress and apoptosis have been shown to contribute to intimal calcification [60]. Currently, it is unclear whether VSMCs can transdifferentiate into an osteochondrogenic phenotype directly or if they first need to de-differentiate into a synthetic [110, 111], or perhaps even the intermediary LGALS3-expressing phenotype [71] mentioned in the section above [56].

1.3.3.5 Other VSMC phenotypes

A number of other potential VSMC phenotypes have been described. In likelihood with the above-discussed phenotypes, they seem to be a result of VSMCs' response to their surrounding environment. When exposed to shear stress *in vitro*, VSMCs have been described to adapt an endothelial cell-like phenotype, expressing markers such as vWF, CD31, VE-cadherin [112], under the control of Notch-signaling [113]. In *in vitro* adipogenic conditions, VSMCs can also transform into an adipocyte-like phenotype, characterized by the expression of adipsin and leptin [114]. The relevance of these phenotypes, and whether they are present in human atherosclerotic disease is however unclear [56].

1.4 ABDOMINAL AORTIC ANEURYSMS

An arterial aneurysm is defined as a permanent, focal, full-thickness dilatation of an artery of more than 50% than its normal diameter. As the name suggests, abdominal aortic aneurysms (AAA) are located in the abdominal part of the aorta, and are the most common form of true arterial aneurysm, i.e. engaging the full-thickness of the arterial wall and its three layers: intima, media and adventitia [115]. Although the abdominal aorta has segments both above and below the renal arteries, AAAs preferably affect its infrarenal segment [116]. AAA-related death remains the 12-15th leading cause of death in USA, EU, and other European countries [117]. Risk factors include age, male sex, smoking, family history, and hypertension [118], while type 2 diabetes has been shown to associate with a decreased risk [119, 120]. AAAs reported prevalence varies widely [121], although data from recent ultrasonography-based screening studies report a prevalence of 1-2% in 65-year-old men and 0.5% in 70-year-old women [122–124]. AAAs are often described as 'silent killers', as they tend to be asymptomatic, and their diagnosis is often incidental or through systematic screening approaches [117]. The most feared consequence of AAA is its acute rupture, which carries a mortality of up to 90% [125, 126].

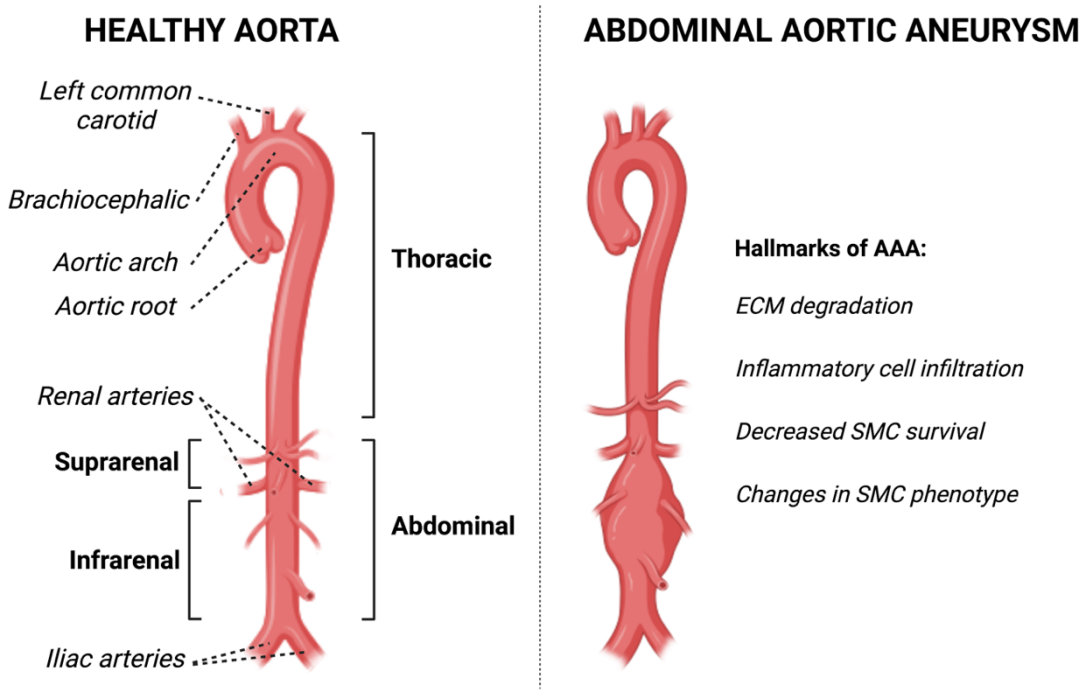

Figure 4. Anatomy of the aorta (left) and hallmark characteristics of human AAA (right). *Illustration by the author.*

1.4.1 Diagnosis and treatment

In clinical practice, an abdominal aorta with a diameter >30 mm is considered aneurysmal among men [127]. To date, there is no effective medical treatment for halting AAA progression or preventing acute rupture. Surgical repair can be performed, either through open surgery, with a prosthetic graft sewn into the aneurysmal part of the aorta, or through endovascular aortic repair (EVAR), where a stent graft is implanted into the aorta via luminal access through the femoral arteries. Compared to open surgery, EVAR is a considerably less invasive procedure, albeit not applicable to all patients, e.g. due to anatomical variations or certain comorbidities. Nonetheless, there still are significant peri-procedural and post-procedural risks such as: consequent endoleaks (incidence 20-50%; defined as "*Persistent blood flow in the aneurysmal sac after deployment of the stent graft*"), graft migration (up to 8%), and/or damage to the access arteries (9-15%). The risk of AAA rupture is positively correlated to the aneurysmal diameter, and size of >55mm is considered critical, as the risk of death due to rupture then exceeds the associated peri-surgical risk [118].

In some countries, screening programs have been put in place to identify previously undiagnosed and potentially fatal AAAs. Screening programs have repeatably been shown to reduce mortality due to rupture of undiagnosed AAA [128] in a cost-effective manner [125, 129, 130]. In Sweden, screening has been offered to 65-year-old men since 2006 and reached national coverage in 2015 [130]. However, many countries still struggle with broad implementation of screening (especially if they lack access to population-wide databases). As a consequence of decreases in the global prevalence of AAA, the efficiency of screening programs can be expected to follow.

1.4.2 Pathogenesis of AAA

The majority of AAA patients suffer from advanced atherosclerosis and the abdominal aorta is a common site of advanced atherosclerotic lesions. Further, atherosclerosis and AAA share several key risk factors, including smoking, age, male gender, and family history. Whether atherosclerosis itself participates in AAA pathogenesis is still controversial [131]. A recent meta-analysis was able to show associations between changes in lipid metabolism-related genes and AAA risk [132]. Atherosclerotic changes and associated chronic inflammation could be hypothesized to contribute, at least in part, to the mechanical integrity loss of the vascular wall [118]. However, a clear causal relationship between these diseases has not been established. In contrast, certain evidence points towards them developing in parallel [133]. In support of this, the transcriptomes of AAA and atherosclerotic arterial disease show distinct patterns with little similarity [134].

The infrarenal abdominal aorta carries certain predispositions for the incidence of aneurysm pathology due to its localization between the renal arteries and the iliac bifurcation. The infrarenal aortic wall lacks *vasa vasorum* in its medial layer [135]. Instead, most of the physiological transport to the medial layer occurs from the lumen through the arterial wall, or under pathological (aneurysmal) conditions through neo-angiogenesis from the adventitial vessels. The medial layer of the abdominal aorta is thus sensitive to hypoxia, and inherently thinner than for example the thoracic aorta. Meanwhile, the lower limitation by the iliac bifurcation introduces disturbances due to pressure-reflective waves [136].

Although we are still far from a complete understanding of the processes involved in AAA pathogenesis, histological and transcriptomic studies of human AAA, as well as molecular studies within its preclinical models have been able to elucidate many important aspects [137]. The hallmarks of AAA pathology are: a) disruption of VSMC plasticity and survival, b) degradation of ECM, and c) inflammatory cell infiltration [138]. Other characteristics are the presence of a chronic intraluminal thrombus (ILT) and adventitial inflammation and/or fibrosis [139]. Fragmentation of ECM through proteases is believed to be the constant driving factor in AAA development and progression [117, 140]. Key established drivers of ECM break-down are serine/cysteine proteases (e.g. plasmin, elastase, cathepsins) and matrix metalloproteinases (MMPs) [141–143].

The significance of the ILT in AAA pathogenesis should not be understated, as it is prevalent in most AAA cases and has been shown to have direct influence over the local milieu within the vascular wall. As it is constantly renewed by circulating blood, it creates and upholds a highly proteolytic and oxidative environment, negatively affecting cells of the intima and media. This leads to the complete lack of an intimal layer (including ECs) in aneurysmal tissue. Further proof that the ILT is involved in AAA progression is that aneurysms with a present ILT are characterized by a thinner arterial wall, loss of medial elastic fibers and SMCs, and increased adventitial inflammation [139]. During its formation and turnover, the ILT recruits red blood cells (RBCs), platelets, neutrophils, fibrinogen, among others. The iron component of hemoglobin, released through RBC hemagglutination, contributes to oxidative stress

through the generation of free radicals [139]. Platelet activation and fibrin formation attract plasminogen, and tissue-plasminogen activator contributes to ECM break-down. Infiltrating neutrophils secrete urokinase-type plasminogen activator, elastase, and other proteases, as well as MMPs. MMPs are secreted as pro-MMPs and are cleaved into active MMPs by proteases (such as plasmin) or undergo auto-activation through interaction with ROS [144]. Elastase degrades fibrillar ECM components, whereas plasmin degrades adhesive glycoproteins (e.g. fibrillin and fibronectin) [117], and mobilizes latent TGF-β stored within the ECM. Loss of adhesive proteins in the media leads to detachment and apoptosis of mesenchymal cells, mainly SMCs (a phenomenon referred to as '*Anoïkis*' [145]). Elastin fragmentation is responsible for progressive dilatation of the vessel, whereas collagen damage reduces the structural integrity (i.e. promotes rupture) [140].

Adventitial response to these events is characterized by chronic inflammation, fibrosis, and neo-angiogenesis. Macrophage accumulation assists in phagocytosis of iron stemming from RBC apoptosis in the luminal parts of the vessel wall. Fibrosis is largely a consequence of ongoing inflammation. Activation of latent TGF-β and TGF-β secretion by cells involved in the resolution of inflammation (e.g. M2 macrophages and regulatory T-cells) trigger an adventitial healing response. Consequently, adventitial fibroblasts produce collagens and other ECM components, which stabilizes the vessel wall and protects it from rupture [139, 146, 147]. Relative hypoxia of the medial and inner adventitial layers within a AAA context stimulates VEGF secretion by adventitial macrophages and medial SMCs, which drives neo-angiogenesis. An extensive adventitial response can sometimes lead to so-called '*inflammatory AAAs*', which, although rare, are characterized by adhesion of the aortic wall to neighboring organs [117].

1.5 ANIMAL MODELS OF VASCULAR DISEASE

1.5.1 Mouse models of atherosclerosis

> ***"An ideal animal model of atherosclerosis resembles human anatomy and pathophysiology and has the potential to be used in medical and pharmaceutical research to obtain results that can be extrapolated to human medicine. Moreover, it must be easy to acquire, can be maintained at a reasonable cost, is easy to handle and shares the topography of the lesions with humans."*** (Emini Veseli *et al. Eur J Pharmacol* 2017) [148]

The idea for mimicking atherosclerotic disease in animals was first tried by Ignatowski in 1907 [149]. By feeding rabbits with food normally consumed by humans (full-fat milk, eggs, meat), he could trigger the development of atherosclerotic plaques [150]. His work soon gave rise to several other studies into mechanisms of atherosclerosis through the development of animal models. In 1912, Anichkov and Chelatov reproduced his experiments, linking atherosclerosis to dietary cholesterol [151, 152]. Over the years, several different animal species have been used to study atherosclerosis [148]. Due to their small size, rapid reproduction, ease of genetic

manipulation, and low housing costs, mice have become the primary model to study atherosclerosis [148]. However, wild-type mice are not spontaneously susceptible to atherosclerosis development as their lipid profiles differ significantly from humans [148]. To better mimic cholesterol profiles of humans, transgenic mice were developed in the 1990s – in particular apolipoprotein E-deficient (*Apoe*$^{-/-}$ [153–155]) and LDL-receptor deficient (*Ldlr*$^{-/-}$ [156, 157]) mice. Apolipoprotein E (APOE) is a ligand for receptors that clear chylomicrons and very low-density lipoprotein (VLDL) particles [158], and its deficiency leads to sharp increases in plasma levels of these cholesterol fractions [148]. Similarly, LDL-receptor (LDLR) is a membrane receptor that mediates endocytosis of LDL-particles [159], levels of which are elevated in *Ldlr*$^{-/-}$ mice [148]. In humans, its deficiency is linked to a variant of familial hypercholesterolemia, a heritable dyslipidemic syndrome [160]. In both models, cholesterol levels can be further increased by feeding the animals a high-fat / Western-type diet rich in cholesterol [148]. The advantage of the *Apoe*$^{-/-}$ model is that lesions develop early, even on a normal ('*chow*') diet. A cholesterol-rich diet can further accelerate this, increasing lesion size and foam cell presence [161]. There are, however, certain disadvantages that warrant consideration. LDL is the primary cholesterol-carrying particle implicated in human atherosclerosis and only mildly elevated in *Apoe*$^{-/-}$ mice [148, 161]. APOE is also involved in other processes important for atherosclerotic plaque development, such as cholesterol efflux in macrophages [162]. Therefore, it also likely contributes to atherosclerotic plaques in other ways than its effects on circulating cholesterol profiles [148, 161]. All considered, *Apoe*$^{-/-}$ currently remains the most frequently used animal model for researchers studying atherosclerosis [148, 161]. While other models have been described, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice have been the most popular in the past decades [161]. Their characteristics are briefly characterized in **Figure 5**.

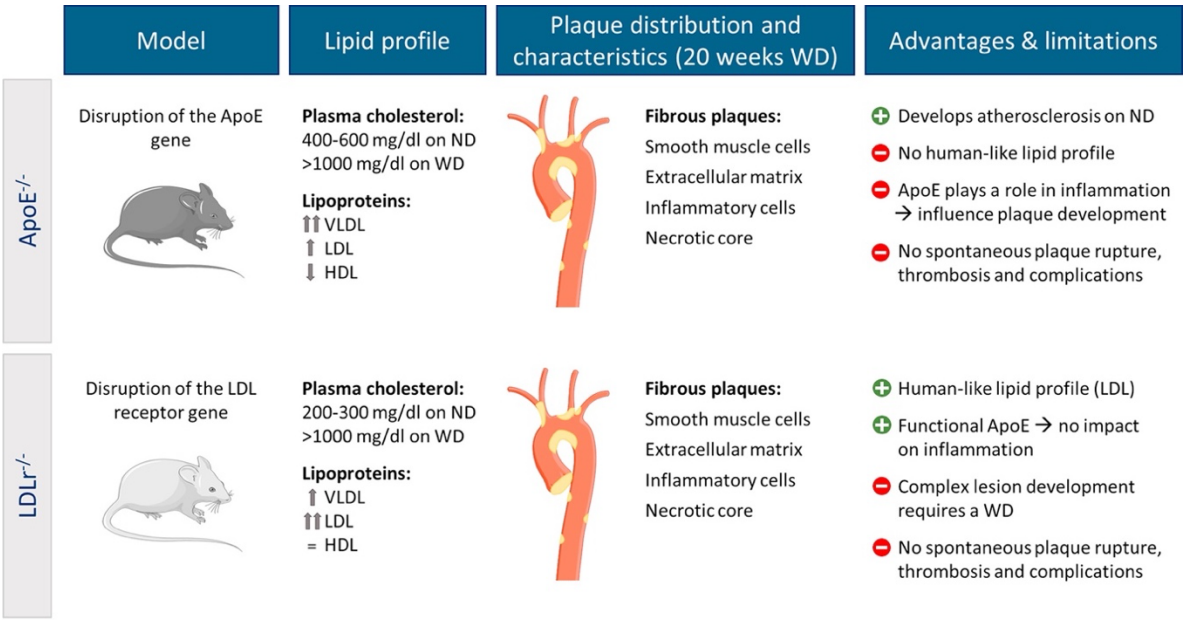

Figure 5. Characteristics of the two most popular mouse models of atherosclerosis. *Figure reproduced with permission from Emini Veseli et al.* [148]. Abbreviations: WD – western diet, ND – normal diet.

1.5.1.1 Plaque rupture in mouse

Disadvantageous for virtually all mouse models is the absence of spontaneous plaque rupture [148], a hallmark of human atherosclerotic disease that heavily contributes to its associated morbidity and mortality [49]. The reason for this is not entirely known. The small size of mouse vessels is likely a contributing factor, as a decrease in vessel diameter leads to an exponential increase of surface tension, making rupture less likely [163].

Several methods of inducing rupture of carotid plaques have been described, although none of them can truly mimic human pathophysiology [148]. An ideal model would correctly mimic human plaque morphology, have a balanced rate of rupture, result in the formation of a luminal thrombus, and have human-like complications (e.g. stroke) [148]. At least two models described in the past decades have closely approximated several of the mentioned characteristics, both in mice with *Apoe*$^{-/-}$ background. A popular model, described by Sasaki *et al*. in 2006 [164], consists of partial ligation of the left common carotid artery in 9-10-week-old *Apoe*$^{-/-}$ C57BL/6J mice and simultaneous introduction of a high-fat diet, which induces neointimal hyperplasia and formation of lipid- and collagen-rich plaques. Four weeks later, a plastic collar is placed around the vessel, which in isolation had been described to lead to plaque formation [165]. Through the combination of these two techniques, the formation of vulnerable plaques can be induced, further mimicking human vulnerable plaques through intraplaque hemorrhage (31-47%), plaque rupture (29-63%), and formation of a fibrin-containing thrombus (17-42%) [148, 164]. Another interesting model was recently described by Chen *et al*. [166]. The authors performed '*tandem*' partial-ligations (spaced 3 mm apart) of the common carotid artery in *Apoe*$^{-/-}$ C57BL/6J mice fed a high-fat diet. They created the model based on a computational fluid dynamics analysis, given that atherosclerotic plaques are known to develop in areas of low-shear stress, which is also the described mechanism driving plaque development and progression in the ligation + cuff model. After seven weeks, they were able to observe an impressive amount of human-like plaques – rich in lipids, macrophages/foam cells, having necrotic-cores covered with thin fibrous caps, displaying neovascularization and outward remodeling. In addition, some of these plaques presented with intraplaque hemorrhage (51%), rupture (32%), and the presence of a thrombus (undisclosed). Few studies have thus far sought to reproduce this model, but it might prove important in future studies of atherosclerosis if successfully reproduced. Nonetheless, certain limitations are likely to remain. One of the major challenges of these inducible plaque rupture models is that although local thrombosis occurs because of plaque rupture, embolic consequences such as stroke are more or less completely absent [148]. This underlines the fact that although the models may closely mimic specific characteristics of human disease, caution is needed when trying to extrapolate the results into clinically relevant conclusions.

1.5.2 Animal models of AAA

To better understand AAA pathophysiology and the mediators involved, several mouse models of AAA have been developed [167, 168]. While human AAA specimens can be used for histological and transcriptomic studies, animal models allow experimental evaluation of research hypotheses *in vivo*. An ideal model should mimic the defining histological characteristics and course of human AAA pathology. Although none of the available mouse models fulfill these criteria, several have become popular within the AAA research field and are constantly refined [169]. Due to different mechanisms of AAA induction, and consequently different AAA characteristics, they can be used to focus on specific parts of AAA pathogenesis. Properties of main mouse models in relation to human disease are summarized in **Table 1**. Two AAA models popular within the field and relevant to this thesis are described below. The characteristics of the $CaCl_2$ model are only briefly summarized for comparison purposes and have been thoroughly reviewed by Busch *et al.* [167, 168]. General limitations relating to the use of animal models and resulting methodological considerations are further discussed in **section 4.5.2**.

	Human AAA	PPE	ePPE	AngII	$CaCl_2$
Fusiform growth	+	+ *	+ *	-	+
Fibrosis	+	+	+	+	+
Rupture	+	-	- (late *) #	+ (early) *	-
Wall dissection	+	-	-	+ *	-
Slow, chronic development	+	-	-	-	-
ILT	+	-	- #	-	-
IMT	+	-	-	+	-
Atherosclerosis	+	-	-	+	-
Calcification	+	-	-	-	+
Angiogenesis	+	+	-	+	+
Elastic fiber loss	+	+	+	+	(+)
Inflammatory response	+	+	-	+	+

Table 1. Comparison of human AAA characteristics and common mouse AAA models. Adapted from Sénémaud *et al.* [167] and Busch *et al.* [168]. Abbreviations: ePPE/PPE – (external) porcine pancreatic elastase model, AngII – angiotensin II model, $CaCl_2$ – $CaCl_2$ model, ILT – intraluminal thrombus, IMT – intramural thrombus. # – present in ePPE in combination with anti-TGF-B treatment. * – introduced or exacerbated by BAPN. Clarifications: PPE/ePPE in combination with BAPN (ß-aminopropionitrile) leads to larger aneurysms. ePPE does not rupture unless combined with anti-TGF-B treatment, or BAPN (late rupture). AngII ruptures early and rupture rate is increased with BAPN. $CaCl_2$ effect on elastic fibers is mostly concentrated to the external parts of the vessel.

1.5.2.1 Angiotensin II infusion in Apoe$^{-/-}$ mice

First described in 2000 [170], this model is today the most popular mouse model of AAA and the only well-established model of AAA rupture. Genetically modified *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ adult mice are infused with high-doses of angiotensin II (AngII; 1000 ng/kg per minute) via a subcutaneous osmotic pump during a 28-day infusion period. During this period, most of the mice develop significant suprarenal aneurysms, and approximately 20% die due to rupture [171]. This model mimics certain aspects of human AAA disease, such as medial elastolysis, progressive aneurysm expansion, macrophage infiltration, and thrombus formation [172–175], but there are important differences to human AAA pathology. Firstly, the aneurysm pathogenesis has recently been highlighted to occur through intimal micro-tears, often in connection to ostia of major branch arteries [172]. This leads to the formation of an intramural hematoma and subsequently an intramural (rather than intraluminal) thrombus, which results

in the development of a so-called 'dissecting AAA' [175, 176]. Secondly, the supra-renal aneurysm location and quickly progressing luminal dilatation significantly contrast human disease [172]. Nonetheless, this model allows experimental studies of possible AAA rupture mechanisms, which is not possible using other models. Due to the use of mice on a hyperlipidemic genetic background, this model also allows studies of AAA in the presence of atherosclerosis.

Recently, certain modifications to the original model have become increasingly popular [169]. One of these is the administration of AngII together with an anti-TGF-β antibody, allowing the use of normolipidemic C57BL/6 mice instead of the considerably more expensive genetically modified *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice [177]. This modification also leads to a more rupture-prone AAA formation [178]. Another modification is the addition of BAPN (ß-aminopropionitrile), a lysyl oxidase inhibitor, to drinking water. While BAPN alone has been used to induce thoracic aortic dissection in juvenile mice, in combination with AngII infusion it increases rupture and dissection rates of the AAA model. When combined with PPE (described below), it leads to larger AAA diameters [168].

1.5.2.2 Porcine pancreatic elastase infusion in wild-type mice

This model consists of transient infusion of porcine pancreatic elastase (PPE) into an isolated segment of the abdominal aorta. Originally developed in 1990 using Wistar rats [179], it was translated to mice in the early 2000s [141]. The procedure was later thoroughly described by Azuma *et al.* [180] and shown to reproduce well in C57BL/6 mice. The acute response (0-7 days) involves transmural inflammation (involving ECs, SMCs, and fibroblasts) and neutrophil recruitment [167]. Aneurysm forms after approximately two weeks and is characterized by elastic and collagen fiber destruction, adventitial infiltration by macrophages, and increased MMP activity. Due to the impressive mimicry of human AAA progression, including medial elastic fiber breakdown, VSMC death, endothelial damage, and adventitial inflammation, this model has become extensively popular in experimental studies aiming to limit AAA progression. However, PPE-induced aneurysms eventually stabilize and seldom rupture, likely due to the transient nature of the initial stimulus. It must be noted that this mouse model of AAA is surgically challenging and requires extensive microsurgical expertise to achieve reproducible results. An alternative and more accessible to perform approach has been proposed, involving periadventitial administration of PPE ('ePPE' in **Table 1**) [181], but it results in significantly smaller and less reproducible aneurysms [167]. Recently, a new approach to the PPE model has been described [182]. Here, through a combination of topical application of elastase and systemic inhibition of TGF-β, significant and reproducible aneurysms were achieved with an approximate rupture rate of 50%. Interestingly, the authors argue that the rupture mechanism was not dependent on medial dissection, a significant limitation of the aforementioned AngII infusion model. In addition, upon histological analysis, most of the mice showed the presence of an ILT. If these findings can be replicated, this modified model could undoubtedly be of value for future experimental AAA research [183].

1.5.2.3 The need for large preclinical animal models

The obvious limitation to the above-mentioned (and other) small animal models is that they do not entirely recreate the pathological basis for AAA human disease. As in other medical research fields, medical therapies proven to limit AAA progression in rodents have failed to affect human AAAs [184, 185]. This has called into question the translational feasibility of these models when aiming to develop novel therapeutic strategies. Murine models, although undoubtedly essential tools, should therefore primarily be seen as a first step of studying AAA disease *in vivo.* To aid the translation of preclinical findings into clinically relevant conclusions, efforts have been made to develop large-animal models of AAA [186]. Such models have been described in dogs [187], rabbits [188], and pigs [189], including miniature Yorkshire pigs [190]. In most of these proposed models, induction of a significant AAA was only possible by combining two or more stimulants (elastase, collagenase, $CaCl_2$, or flow alternations). In addition, few (if any) of these models have been shown to be consistently reproducible in experimental studies, likely due to their complexity and high costs. To address this issue, our group has recently evaluated the PPE infusion model in wildtype and *LDLR*$^{-/-}$ Yucatan mini-pigs [191]. While the wild-type animals did not develop significantly sized aneurysms (as had been reported before [192]), we were able to successfully induce relevant AAAs (>1.5-fold diameter increase) in *LDLR*$^{-/-}$ animals 28 days after instillation. The advantage of the Yucatan mini-pig over the majority of other animal species is the fact that their physiology can be considered more human-like, through similar body weight (approximately 75-80 kg), blood pressure, heart rate, and overall dimensions (size) of the cardiovascular system (heart and large arteries, such as the aorta and the carotid arteries) [191, 193].

1.6 GAPS IN KNOWLEDGE

If left undiscovered or untreated, the potentially devastating consequences of AAA make it an important disease to study. The asymptomatic onset and progression of the disease can in many cases make rupture the debut symptom, which is associated with low survival. There is currently no pharmacological treatment for AAA and surgery remains the only option for patients at risk of rupture. During the last decade, multiple ncRNAs (non-coding RNAs) have been found to regulate vascular pathologies in general and AAA disease in particular [137]. A pharmacological (ncRNA-based) treatment would benefit patients who cannot or should not undergo surgery due to the risks involved. In many of these studies, experimental inhibition or overexpression of these molecules has been shown to halt AAA progression. Whether these findings can be translated into AAA human pathology remains to be seen. To facilitate this, novel preclinical models with better physiological and anatomical relevance to human disease will be of value.

Similarly, given many intriguing reports on the involvement of ncRNAs in atherosclerosis, particularly in the regulation of VSMC fates, ncRNAs may emerge as potential novel targets for medical treatment of atherosclerotic disease. They could prove key to both understanding and impacting early atherogenesis, including genetic risk factors, but perhaps most importantly they could contribute to better management of late-stage atherosclerotic lesions.

In the past two decades, extensive efforts have been made to identify biomarkers of AAA disease and its progression [194]. Certain features such as ILT-volume or fluorodeoxyglucose uptake have been shown to correlate with AAA progression in retrospective studies [195, 196]. Peak wall stress has also been described as a predictor of AAA rupture risk [197, 198]. However, only circulating biomarkers can be considered feasible when looking for non-radiological ways to diagnose AAA or identify patients at risk for AAA. Past studies of circulating biomarkers have focused on molecules involved in hemostasis (e.g. D-dimer [199], fibrinogen [200]) and arterial wall structural integrity (e.g. elastin [201, 202], MMPs and TIMPs [203–205]). Molecules involved in inflammation (e.g. IFNγ, interleukins [206]) and risk factors (e.g. serum triglycerides [207]) have also been studied, although given the severe atherosclerotic burden of many AAA patients, they are likely to be inherently less specific. Despite these efforts, no circulating protein biomarker of AAA has yet made it into clinical practice [194]. In addition, while females with AAA present with worse intervention outcomes, in contrast to men they do not benefit from ultrasound screening due to the lower prevalence of the disease [208–210].

Using ncRNAs as biomarkers has several unique advantages: many are tissue/cell-type specific, they are detectable with high sensitivity and specificity, one can perform network analysis (of ncRNA and putative mRNA targets), and they are stable in biofluids [211, 212]. The latter is especially true for the more recently characterized circular RNAs, which were not within the scope of this thesis.

1.7 NON-CODING RNAS IN VASCULAR DISEASE

The central dogma of molecular biology states that DNA is transcribed into mRNAs, which in turn are translated into proteins. Today, we know that while 70-90% of our genome is transcribed [213, 214], only approximately 2% effectively get translated into proteins [215]. Thus, considerable effort has been put into studying RNA species other than mRNA during the past two decades [61]. While the functions of tRNAs and rRNAs have long been known, other classes have emerged, previously considered 'transcriptional junk' [216, 217]. These are referred to as ncRNAs – as they do not code for proteins but rather extend their effects through transcriptional or post-transcriptional regulation [218]. These molecules have been characterized by their length, function, and genomic location. In this thesis, ncRNA subclasses microRNAs and long non-coding RNAs have been studied. Another type of ncRNA, studies of which have recently gained much traction, are circular RNAs [219].

1.7.1 MicroRNA

Among the shorter ncRNAs, and currently the most well-studied, are microRNAs (miRNAs), ~22-nucleotide single-stranded RNAs. Although described already 1993 in *Caenorhabditis elegans* [220, 221], it was not until the early 2000s that they were shown to be highly conserved across species and relevant in humans [222]. To this date, miRNAs have been shown essential for human development, cell differentiation, and homeostasis [223], and the human genome encodes for approximately 1000 miRNAs [224, 225]. Their deregulation

has been associated with the pathogenesis and progression of many diseases, and it is estimated that miRNAs regulate the translation of more than 60% of protein-coding genes [218]. During the last decade, many miRNAs have been described as highly relevant in CVD [225–227]. They have been shown to regulate SMC phenotype and apoptosis, matrix production and remodeling, TGF-β and inflammatory signaling, and EC function [226, 228, 229].

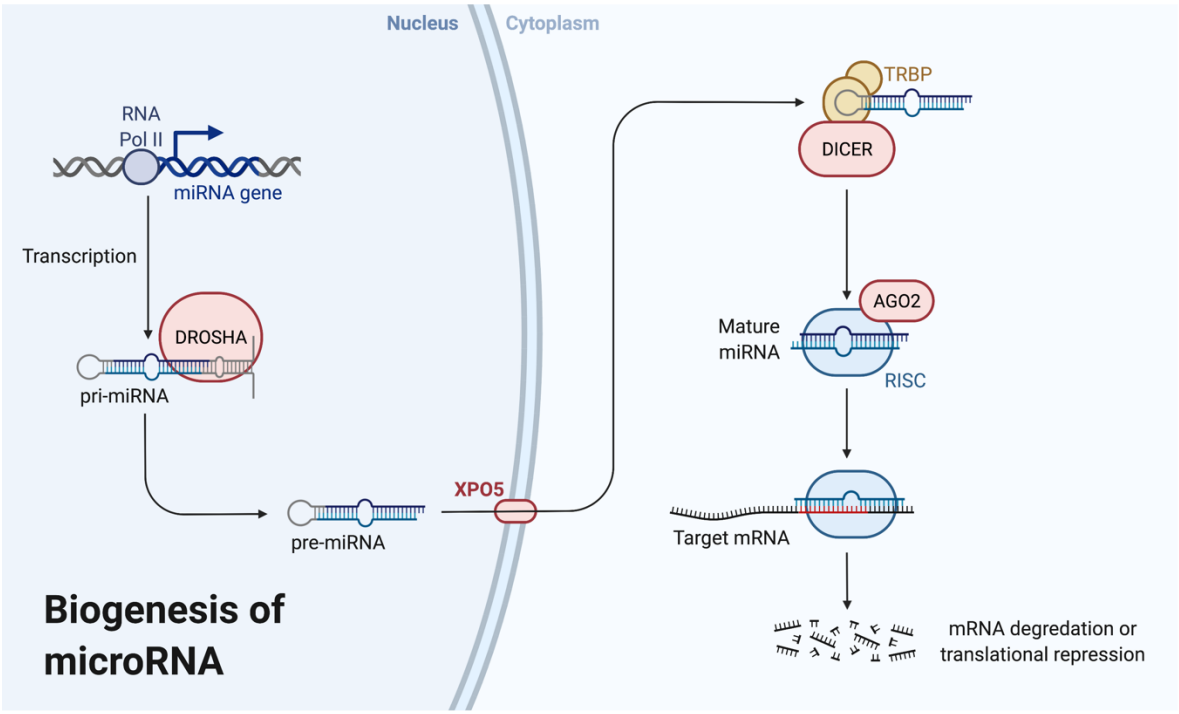

Figure 6. microRNA biogenesis and main principles of function. *Illustation by the author.*

The biogenesis of miRNA consists of several steps (**Figure 6**). Hairpin-shaped primary miRNA (pri-miRNA) transcripts are transcribed mainly by Polymerase II and then processed into miRNA precursors (pre-miRNA) by nuclear RNAse III enzyme Drosha [230] assisted by the protein DGCR8 [231, 232]. The remaining hairpin loop of the pri-miRNAs is then cleaved by cytoplasmic RNAse III enzyme Dicer to a specific length, typically 21-23 nucleotides [233, 234]. This results in a mature miRNA duplex, of which one strand is loaded onto an AGO protein, most commonly Ago2 [235]. The strand preference of Ago2 is believed to be based on certain features of the 5' nucleotide of the miRNA strand, although many miRNAs will exist in both strand (3p and 5p) variants [236–238]. The AGO-miRNA complex will then associate with target mRNA, forming the miRNA-induced silencing complex [235]. This is the primary regulatory function of miRNAs, as they initiate mRNA decay or exhibit translational repression through inhibition of ribosomal binding [239].

miRNAs are known as fine-tuners rather than absolute regulators in the sense that they can decrease protein production by approximately two-fold [240]. In addition, miRNAs are largely unspecific as they interact with mRNAs through complementary binding via their '*seed sequence*', 6-8 nucleotides in length (usually located at nucleotides 2-8 from the 5' end) [241]. In effect, a key characteristic of miRNAs is that they regulate not only specific genes but have often been found to have multiple targets within specific functional networks [242]. This

property might be particularly interesting in the context of vascular disease, due to its highly sensitive nature [243], which has made the discovery of traditional drugs challenging [244].

In AAA, several miRNAs have been shown to regulate ECM-composition (miR-29, 181, 195), SMC survival (miR-21), SMC differentiation (miR-143/145 cluster), inflammation (miR-24, 33, 181b), TGF-ß signaling (miR-181b, 195) as well as the expression of MMPs or tissue inhibitors of matrix metalloproteinases (TIMPs; miR-181b, 195, 205). Modulation of aforementioned miRNAs in experimental AAA models led to reduced AAA progression.

1.7.2 Long non-coding RNA

In recent years, focus has shifted towards ncRNAs with longer sequences. Among these are long non-coding RNAs (lncRNAs; >200 nucleotides in length). Compared to miRNAs, they are less abundant and less conserved among species [217]. In further contrast to miRNAs, which tend to exert their function through post-transcriptional inhibition of target mRNAs in the cytoplasm, functional lncRNA are found both in the cytoplasm and the nucleus. This gives them the ability to not only interact with other RNAs but also affect the chromatin dynamics and translation through RNA-protein interaction [245]. In addition, lncRNAs can regulate miRNA expression by acting as host genes for their transcription or by competing for complementary binding with miRNA target genes ('*miRNA sponges*') [246–248]. These and other key mechanisms of lncRNA function are summarized in **Figure 7**.

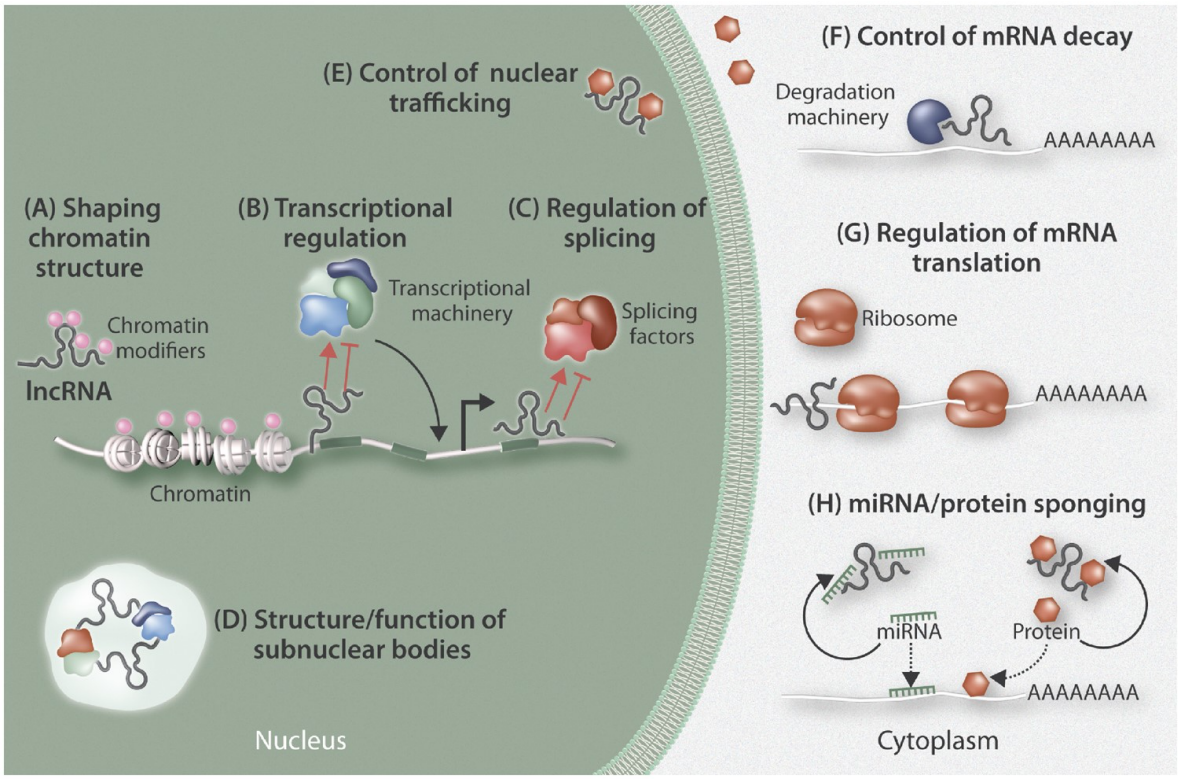

Figure 7. Different described action mechanisms of lncRNA. *Reproduced with permission from Fasolo et al.* [248].

Only a handful of lncRNAs have thus far been implied in CVD, many more still likely to be discovered [249]. This is partly due to a relatively small number of lncRNAs with currently known biological functions (<1% of annotated lncRNAs [250]). In contrast to annotation of protein-coding genes, which can be identified by their open reading frame, lncRNAs lack known universal sequence features. Intriguingly, several lncRNAs have been described to encode short functional peptides of functional importance [251]. The relatively low abundance and weak evolutional conservation of lncRNAs further complicate the discovery and annotation of novel transcripts [252, 253]. Consequently, as new techniques are being developed, such as long-read sequencing technologies and novel annotation algorithms, our understanding of lncRNA biology will likely significantly increase [254].

During the past decade, key roles of lncRNAs in atherosclerosis and AAA have been characterized. Genome-wide genetic association studies have identified atherosclerotic disease-associated genetic variants in the *Chr9p21* locus to overlap the sequence coding for lncRNA *ANRIL* [255]. Increased expression of *ANRIL* is considered atherogenic, and its levels in plaques (but also in circulation) correlate to atherosclerotic severity [256–258]. Even though *ANRIL* is one of the most studied lncRNAs in CVD, its exact molecular function remains elusive, and several mechanisms have been proposed [259]. *ANRIL* is also particularly interesting as increased levels of its circular isoform (circular RNA) seem to cause opposite effects compared to its linear counterpart, highlighting the intriguing complexity of the ncRNA landscape [260]. Several other lncRNAs have been described to functionally regulate VSMCs, e.g. *lincRNA-p21* [261], *SENCR* [262], *MALAT1* [263], *SMILR* [264] and *CARMN* [265]. The latter, originally thought to be a host gene for the miRNA-143/145 cluster, was recently described as able to directly (and independently of miRNA-143/145) interact with MYOCD, facilitating its binding of SRF [266]. *CARMN* is also a good example of a lncRNA with high tissue specificity, expressed solely in the vasculature [266].

Our group has recently described H19 to be the first lncRNA implicated in AAA development [191]. Its expression was increased in AngII and PPE mouse models of AAA, the PPE *LDLR*$^{-/-}$ Yucatan mini-pig model (described in **section 1.5.2.3**) and human AAA patient tissue. H19 was also shown to trigger transcription of *HIF1α* via recruitment of the SP1 transcription factor to the promoter region. In the cytoplasm, H19 interacted with HIF1α, induced p53 activity, and was associated with increased SMC apoptosis and decreased SMC proliferation and migration.

Due to their relative high tissue specificity, ability to regulate key features of VSMCs, and their apparent implication in CVD, lncRNAs shown to be involved in atherosclerosis and AAA pathogenesis seem like suitable candidates for potential treatment strategies and potential disease biomarkers [267].

1.7.3 Delivery of ncRNA based therapeutics

Therapies based on inhibition of disease-relevant ncRNAs are feasible by supplying antagomiRs (microRNA inhibitors) or GapmeRs (inhibitors of lncRNAs), which are synthetic antisense oligonucleotides (ASOs) that bind to the up-regulated ncRNA and silence it. Alternatively, by using lncRNA- or miRNA-mimics, it is possible to enhance the expression of down-regulated ncRNAs [267, 268].

One major disadvantage of such treatment strategies, utilizing systemic intravenous, subcutaneous or intramuscular injections of ncRNA-inhibitors, are the documented off-target effects of these ASOs on other organ systems. In fact, ncRNA-inhibitors tend to assimilate to a much greater extent in organs such as the liver, kidney, lung, and spleen than in the targeted cardiovascular system [269]. Therefore, tools and techniques for local or cell-type-specific delivery would be of great value to make such treatments feasible. In one such effort, our group has recently described local delivery of a miR-21 mimic using ultrasound-targeted microbubbles into carotid plaques to induce a more stable plaque phenotype [270].

2 RESEARCH AIMS

The overall objective of the thesis was to extensively evaluate the role and therapeutic potential of novel ncRNAs in vascular disease development and progression. We hypothesized that modulation of these (or previously described) ncRNAs through local delivery mechanisms might prove to be an efficient method to halt disease progression. In addition, we aimed to study the potential of ncRNAs (primarily miRNAs) as diagnostic and prognostic biomarkers for AAA disease.

The specific aims were:

Study I. To explore the functional role of long non-coding RNA *MIAT* in human atherosclerotic disease.

Study II. To explore the functional role of a human disease *NUDT6,* a natural antisense transcript of *FGF2,* in experimental in vivo and in vitro models of vascular disease.

Study III.

a. To evaluate the potential protective role of multi-kinase inhibitor lenvatinib on AAA disease phenotype and progression.
b. To evaluate the feasibility of targeted drug delivery to the aortic tissue in a translational mini-pig model of AAA disease.

Study IV. To identify potential miRNA biomarkers of AAA disease and study their involvement in the pathogenesis of the disease.

3 MATERIALS AND METHODS

3.1 STUDY RATIONALE

For **study I**, we performed gene expression profiling in tissues from patients with advanced carotid artery atherosclerotic lesions from the Biobank of Karolinska Endarterectomies, comparing them to non-atherosclerotic iliac artery controls. We found increased expression of lncRNA *MIAT* (*Myocardial Infarction Associated Transcript;* previously also known as *Gomafu)*. *MIAT* had previously been implicated in patients with myocardial infarction, as well as in endothelial inflammation in diabetic retinopathy. A subsequent separate analysis of an independent cohort of patient plaques from the Munich Vascular Biobank showed increased expression of *MIAT* in fibrous caps in ruptured/unstable (cap thickness ≤200 μm) compared to stable (cap thickness >200 μm) plaques.

In **study II**, we employed a hypothesis-driven approach to look at natural antisense transcripts (NATs) of known growth factors that play key roles in both carotid atherosclerosis and AAA. While direct inhibition of growth factors is a clinically utilized therapeutic strategy for the treatment of certain conditions (e.g. cancer), there are scenarios where instead an increased expression of specific growth factors is desirable. We hypothesized that targeting the NAT, and thus a potential transcriptional and/or translational repressor of the growth factor in question, could be used to achieve this. In fibrous caps of stable and unstable/ruptured plaques from the Munich Vascular Biobank (described in the paragraph above) and in aortic tissues from AAA patients and healthy organ donors, we found *FGF2* and its NAT *NUDT6* to be differentially regulated in opposing directions. Expression of *FGF2* was decreased in AAA/unstable plaques, whereas *NUDT6* was increased. As we considered *FGF2* to be beneficial in both AAA and advanced atherosclerosis, we wanted to study whether *NUDT6* inhibition could turn out to be a potential treatment strategy for stabilization of these diseases.

Hypoxic mechanisms and associated angiogenesis have been implicated in AAA pathogenesis [271–273]. Hypoxia-induced VEGF signaling drives angiogenesis and is a known phenotypic modulator of SMCs [274]. Therefore, in **study III**, we employed a drug repurposing approach. Lenvatinib, a multiple kinase inhibitor developed as an anti-cancer medication, is known to target the main receptor of VEGF (VEGFR2) [275].

As the above approaches turned out successful in murine models, we attempted to bridge the translational gap between mice and humans, by using a more human-mimicking, novel *LDLR*$^{-/-}$ mini-pig models of atherosclerosis and AAA, recently described by our group. The therapeutic approaches attempted in **studies II and III** were also deemed promising candidates for local delivery through the use of drug-eluting balloons (DEBs).

Finally, in **study IV**, we sought to identify disease-relevant circulating miRNA biomarkers for AAA through a non-biased approach using multiple large-sized cohorts of AAA patients with different characteristics, and controls. The need for such a study was identified based on the

fact that few such studies had been performed and validated on sufficiently large cohorts, and that few studies had attempted to directly show functional relevance of discovered miRNAs.

Table 2. Overview of studied mechanisms, outcomes and methods in each of the studies.

Study	*Target & studied disease*	*Role in disease*	*Animal models*	*Experimental outcome*	*Molecular methods*
I	***MIAT*** Carotid artery atherosclerosis	Mediates VSMC phenotypic switching through induction of KLF4	*Apoe*$^{-/-}$ *Miat*$^{-/-}$ C57BL6 mice (ligation+cuff) *LDLR*$^{-/-}$ Yucatan mini-pigs	Inhibition reduces atherogenesis, increases experimental plaque vulnerability	Laser-capture microdissection, MA, qPCR, WB, ISH/FISH, IHC/IF, RIP *In vitro:* RNA/plasmid (co)transfection, luciferase reporter assay cell fractionation, live cell imaging, primary cell isolation *In silico:* lncRNA-DNA target prediction
II	***NUDT6*** Carotid artery atherosclerosis, AAA	Inhibits FGF2; Detrimental to atherosclerotic plaque stability and AAA, through reduced proliferation of VSMCs	*Apoe*$^{-/-}$ C57BL6 mice (ligation+cuff, AngII AAA) C57BL6 mice (PPE AAA) *LDLR*$^{-/-}$ Yucatan mini-pigs (PPE AAA, DEB, UTMD)	Inhibition stabilizes experimental plaques, augments AAA progression	Laser-capture microdissection, MA, qPCR, WB, ISH, IHC, RNA-pulldown *In vitro:* RNA transfection, live cell imaging
III	**VEGF-signaling (VEGFR2)** AAA	Mediates angiogenesis, phenotypic switching of VSMCs	*Apoe*$^{-/-}$ C57BL6 mice (ligation+cuff, AngII AAA) C57BL6 mice (PPE AAA) *LDLR*$^{-/-}$ Yucatan mini-pigs (PPE AAA, DEB)	Inhibition through lenvatinib augments AAA progression, preserves SMC contractile phenotype	MA, qPCR, WB, IHC/IF In vitro: live cell imaging, cell contraction assay, primary cell isolation *In silico:* Pathway ORA
IV	**miR-15a-5p** AAA	Circulating biomarker; Inhibits proliferation of VSMCs, reduces transdifferentiation of VSMCs to an inflammatory phenotype	*Apoe*$^{-/-}$ C57BL6 mice (ligation+cuff, AngII AAA) C57BL6 mice (PPE AAA)	Inhibition augments AAA progression	MA, RNA-seq, qPCR, FISH, IF *In vitro:* miRNA-transfection, live cell imaging *In silico:* miRNA-target prediction, GSEA

Abbreviations: MA – microarray; RNA-seq – RNA sequencing; qPCR – reverse transcription quantitative polymerase chain reaction; WB – western blot; (F)ISH – (fluorescent) in situ hybridization; IHC – immunohistochemistry; IF – immunofluorescence; RIP – RNA immunoprecipitation; ORA – over-representation analysis; GSEA – gene-set enrichment analysis; PPE AAA – PPE perfusion AAA model, AngII AAA – AngII infusion AAA model; UTMD – ultrasound targeted microbubble destruction; DEB – drug-eluting balloon.

3.2 MATERIALS

3.2.1 Patient populations

All material used for histological, gene expression and protein expression analyses of carotid atherosclerotic plaques stems from either the Biobank of Karolinska Endarterectomies (BiKE) or Munich Vascular Biobank (MVB). All material used for histological, gene expression and protein expression analyses of AAA in **studies II and III** stems from MVB. In **study IV**, material for histological analyses stems from MVB, material for gene expression stems from MVB and Stockholm AAA Biobank (StAAAB), patient plasma/serum stems from Second manifestations of ARTerial disease (SMART) cohort, Stockholm AAA screening cohort, StAAAB or Vienna AAA cohort. All patients provided written and informed consent approved by the responsible ethics committees at each site.

3.2.1.1 Biobank of Karolinska Endarterectomies (BiKE)

BiKE and its sampling protocols have been described in detail by Perisic *et al.* [276]. Briefly, patients with symptomatic or asymptomatic, high-grade (NASCET >50% [277]) carotid artery stenosis were enrolled. Exclusion criteria were atrial fibrillation and/or major stroke. Carotid plaques were sampled during CEA. Each carotid plaque was divided in two parts, one from which RNA was extracted and one used for histology. Control arteries were obtained from organ donors and included nine non-atherosclerotic iliac arteries and one aorta.

3.2.1.2 Munich Vascular Biobank (MVB)

MVB and its sampling protocols have been described in detail by Pelisek *et al.* [278]. Briefly, tissue samples were collected during open AAA repair or CEA. Specimens used for histological analyses were fixated in 4% paraformaldehyde for 48h and after that embedded in paraffin. For RNA analysis, fresh frozen specimens were stored at -80°C. For some of the patients (and if technically possible), in addition to the aneurysmal tissue sample, a proximal region of non-aneurysmal aortic tissue was sampled. Control aortic tissues were taken from organ donors.

Patient-derived cells from AAA or atherosclerotic tissue samples were collected during open AAA repair or CEA and stored short-term in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at +4°C. Then, the adventitia and – if possible, the endothelium was removed, and the tissue was incubated at 37°C, 5% CO_2 on collagen-coated Petri dishes in SMC Growth Medium (PELO Biotech, Planegg, Germany). Primary culture of patient-derived cells was used until passage 7.

3.2.1.3 Second manifestations of ARTerial disease (SMART)

The rationale and design of the SMART study cohort, and AAA definition and policy, have been described in detail before [279, 280]. Briefly, patients aged 44-80 with clinically manifest vascular disease or cardiovascular risk factors were enrolled at University Medical Center Utrecht, The Netherlands. Patients received a standardized vascular screening, including a

health questionnaire, laboratory assessment, and ultrasonography. Based on the power calculation in **study IV**, we aimed to include ≥200 patients with ultrasonographically defined AAA (aortic diameter >30mm). To maximize the chance of discovery, we proceeded with 200 patients whose AAAs were deemed clinically relevant (aortic diameter >40mm). In addition, from SMART, we also included 200 control patients matched for age, gender, CVD risk profile, and medications. For both AAA and control patients, we retrieved 200 μl of plasma, stored at -80°C between original sampling and final analysis.

3.2.1.4 Stockholm AAA Biobank (StAAAB)

The design of StAAAB has been described by Lindquist Liljeqvist *et al.* [281]. We received access to the transcriptomic profiles of patients enrolled in that study, i.e. patients undergoing open AAA repair. In addition, we received already extracted RNA from medial and adventitial tunicas of ILT-covered patient aortas (n=20), which had previously been used to establish the aforementioned transcriptomic profiles. For these patients, we were also provided ≥200 μl of plasma sampled ahead of surgery. All samples were stored at -80°C between original sampling, extraction, and final analysis.

3.2.1.5 Stockholm AAA screening cohort

As part of StAAAB, during recent years, plasma from 65-year-old men from the Stockholm region undergoing AAA screening has been collected as recently described by Villard *et al.* [282]. Patient demographics were recorded by a structured questionnaire. Screening, plasma sampling, and questionnaire were all collected on the same day. AAA was defined as aortic diameter ≥ 30mm. Subjects not diagnosed with AAA (aortic diameter <30mm) were sampled in a corresponding fashion and served as controls. Exclusion criteria were inadequate information provided in the structured questionnaire or unattainable blood sample. In total, we received access to 68 plasma samples (≥200 μl) from AAA patients and 35 from non-AAA controls. The plasma samples were stored at -80°C between original sampling and final analysis.

3.2.1.6 Vienna AAA cohort

The Vienna AAA longitudinal follow-up protocol has been recently described by Eilenberg *et al.* [283]. In brief, AAA patients without indication for surgical repair were recruited through the Division of Vascular Surgery at Vienna General Hospital. The exclusion criteria were recent (<1 year) tumor and/or chemotherapy, systemic autoimmune or hematological disease, and organ transplantation. Patient demographics were recorded by a structured questionnaire. From this study population, we gained access to serum samples (≥200μl) from 28 patients, as well as their baseline and 6-month follow-up AAA diameter measurements. The serum samples were stored at -80°C between original sampling and final analysis.

3.2.2 Animals

3.2.2.1 C57BL/6 wildtype and Apoe$^{-/-}$ mice

Apoe$^{-/-}$ mice were used in mouse models requiring dyslipidemic conditions. In **studies II and III**, C57BL/6J background mice were used, in **study IV** C57BL/6N mice were used, and in **study I** C57BL/6J mice were used for initial experiments, but C57BL/6N mice were required for the development of the double-knockout (*Apoe*$^{-/-}$ *Miat*$^{-/-}$) model (described further down). C57BL/6J *Apoe*$^{-/-}$ mice were purchased from Taconic Biosciences (Silkeborg, Denmark). C57BL/6N *Apoe*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice denamed wild-type in the thesis and constituent studies were on a C57BL/6J background and purchased from Taconic Biosciences.

3.2.2.2 C57BL/6N Apoe$^{-/-}$ Miat$^{-/-}$ double-knockout mice

Miat$^{-/-}$ mice (originally denoted *Gomafu* CDB1347K) on C57BL/6N background were a kind gift from Professor Nakagawa at RIKEN Center for Life Science Technologies, Kobe, Japan [284]. These *Miat*$^{-/-}$ mice were intercrossed with C57BL/6N mice creating *Apoe*$^{-/-}$ *Miat*$^{-/-}$ double-knockout mice. Consequently, *Miat*$^{-/-}$ mice maintained on a C57BL/6N background with heterogenous breeding served as single-knockout controls.

3.2.2.3 Mouse housekeeping

All mice were housed and experiments were performed at the animal facilities provided by the Comparative Medicine unit at Karolinska Institutet. The only exceptions were AngII AAA C57BL/6J *Apoe*$^{-/-}$ mice in **study III**; these experiments were performed by collaborators in Austria, Vienna. Animals were housed under standard conditions (temperature 22°C, humidity 56%, 12-hour light/12-hour dark cycle). Mice were co-housed in cages enriched in accordance with animal welfare legislation. Mice were attended daily by the animal facility staff and had free access to food and water. All mice were fed chow-diet (R36; Lantmännen, Stockholm, Sweden). Upon experiment end, mice were euthanized through CO_2 inhalation, exsanguinated by heart puncture, and perfused with 4°C PBS before organ harvesting.

3.2.2.4 *LDLR$^{-/-}$ Yucatan mini-pigs*

LDLR$^{-/-}$ Yucatan mini-pigs were provided by Exemplar Genetics (Coralville, IA, USA). The pigs were housed in animal facilities at Technical University Munich, co-housed in groups of 2-4 under conventional hygienic conditions. Environmental enrichment was provided and general conditions were: temperature 19 ± 2°C; humidity 50–60%; 12-hour light/12-hour dark cycle. The mini-pigs received an acclimatization period of at least nine days before undergoing surgery. They were fed with a pelleted high-fat diet (Altromin, Lage, Germany) twice a day and received water *ad libitum*. Before anesthesia, the mini-pigs were fasted for 12 hours with free access to water. Blood sampling and other procedures were performed under anesthesia. Upon experiment end, animals were sacrificed using pentobarbital (>50mg/kg) and 40ml of 1M KCl solution.

3.3 ANIMAL MODELS

3.3.1 Mice

3.3.1.1 Mouse anesthesia

All experiments (including serial ultrasound measurements, if applicable) were performed under anesthesia. Mice were placed under 2% isoflurane anesthesia. Prior to the procedure, anesthesia depth was verified by toe pinch. After procedure completion, mice were left to recover in open enriched cages under an infra-red lamp to minimize body temperature loss. Mice were then observed until fully recovered from the procedure and no signs of distress were visible. Upon complications, mice were either provided additional analgesia or terminated, in accordance with the ethical permit protocols.

3.3.1.2 Inducible carotid plaque rupture model

For studies of plaque vulnerability and rupture, we used a modified ligation and cuff model in *Apoe*$^{-/-}$ mice [285, 286]. In this approach, a conical rather than symmetrical cuff is used to induce shear-stress more efficiently, as proposed by Kuhlmann *et al.* [287]. By combining this with prior common carotid artery ligation, plaque development and subsequent rupture can be induced. In contrast to the ligation and cuff model described by Sasaki *et al*. [164], our approach does not require the mice to be fed a high-fat diet [270, 285].

Briefly, 10-12-week-old male *Apoe*$^{-/-}$ mice are anesthetized with 2% isoflurane. Via medial neck incision, the right common carotid artery is dissected from surrounding connective tissue. Directly below carotid bifurcation, a 5-0 Vicryl (Ethicon, Bridgewater, NJ, USA) suture is used to partially ligate the artery. On day 28 after ligation, a cone-shaped plastic 1.7mm long cast consisting of two rigid halves (Promolding BV, Rijswijk, Netherlands) is placed around the common carotid artery, proximal to the ligation site. The internal lumen of the cuff ranges from 300µm (proximal) to 150µm (distal). After four days, animals were sacrificed, and after PBS perfusion, the right common carotid artery was macroscopically inspected for signs of plaque rupture.

3.3.1.3 Angiotensin II infusion AAA model

The advantages/disadvantages and characteristics of different models of murine AAA are thoroughly discussed in **section 1.5.2**. Briefly, C57BL/6J *Apoe*$^{-/-}$ mice were placed under anesthesia. Osmotic minipumps releasing AngII at 1,000 ng/kg min^{-1} (sized 20 x 6mm) were implanted subcutaneously through a small dorsal flank incision. Using ultrasound (Vevo 2100, Visualsonics, Toronto, Canada), maximum infrarenal aortic diameter was measured under anesthesia at baseline, day 7, day 14 and day 28 after pump implantation. After the final diameter measurement, the anesthetized mice were sacrificed through heart puncture.

3.3.1.4 Porcine pancreatic elastase perfusion AAA model

Briefly, 10-week-old male C57BL/6J WT mice were placed under anesthesia. After careful dissection of the aorta from surrounding tissue, proximal and distal temporary silk ligatures

were placed. A microcatheter was then inserted into the aortic lumen, and the lumen was perfused with PPE (Sigma-Aldrich, Burlington, MA, USA) at a concentration of 2U/mL for 10 minutes. After thorough flushing of the aorta with saline, a single knot 10-0 Prolene (Ethicon) suture was used to seal the catheter entry site. The abdomen was closed layer by layer with 5-0 Vicryl (Ethicon) running sutures.

3.3.1.5 Tissue harvesting and blood sampling

After euthanasia through CO_2 inhalation, blood was collected through heart puncture into EDTA containers and centrifuged at 10,000xG for 10 minutes. Plasma was transferred to new Eppendorf tubes and stored at -80°C. Relevant organs were snap-frozen or embedded in Optimal Cutting Temperature Cryomount compound (OCT, Histolab, Gothenburg, Sweden) and thereafter stored at -80°C.

3.3.2 Mini-pigs

3.3.2.1 Yucatan mini-pig LDLR$^{-/-}$ pancreatic elastase perfusion AAA model

The advantages of using Yucatan mini-pigs are discussed in **section 1.5.2.3**. In summary, these animals possess physiological characteristics that can be likened to humans, with similar body weight, blood pressure, and heart rate. This is in contrast to most commonly used murine AAA models. Through this approach, the translational potential of interventional studies can be further enhanced. It also allows for the use of drug delivery tools with translational relevance for human patients, such as drug-eluting stents/balloons.

Anesthesia, analgesia, peri- and postoperative monitoring are all thoroughly described in the supplemental material of **study III.** Prior to surgery, the baseline aortic diameter was measured. The porcine aorta was exposed via left lateral flank access. The aorta was clamped over 3-4 cm. A PTA balloon (Medtronic, Minneapolis, MN, USA) was used to predilate the aorta. PPE (Sigma-Aldrich) was perfused at 10U/mL for 10 minutes through a blunt 5G needle. After flushing with heparinized saline (1000 U/L), the aortic incision was closed with a 5-0 Prolene suture (Ethicon) before the clamps were released. The surgical wound was closed layer-by-layer and covered with a spray-on dressing.

Repeated diameter measurements were performed at day 0, day 7, day 14, and day 28. Blood was sampled at day 0, day 7, and day 28. If applicable, on day 7, a drug-eluting balloon was spray coated with the therapeutic of choice and applied to the aneurysmal part of the porcine aorta (described in **studies II and III**). Animals were sacrificed on day 28 post AAA-induction.

3.4 KEY MOLECULAR METHODS

Detailed methods and protocols have been thoroughly described in the published papers (**studies I and III**) / attached manuscripts (**studies II and IV**), and supplemental materials. Key methods and methodological considerations are described below. Methodological limitations are discussed in **section 4.5**.

3.4.1 Genomics & bioinformatics

3.4.1.1 RNA isolation and RT-qPCR

Sample handling, RNA/protein extraction, are thoroughly described in the methods sections of each constituent study. RNA was extracted using manufacturer protocols, depending on material type, using one of the following kits: miRNeasy Micro Kit, miRNeasy Mini Kit, miRNeasy Advanced Serum/Plasma Kit, miRNeasy Advanced Tissue/Cells Mini Kit (all Qiagen, Hilden, Germany). For plasma/serum samples, cel-miR-39 was spiked in as a control during the extraction. The concentration of total RNA was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For miRNA analysis, RNA was diluted in RNAse-free water to a concentration of 2 ng/µl. cDNA synthesis was performed using TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific), using 10 ng of total RNA, with reverse transcriptase (RT) primers specific for each miRNA. For serum/plasma samples, RNA concentration was not measured, and 2 µl of the total elute (20 µl, extracted from 200 µl human or 100 µl mouse serum/plasma) was used for each miRNA RT reaction. For gene expression analysis, 200-1000 ng total RNA was used for cDNA synthesis using the TaqMan High-Capacity cDNA Transcription Kit. Expression of mRNA/miRNA was quantified using TaqMan FAM/VIC-labeled assays on the QuantStudio 6/7, 7900HT and StepOnePlus RT PCR systems (all Applied Biosystems, Thermo Fisher Scientific). Differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$.

3.4.1.2 Gene expression / miRNA microarrays

Applied Biosystems OpenArray technology was used to assay relative plasma miRNA expression in **study IV**, further explained in the methods section of the manuscript. In this approach, microchips containing wells coated with TaqMan probes for 758 different miRNAs were loaded with cDNA synthetized using a RT primer mix containing primers specific to these miRNAs. The OpenArray chips were cycled and processed on the QuantStudio 12K Flex System (Applied Biosystems). In **study III**, we used Affymetrix GeneChip Mouse Transcriptome Assay 1.0 (Thermo Fisher Scientific) to profile gene expression changes in the aortas of mice treated with lenvatinib.

3.4.1.3 RNA sequencing

In **study IV**, to identify potential targets of miRNA-15a-5p (miR-15a), we sequenced RNA of primary human aortic SMCs, harvested 48h post-transfection with miR-15a-mimic (n=3), miR-15a-inhibitor (n=3) or scrambled control oligo (n=3). Library preparation and RNA sequencing were performed by a commercial vendor (Novogene UK Company Ltd, Cambridge, United

Kingdom). Libraries were sequenced on the Illumina NovaSeq PE150 platform (Illumina, San Diego, CA, USA) and >40M paired-end reads were generated for each sample. Reads were cleaned, mapped, aligned, and analyzed for differential expression by the commercial vendor. Downstream bioinformatic analyses were independently performed by the author.

3.4.1.4 miRNA / lncRNA target prediction

For **study IV** utilized available *in silico* miRNA target prediction data from DIANA microT-CDS [288, 289], miRDB 6.0 [290, 291], miRWalk 3.0 [292], as well as experimentally validated targets from miRTarBase [293]. In **study I**, we looked at interactions of lncRNA and DNA / proteins. DNA-lncRNA interactions were predicted using LongTarget v2.1 [294]. Protein-lncRNA interactions were predicted using RegRNA v2.0 [295].

3.4.1.5 Data integration and tools for data interpretation

Microarray and RNA sequencing experiments create large amounts of data. In these experiments, thousands of differentially expressed genes can be identified. Many different bioinformatic tools have been developed to aid in converting this information into biologically relevant conclusions. Some examples include clustering, overrepresentation, enrichment, and network analyses. In the presented studies, the most common approach was analysis of gene enrichment/overrepresentation in known signaling pathways and in sets of genes that give rise to certain types of biological responses such as inflammation, apoptosis, or hypoxia.

All data analysis was performed using R. Bioconductor is a collection of libraries for R pertaining to the analysis of bioinformatics data. Among the most commonly used libraries were limma, clusterProfiler, HTqPCR, ggplot2 [296–299].

3.4.2 Histology

3.4.2.1 Tissue preparation and imaging

Tissues used for histological analyses were either fresh frozen and embedded in OCT (Histolab) or formalin-fixed and paraffin-embedded (FFPE). Fresh frozen tissues were cut into 6 µm thick (mouse carotid) or 8 µm thick (mouse aorta) sections onto positively charged SuperFrost Plus slides (Menzel Gläser, Braunschweig, Germany), dried and stored at -80ºC. Paraffin-embedded tissues were cut into 5 µm thick sections. Slides from fresh frozen tissues were thawed, once again dried, and fixed in 4% paraformaldehyde for 10 minutes. Basic tissue morphology was visualized through either hematoxylin and eosin or elastic van Gieson stains. Imaging was performed using either a Leica Microsystems TCS SP8 (Wetzlar, Germany) confocal microscope or Olympus SLIDEVIEW VS200 (Tokyo, Japan) slide scanner.

3.4.2.2 Laser capture microdissection

In studies of fibrotic caps in plaques, especially in **study II**, localized changes in gene expression can be ‘drowned out’ by other unaffected parts of the tissue when analyzing bulk gene expression of whole lesions. Therefore, a laser capture microdissection approach was used

to extract RNA solely from the histological areas of interest. Up to 10 consecutive slides per patient were micro-dissected and put into RLT buffer (Qiagen), whereafter RNA extraction was performed according to standard protocols.

3.4.2.3 Immunohistochemistry / immunofluorescence

Immunoassays were used to identify proteins in tissue sections from patients and experimental animals. Fresh-frozen sections were processed directly, while FFPE sections were first deparaffinized in xylene and rehydrated in decreasing serial ethanol dilutions. For FFPE sections, antigen retrieval was performed by heating in a citrate buffer. Subsequently, serum blocking and H_2O_2 blocking (only in immunohistochemistry, to quench endogenous peroxidase activity) were performed. Slides were incubated with primary antibody (except for negative control slides where this step was omitted) and thereafter with an HRP-conjugated (immunohistochemistry) or fluorophore-conjugated (Alexa, Thermo Fisher Scientific) secondary antibody (immunofluorescence). For immunohistochemistry, HRP-activity was visualized by incubating the slides with AEC or DAB substrate and nuclei counterstained with hematoxylin. For immunofluorescence, nuclear counterstaining was performed through incubation with DAPI.

3.4.2.4 Fluorescent in situ hybridization (FISH)

FISH was used to visualize genes and miRNA in tissue sections from patients and experimental animals. Sections were deparaffinized and rehydrated as per above (FFPE) or processed directly (fresh frozen). H_2O_2 blocking was performed, and sections were predigested/permeabilized using proteinase-K (in a concentration recommended by Exiqon for respective section type) for 10 minutes at 37ºC. The double-DIG-labelled miRNA *in situ* hybridization (ISH) probes (Exiqon, Vedbæk, Denmark) were hybridized for 2h at 30ºC below their manufacturer-specified melting temperature (T_m). Scrambled control probes were used as negative control. Stringency washes were performed in serial dilutions of saline-sodium citrate (SSC) buffer at room temperature. After blocking, sections were incubated with a secondary anti-DIG-POD (HRP) antibody (Sigma-Aldrich), after which the signal was visualized by incubation with an Alexa-TSA (Thermo Fisher Scientific) substrate.

In situ hybridization of lncRNA was performed using the RNAscope Multiplex Fluorescent v2 Assay (ACD, Bio-Techne, Minneapolis, MN, USA) according to the manufacturer's instructions. The advantage of the RNAscope approach over standard FISH is that it requires two independent probes to hybridize to the target RNA for amplification to occur. This minimizes the issue of non-specific hybridization that can often be an issue with standard FISH protocols. RNAscope also includes a signal-amplification step, where a tree-like scaffold is built on top of the two specific probes, each part of the scaffold conjugated to an HRP-enzyme. Thereafter, the signal can be visualized by a standard fluorophore (Alexa/Opal)-TSA incubation, activated upon contact with HRP.

3.4.2.5 Multiplexing

The FISH protocol can be combined with immunofluorescence to aid in assessing colocalization of lncRNA/miRNA and e.g. cell-type-specific protein markers. The standard FISH protocol was followed until after Alexa-TSA substrate incubation. Thereafter, immunofluorescence was performed, starting with the primary antibody incubation step. One disadvantage of this approach is that it excludes antigen retrieval in citrate buffer solution and instead includes a proteinase-K pre-digestion step. Due to this, certain primary antibodies may be incompatible with this approach due to epitope unavailability/loss. In multiplex protocols, extra care was taken to ensure no signal carryover between different fluorophores. For this reason, apart from standard negative control slides that were always included in all histological studies, additional single-stain negative controls were included when staining in multiplex.

3.4.3 Cell culture

3.4.3.1 General conditions

Cells were cultured in incubators at 5% CO_2 and atmospheric O_2 levels. All experiments were performed under sterile conditions in laminar flow hoods. All *in vitro* experiments were performed in triplicates or more. Methods for RNA isolation are described in **section 3.4.1.1**. Protein lysates were harvested using RIPA buffer (Sigma-Aldrich) supplemented with phosphatase inhibitors, protease inhibitors, and EDTA and stored at -20ºC.

Cell types used were primary aortic and carotid SMCs (Cell Applications, San Diego, CA, USA), HEK293 cell-line (Public Health England Culture Collections, Salisbury, United Kingdom), THP-1 (gift from the Vascular Surgery Unit at Karolinska Institutet), immortalized VSMC cell-line (Applied Biological Materials, Richmond, Canada), as well as primary patient cells from MVB. For primary cells, cell passages no later than passage 7 were used.

3.4.3.2 Isolation of primary cells

Primary mouse aortic SMCs were isolated from pooled mouse aortas and propagated in DMEM medium (Thermo Fisher Scientific). Primary patient carotid/aortic SMCs were isolated from tissue samples collected during open AAA repair or CEA. They were stored in DMEM medium (Thermo Fisher Scientific) short-term at 4ºC, whereafter adventitia and endothelium were removed and remaining tissue incubated to propagate on collagen-coated Petri dishes in SMC Growth Medium (PELO Biotech).

3.4.3.3 Transfection

In all constituent studies, transfection of ASOs or miRNA-mimics was performed to study the effect of gene expression changes on functional phenotypes. Transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific). All RNA transfection experiments were controlled by parallel transfection of scrambled constructs of the same chemistry. All overexpression experiments (except for miRNA) were performed through transient transfection with expression plasmids, using Lipofectamine 3000 (Thermo Fisher Scientific).

Plasmid experiments were controlled through parallel transfection with an empty vector of the same backbone.

3.4.3.4 Live-cell imaging (cell migration, proliferation, apoptosis)

Studies of cell dynamics were performed using an automated live cell imaging system (IncuCyte Zoom, Sartorius, Göttingen, Germany). An image masking algorithm was trained to study proliferation for the particular cell type. Images were automatically taken every second hour, whereafter the IncuCyte Zoom software calculated confluency for each timepoint. For apoptosis studies, the cell culture medium was supplemented with either a Caspase 3/7 Apoptosis reagent (Sartorius, Göttingen, Germany) in a 5 μM concentration or an Annexin V-Alexa Fluor™ 594-conjugate (Thermo Fisher Scientific) in a 1:100 dilution. Thereafter apoptosis could be monitored by automated observation of the number of cells fluorescently labeled by the reagent. For cell migration studies, cells were plated and grown to a 90% confluency in IncuCyte ImageLock 96-well plates (Sartorius). The ImageLock plates have optically clear bottoms with physical markings allowing for a reproducible objective lock of the automated IncuCyte Zoom live cell imaging system. Before imaging, an identical wound was created in all wells using the IncuCyte WoundMaker (Sartorius). The kinetics of wound closure were then continuously (every second hour) monitored.

3.4.4 RNA interactions

Co-localization in histological tissue sections (described in **section 3.4.2.5**), or other approaches such as proximity ligation assays, can sometimes be considered indirect evidence of interplay between genes or proteins. However, these techniques do not prove that an actual interaction takes place. In our studies, we used the following methods to establish evidence of interaction between different molecules.

3.4.4.1 Luciferase reporter assay

The luciferase reporter assay can be used to establish either transcriptional (e.g. for lncRNAs) or post-transcriptional (e.g. for miRNAs) effects on target genes. Cells are transfected with an expression plasmid containing the firefly luciferase gene (Active Motif, Carlsbad, CA, USA) under control of a target promoter (for studying effects on transcription) or containing a target 3'UTR region (for studying post-transcriptional mechanisms of miRNAs). 24h after simultaneous transfection with the ncRNA of interest, the luciferase signal can be quantified by lysis of the cells, the addition of a luciferase substrate, and subsequent read-out in a luminometric microplate reader.

3.4.4.2 RNA immunoprecipitation

The binding of RNA to proteins of interest can be studied through RNA immunoprecipitation. Briefly, upon immunoprecipitation using an antibody designed to target a specific protein, RNAs that bind to this protein can be expected to co-precipitate into the immunoprecipitated protein-enriched fraction. Observed enrichment of RNA expression in the protein-enriched

fraction constitutes proof that these molecules can interact. However, this does not explicitly imply a direct interaction, as other co-interactors might mediate in this process.

3.5 STATISTICS

3.5.1 Analysis of experimental data

GraphPad Prism 9 (San Diego, CA, USA) was used to perform statistical inference on experimental data. Differences between two groups were primarily analyzed by a two-sample (Student's) t-test, either unpaired or paired depending on the study design. Normality was assumed for measurements expected to follow a normal distribution (according to the central limit theorem) and, if needed, verified using D'Agostino-Pearson or Shapiro-Wilks normality tests. Q-Q plots were used to visually inspect the data normality. If a normal distribution could be achieved through appropriate data transformation, this approach was preferred. For example, gene expression data was compared using the log2 expression values and then transformed into fold-change for presentation purposes. Otherwise, the nonparametric Mann-Whitney U test was chosen. Equal variances were similarity first hypothetically evaluated, inspected on a homoscedasticity plot, and thereafter tested using the F-test of equality of variances. If the variance was different between groups, Welch's t-test was used. Welch's t-test was also preferred if sample sizes were unequal. Statistical comparison of frequencies was performed using the chi-square test. Two-sided $p<0.05$ was considered statistically significant. One-sided $p<0.05$ was considered significant when there was a clear prior hypothesis on the direction of change, e.g. in validatory experiments. Throughout this thesis and its constituent studies, significance levels are depicted as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

When comparing more than two groups, an analysis of variance (ANOVA) approach was used, in either one- or two-way design, depending on the number of analyzed factors. In addition, if a subject was assayed multiple times over time, a repeated-measures design was used. Normality and variance assumptions were assessed visually (through Q-Q and homoscedasticity plots). If significant divergence was observed, Shapiro-Wilk's and Levene's tests were additionally performed. Post hoc multiple comparisons of ANOVA analyses were performed using the Tukey method, or using Fisher's LSD test with subsequent Šidák (Dunn-Šidák) or Holm-Šidák corrections.

Linear regression was used to study the association between continuous variables, and a t-test was used to assess its significance. Pearson correlation coefficient (r) was calculated to quantify the degree of correlation.

3.5.2 Analysis of transcriptomics data

All analyses of data stemming from high-throughput transcriptomics experiments (microarrays and RNA sequencing) were performed using R and selected Bioconductor libraries. Differential expression analysis of microarray data was performed using limma or limma-dependent libraries. Differential expression analysis of RNA sequencing data was performed

using DESeq2 or edgeR. Overrepresentation analyses (ORA) and gene set enrichment analyses (GSEA) were performed using clusterProfiler. Resulting p-values from all above-described approaches (differential expression, ORA, GSEA) were adjusted for false discovery rate using the Benjamini-Hochberg approach. Specific approaches, including data normalization strategies, are more thoroughly explained in each constituent study.

3.6 ETHICAL CONSIDERATIONS

To allow evaluation of our research hypotheses, there is an inherent need for use of animal models, for all of which (murine AngII / PPE models, Yucatan mini-pig PPE models) we currently hold updated ethical permits. Nonetheless, when designing and performing animal experiments, it is important that Russell & Burch's 3R [300, 301] are taken into consideration. For the diseases studied, animal experiments cannot be entirely replaced by *ex vivo* or *in vitro* approaches. The compounds being evaluated have not yet been tried in humans, and it would therefore be unethical to progress with such unless effects have been thoroughly verified in animal studies. Reduction is performed through careful analyses of human AAA tissues and/or blood samples, to only perform animal experiments deemed relevant to human vascular disease. In addition, calculations regarding the sample size of animal experiments are based on previous experience and complemented by power analyses to ensure that the smallest possible number of animals are used while still being able to reach statistically sound conclusions. Refinement is performed through Karolinska Institutet established protocols, including careful monitoring of animals' well-being by researchers in our group and animal facility staff (at least once daily). All models are based on the latest knowledge within the field. To further minimize suffering, all invasive procedures are performed under general anesthesia, and the animals receive pre-procedure analgesics routinely and during 24-48h post-procedure or later if required (described in more detail in previous sections).

When working with external biobanks to access patient samples or tissues, care was taken to ensure that all ethical permits were in check and that there was a proper protocol for patient consent, in line with the Declaration of Helsinki [302].

Upon publication, data from bioinformatic experiments will be made available in public repositories to ensure transparency and maximize the impact of public funds and effort put into our work. All constituent studies have been, or will be, published in open-access journals to maximize data availability.

The studies we perform are explorative and will not necessarily instantly contribute to changes in clinical practice. Nonetheless, through the use of novel, more relevant models, we aim to facilitate the translation of our research findings into clinical trials and in the long term into clinical practice. Regardless of whether this turns out successful, our findings will contribute to a better understanding of the diseases studied and thus facilitate continued research into the pathologies in question.

4 RESULTS AND DISCUSSION

4.1 SUMMARY OF KEY RESULTS

Results have been independently reported and discussed in the published papers (**studies I and III**) / attached manuscripts (**studies II and IV**). Key findings of each of the studies are summarized below and are thereafter discussed through the lens of vascular disease and important aspects of pathogenic processes that characterize atherosclerosis and/or AAA.

4.1.1 Study I

Expression of lncRNA *MIAT* is increased in vulnerable carotid plaques from patients. *In vitro*, *MIAT* expression can be induced in VSMCs through stimulation with oxLDL and lipoprotein(a). We show *MIAT* to participate in phenotypic switching of VSMCs. We suggest the following mechanisms for its role: a) through interaction with the *Klf4*-promoter and induction of ELK1-phosphorylation, *MIAT* promotes de-differentiation of VSMCs into more inflammatory macrophage-like phenotypes; b) through activation of the EGR1-ELK1-ERK pathway, *MIAT* promotes proliferation and survival of VSMCs. In addition, we show that *MIAT* participates in the regulation of macrophage-specific processes: a) it facilitates the activation and nuclear translocation of NF-κB; b) it promotes expression of transporters (*CD36*, *SRA*) involved in oxLDL uptake through a yet unknown mechanism. Overall, *MIAT* seems to drive key processes in the development of atherosclerotic plaques and their progression towards more advanced stages. However, it does also promote the stability of very advanced plaques through its positive effects on VSMC survival and therefore fibrous cap thickness. Lastly, we were able to observe the presence of the above-described effects in *LDLR*$^{-/-}$ Yucatan mini-pigs, increasing their translational feasibility.

4.1.2 Study II

In laser-capture micro-dissected fibrous caps of patient carotid plaques, we found *FGF2* to be down-regulated and its natural antisense transcript *NUDT6* up-regulated in fibrous caps of unstable (cap thickness ≤ 200 μm) vs stable (cap thickness > 200 μm) lesions. This was also true in aortic tissues from AAA patients compared to organ donor controls. Stimulation by either oxLDL or AngII led to *in vitro* increase of *NUDT6* in human carotid or aortic VSMCs respectively. *In vitro* overexpression of *NUDT6* led to a repression of *FGF2* in human VSMCs and consequently decreased their proliferation and increased apoptosis. In the mouse model of plaque rupture by carotid ligation and cuff placement, *in vivo* inhibition of *Nudt6* led to a decreased plaque rupture rate. In both the PPE perfusion and AngII infusion mouse models of AAA, *Nudt6* inhibition limited aneurysm growth. We also evaluated *NUDT6* inhibition in our translational porcine AAA model in *LDLR*$^{-/-}$ Yucatan mini-pig model receiving PPE perfusion. While we could not observe any significant effects on AAA diameter, analysis of aortic tissues showed successful inhibition of *NUDT6*, a consequential increase of *FGF2* and *MYH11* – suggesting increased VSMC survival. In line with this, histological analysis of porcine tissues showed more FGF2-positive and SMA-positive cells in *NUDT6* inhibited animals.

4.1.3 Study III

We evaluated lenvatinib, a multi-kinase inhibitor known to inhibit VEGFR2, as a potential therapeutic agent for patients with AAA. In both PPE and AngII models of murine AAA, treatment with lenvatinib led to less AAA diameter growth. In addition, local (as opposed to systemic) delivery of lenvatinib through surgical reintervention yielded similar results. To ensure translational feasibility, we also employed our *LDLR*$^{-/-}$ Yucatan mini-pig PPE AAA model, where DEB-mediated local delivery of lenvatinib successfully halted AAA progression. Studies using microarray technology, immuno-histomorphological analysis of in vivo experiments, and cell culture models (including primary patient-derived cells) indicated that lenvatinib keeps VSMCs in their contractile phenotype and thus restores vessel wall integrity upon aneurysm induction.

4.1.4 Study IV

We identified miR-15a as up-regulated in plasma from patients with AAA compared to controls. Its expression was similarly up-regulated in plasma of PPE and AngII murine AAA model mice and aneurysmatic aortic tissues from patients. Through analysis of material from three additional independent AAA patient cohorts, we were able to: a) confirm that miR-15a levels are higher in plasma of patients with large aneurysms vs control; b) observe that levels of miR-15a in tunica media correlate with AAA diameter; c) observe that levels of miR-15a in plasma and serum correlate with AAA diameter. Through *in silico* miRNA target prediction as well as RNA sequencing of human aortic VSMCs transfected with miR-15a modulators, we suggest a role of miR-15a in the modulation of VSMCs towards a more inflammatory phenotype. Lastly, through systemic delivery of miR-15a inhibitors, we were able to limit AAA diameter growth in the murine PPE perfusion model of AAA.

Table 3. A brief overview of study results.

Study	*Target & affected pathways*	*Observed effect on VSMC phenotype*	*Experimental modulation in vitro*	*Experimental modulation in vivo (mice)*	*Experimental modulation in vivo (mini-pigs*)*
I	***MIAT*** ↑ KLF4 ↑ NF-κB ↑ ERK-ELK1-EGR1	Mediates VSMC trans-differentiation into inflammatory macrophage-like phenotype	**KD in hCtSMCs:** ↓ proliferation ↑ apoptosis **KD in MP:** ↓ oxLDL uptake	Inhibition (GapmeR) reduces atherogenesis, increases experimental plaque vulnerability	**Not modulated** *MIAT*, *KLF4*, *EGR1*, and *ELK1* all decreased in mini-pigs with more severe lesions.
II	***NUDT6*** ↓ *FGF2*	Opposed effect to FGF2, which is known to promote de-differentiation of VSMCs into an activated, proliferative phenotype	**OE in hCtSMCs:** ↓ proliferation ↑ apoptosis ↓ migration **OE in hAoSMCs:** ↓ proliferation ↑ apoptosis ↓ migration	**Knockdown (GapmeR)** *Plaque rupture:* ↓ rupture rate *PPE*: ↓ diameter ↑ α-SMA content *AngII*: ↓ diameter ↑ α-SMA content ↑ preservation of elastic lamellae	**Knockdown (GapmeR)** *Aorta:* ↑ α-SMA content ↑ preservation of elastic lamellae
III	**Inhibition of VEGF-signaling (VEGFR2) via lenvatinib** ↓ *VEGF* ↓ ERK ↑ MYH11	Promotes a contractile phenotype through several potential mechanisms	**hAoSMCs:** ↓ proliferation ↓ apoptosis ↓ migration **AAA patient-derived cells:** ↓ proliferation ↓ migration ↑ contractility ↑ contractile gene expression	**Systemic treatment** *PPE*: ↓ diameter ↑ SMC contractility *AngII:* ↓ diameter	**Systemic and targeted (DEB) delivery** ↓ diameter ↑ SMC contractility
IV	**miR-15a-5p** ↑ broad network of inflammatory genes	No established phenotype Pro-inflammatory	**hAoSMCs:** ↓ proliferation ↑ apoptosis (literature)	**Knockdown (antagomiR)** *PPE:* ↓ diameter ↑ α-SMA content	–

Abbreviations: KD – knock-down, OE – over-expression, hCtSMCs – human carotid smooth muscle cells, hAoSMCs – human aortics smooth muscle cells, MP – macrophages, PPE – porcine pancreatic elastase murine AAA model, AngII – angiotensin II infusion murine AAA model, DEB – drug-eluting balloon, (V)SMC – (vascular) smooth muscle cell. * – *LDLR*$^{-/-}$ Yucatan mini-pig porcine pancreatic elastase AAA model.

4.2 KEY ROLES OF VSMCS IN VASCULAR DISEASE

During the last decade, extensive efforts have been made to closer understand the role of VSMCs as involved in key processes present in vascular disease that previously had been attributed to other cell types [56]. Several key publications have been able to more or less confirm what many researchers had long suspected – that the many key processes in atherogenesis and atherosclerotic plaque progression (but also in AAA) can in different ways be attributed to VSMC behavior [303]. This is mediated by the ability of VSMCs to de-differentiate and transdifferentiate into functionally distinct phenotypes. This spectacular plasticity should be seen as a key 'feature' rather than a weakness, especially given that contractile differentiation of VSMCs is not definitive. Much evidence points towards this plasticity being important in the maintenance of vascular wall homeostasis and its ability to adapt to changing, and sometimes pathological, conditions.

In **study I**, we discovered that the lncRNA *MIAT* could positively regulate *KLF4* on a transcriptional level, through a lncRNA-DNA interaction with the *KLF4* promoter. *In silico* analysis revealed potential binding sites for *Miat/MIAT* at the *Klf4/KLF4*-promoter (**Figure 8**). In human VSMCs, oxLDL-induced *MIAT* expression led to increased *KLF4*-promoter activity (**Figure 9**) and subsequently increased nuclear levels of KLF4 (**Figure 10**). Plasmid-based overexpression of *Miat* in murine VSMCs led to a similar increase in *Klf4*-promoter activity (**Figure 9**).

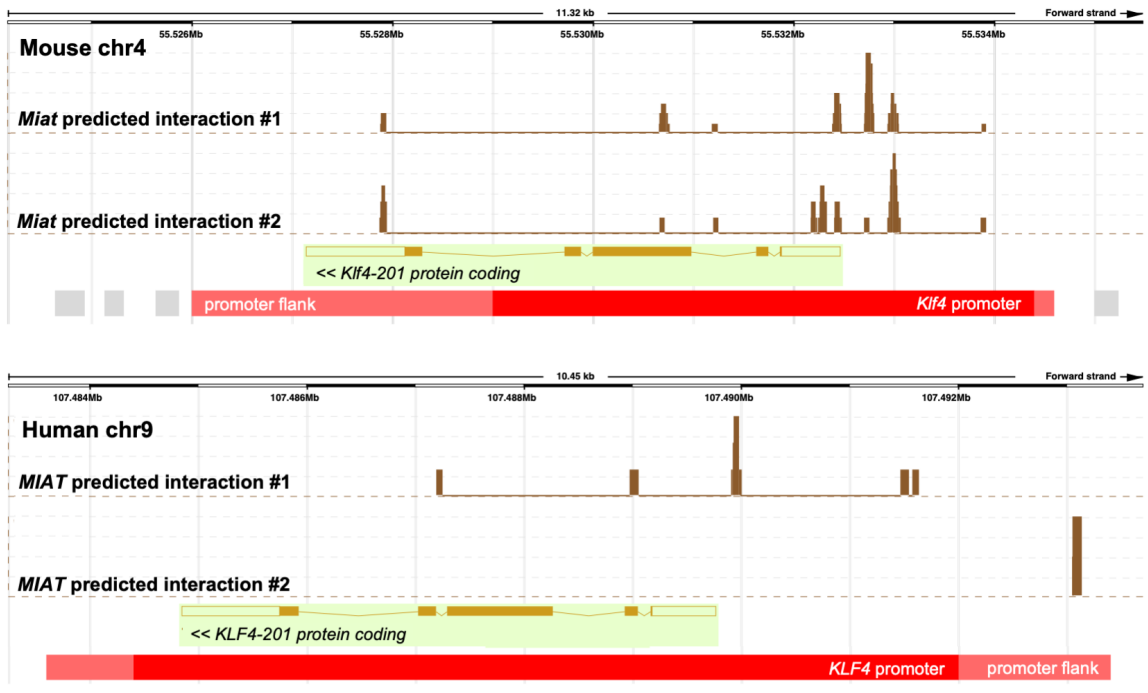

Figure 8. Interaction of *Miat/MIAT* with *Klf4/KLF4* promoter as predicted by LongTarget v2.1. Peaks indicate predicted binding sites of *Miat/MIAT* within Klf4/KLF4 promoter region. Figure 6D in study I.

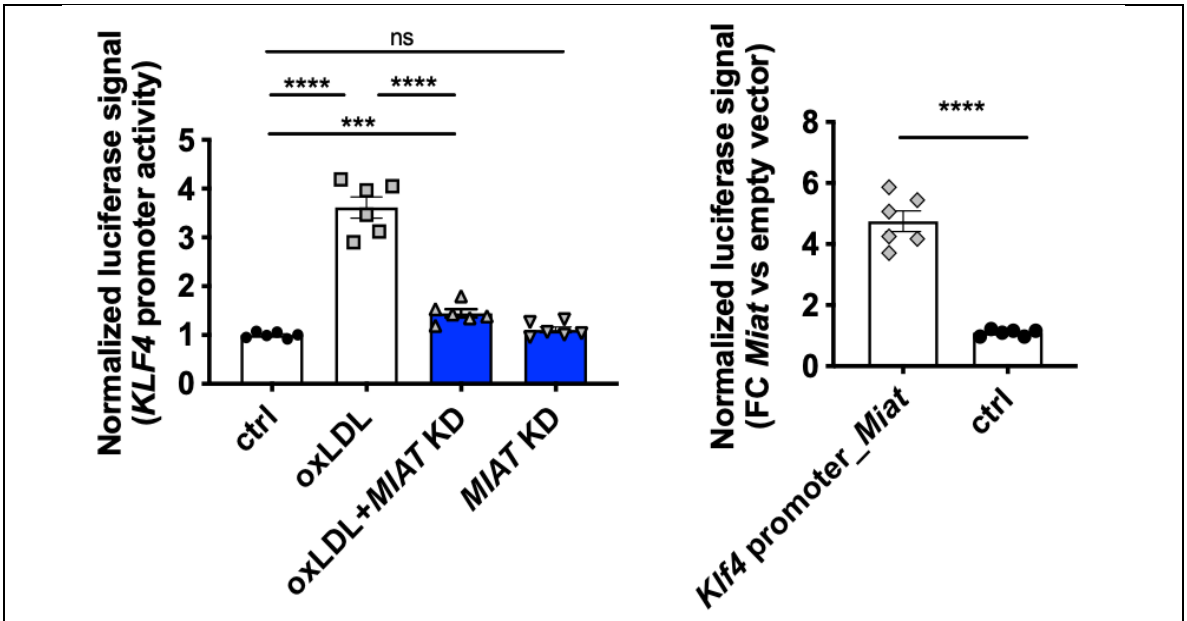

Figure 9. Left: Luciferase reporter assay with human *KLF4* promoter on oxLDL stimulation / *MIAT* knock-down (KD) in human carotid SMCs. **Right:** Luciferase reporter assay with murine *Klf4* promoter (containing *Miat* predicted binding sites; *Klf4 promoter_Miat*) or promoter flanking regions (harboring no predicted *Miat* binding sites; *ctrl*) in mouse aortic SMCs upon *Miat*overexpression (pCAG-*Miat*). *Figures 6E,F from study I.*

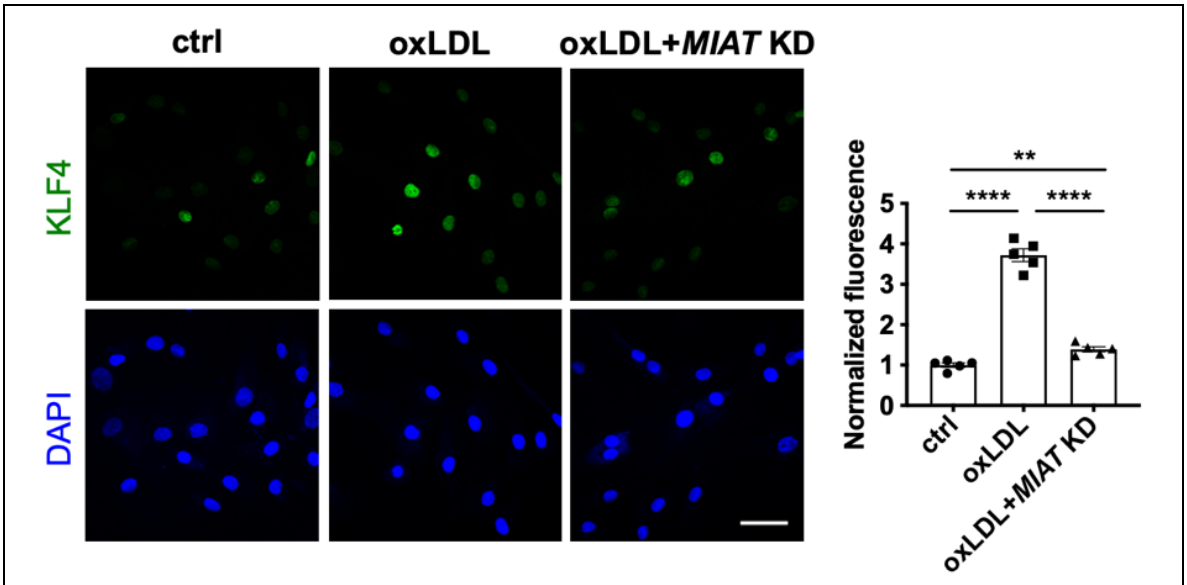

Figure 10. Left: Immunostaining of KLF4 in human carotid SMCs stimulated with oxLDL and with or without *MIAT* siRNA mediated knock-down. **Right:** fluorescence quantification. *Figure 6C from study I.*

KLF4 is a master regulator of VSMC phenotypic switching [68] through its negative role on MYOCD/SRF-mediated transcriptional control of contractile genes. Increased KLF4 expression has been implicated in trans-differentiation of contractile VSMCs into macrophage-like phenotypes [71]. In line with this, upon increased *MIAT* expression after oxLDL stimulation of human VSMCs (**Figure 11**), we observed a sharp decrease in expression of contractile markers *ACTA2, TAGLN, MYH11*, and sharp increases of macrophage markers *CD68, LGALS3* (**Figure 11**). This correlated to increases in expression of not only *KLF4,* but also *ELK1* and *HDAC2* (**Figure 11**), both of which are known to interact with the KLF4-MYOCD-SRF axis to induce contractile phenotype loss of VSMCs. Through further *in silico*

analysis, we identified potential lncRNA-protein interactions with ELK1 and EGR1, both parts of the ERK/MAPK-ELK1-EGR1 pathway. We were able to show that *MIAT* co-precipitated during immunoprecipitation with anti-ELK1 antibodies and nuclear levels of p-ELK1 were decreased upon *MIAT* knockdown in human VSMCs.

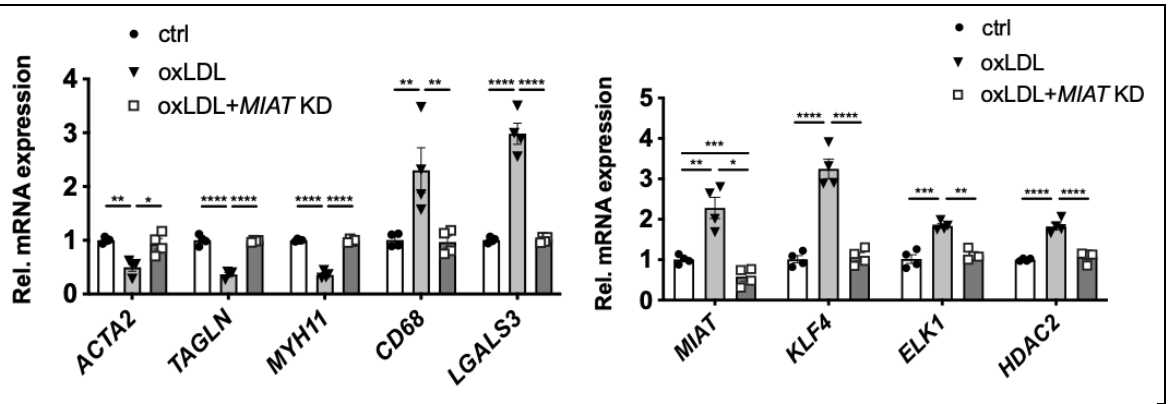

Figure 11. Left: Expression of SMC, phagocytosis and/or macrophage markers in human carotid SMCs stimulated with oxLDL, with or without MIAT knockdown (KD). **Right:** Expression of *MIAT*, transcription factor *KLF4* and its coeffectors *ELK1* and *HDAC2* in human carotid SMCs stimulated with oxLDL and with or without MIAT KD. *Figures 6A,B from study I.*

In **study II** we evaluated the lncRNA *NUDT6*, a NAT of *FGF2*. Increased expression of *NUDT6* leads to repressed levels of *FGF2* and thus also FGF2-mediated signaling. FGF2 is a growth factor implicated in the de-differentiation of VSMCs into a proliferative phenotype [304]. One mechanism for this effect of FGF2 is inhibition of TGF-β [305] through a let-7 miRNA-dependent mechanism [306, 307]. TGF-β-signaling plays a crucial role in the maintenance of VSMC contractile phenotype, and its loss leads to their de-differentiation [308]. TGF-β-signaling is reduced in unstable atherosclerotic plaques and has commonly been described as atheroprotective and a key modulator of vascular repair [309–311]. However, the overall role of TGF-β in vascular disease is complex and elusive, and its beneficial roles are considered to be context-dependent [309]. In AAA, several studies have shown TGF-β-inhibition to exacerbate experimental murine AAAs [312, 313]. If the effects of *FGF2* were to be dependent on its inhibition of TGF-β-signaling, one would therefore likely expect *FGF2* to yield similar results. On the contrary, treatment with FGF2 has been shown to limit the progression of experimental AAAs [314, 315]. In line with this, in study II, we were able to show that inhibition of *Nudt6* and thus resulting *Fgf2* increase lowered experimental plaque rupture rates and limited experimental AAA progression in mice (**Figure 12**). Therefore, other mechanisms are likely to contribute to these effects.

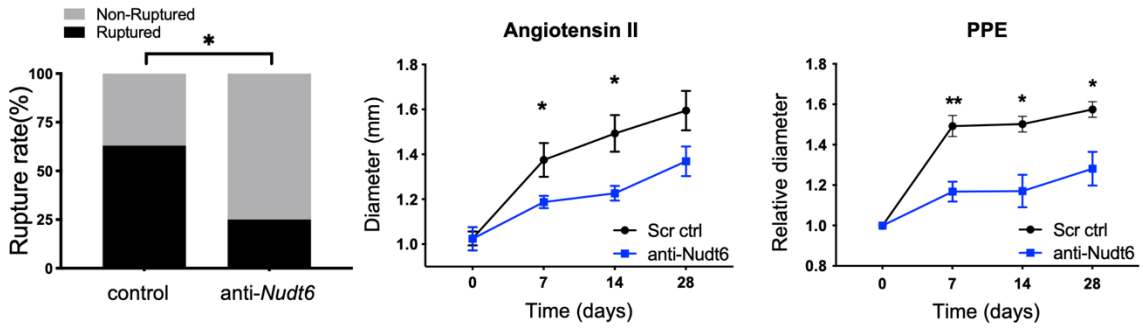

Figure 12. Left: Systemic Anti-*Nudt6* GapmeR treatment of *Apoe*$^{-/-}$ mice (n=20) significantly reduced plaque rupture in the inducible plaque rupture model compared to control (scramble-control treated) animals (n=19). **Middle:** In the angiotensin II model, local anti-*Nudt6* treatment (n=8) via ultrasound targeted microbubble destruction led to significantly lower abdominal aortic diameter and reduced growth compared to control treatment (n=13). **Right:** Systemic anti-*Nudt6* treatment (n=4) in the porcine pancreatic elastase mouse model significantly reduced abdominal aortic diameter and growth rate compared to control treatment (n=5). Mean+SEM. Data was analysed with using multiple t-tests.
Figures 3A,C,E from study II.

The aforementioned studies [314, 315] characterized the effects of FGF2 treatment to be mediated through its mitogenic properties, resulting in increased proliferation, survival of VSMCs, as well as an overall increased VSMC content. Kawai *et al.* [315] also reported sharply increased contractility of harvested aneurysmal aortas in animals treated with FGF2. This suggests that the protective effect of FGF2 is at least in part connected to its positive effect on the maintenance of VSMC contractile phenotype in experimental AAAs. In line with this, our *in vivo* experiments with *NUDT6* modulation in different animal models showed increases in VSMC content (**Figure 13**). *In vitro*, human VSMCs overexpressing *NUDT6* grew slower and displayed increased apoptotic activity (not shown; **figure 4G-H in study II**).

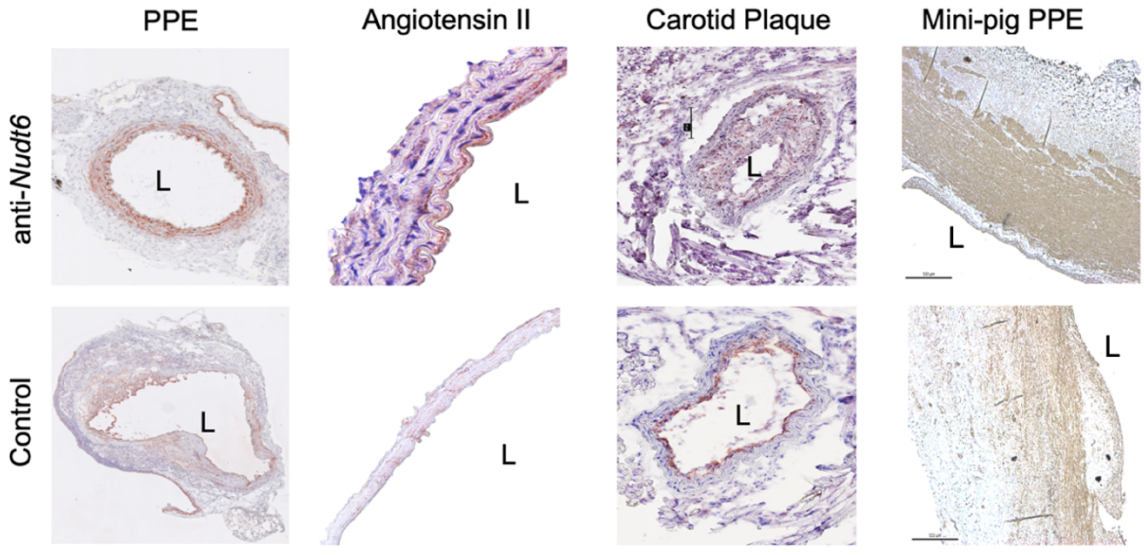

Figure 13. α-SMA immunohistochemical staining in three different murine models of cardiovascular diseases show restoration after anti-*Nudt6* GapmeR administration. **Abbreviations:** L – lumen; PPE – Porcine pancreatic elastase perfusion mouse AAA model; Angiotensin II – Angiotensin II infusion mouse AAA model; Carotid Plaque – mouse inducible carotid plaque rupture model; Mini-pig PPE – Porcine pancreatic elastase perfusion Yucatan mini-pig AAA model. *Figures 6A, S2B from study II.*

VEGF-signaling has also been implicated in the regulation of VSMC phenotype. A study by Liao *et al.* described the ability of VEGF to negatively regulate MYOCD-SRF activity through its induction of JAK-STAT3 signaling [316]. VEGF is otherwise known to be a key mediator

of hypoxia-induced angiogenesis [317]. In addition, it has been described to respond to other conditions present in vascular disease, e.g. increased levels of MCP-1 [318], a cytokine otherwise mainly known to be involved in the recruitment of monocytes into atherosclerotic lesions, and also expressed by macrophage-like VSMCs. Interestingly, JAK-STAT3 signaling has in turn been described to mediate IL-6 induced MCP-1 production by VSMCs [319], which might implicate certain feed-forward activity under atherosclerotic, inflammatory conditions. In **study III**, we showed that experimental murine and porcine AAA model animals receiving lenvatinib, a potent VEGFR2 inhibitor [275], had smaller AAA diameters and increased expression of contractile VSMCs markers (**Figure 14**).

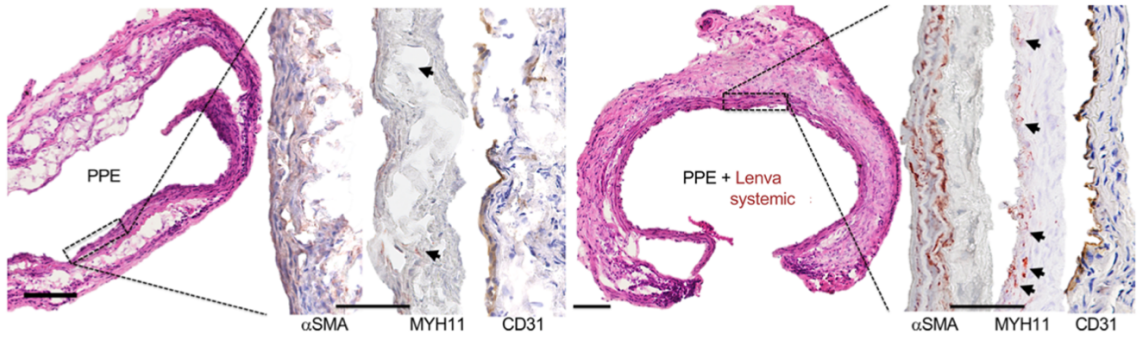

Figure 14. Left: Murine PPE aorta stained for α-SMA, MYH11, CD31. **Right:** Lenvatinib treated murine PPE aorta stained for α-SMA, MYH11, CD31. The overview images were stained using hematoxylin and eosin. *Figures 1D,E from study III.*

Upon treatment with lenvatinib, AAA-patient-derived VSMCs, showed enhanced contractility and had a higher expression of MYOCD and contractility markers (**Figure 15**). This effect could be mediated through the aforementioned VEGF-JAK-STAT3-axis, but when looking for potential other pathways that lenvatinib could be affecting, we noted a decrease in phosphorylation of ERK1-2 upon lenvatinib treatment in patient cells. ERK-signaling has the potential to inhibit MYOCD through several mechanisms. It can phosphorylate ELK1, which then competes with MYOCD for the same binding site on SRF [69], but has also been described to phosphorylate MYOCD, negatively affecting its ability to regulate transcription [320].

In **study IV**, we observed that increased miR-15a, which we identified to be a circulating biomarker of AAA, also seems to be involved in AAA pathogenesis and has the ability to affect the VSMC phenotype *in vitro* and *in vivo*. Through an established inhibitory effect on CDKN2B, its increased expression negatively affects VSMC viability *in vitro* [321]. However, we did not believe that this was the sole action mechanism of miR-15a, as miRNAs in general tend to have many gene targets and can assert regulatory roles on whole networks of genes [322, 323]. In an unbiased approach, we performed RNA sequencing-based gene expression profiling of miR-15a modulated (mimic and inhibitor) aortic VSMCs. By connecting this with previously published gene expression data of AAA patients, we were able to identify a large number of potential disease-relevant targets of miR-15a. However, perhaps most importantly, gene-set enrichment analysis revealed that VSMCs overexpressing miR-15a were of a more inflammatory phenotype (**Figure 16**) and experimental inhibition of miR-15a in the murine PPE model of AAA augmented aneurysm growth (**Figure 17**).

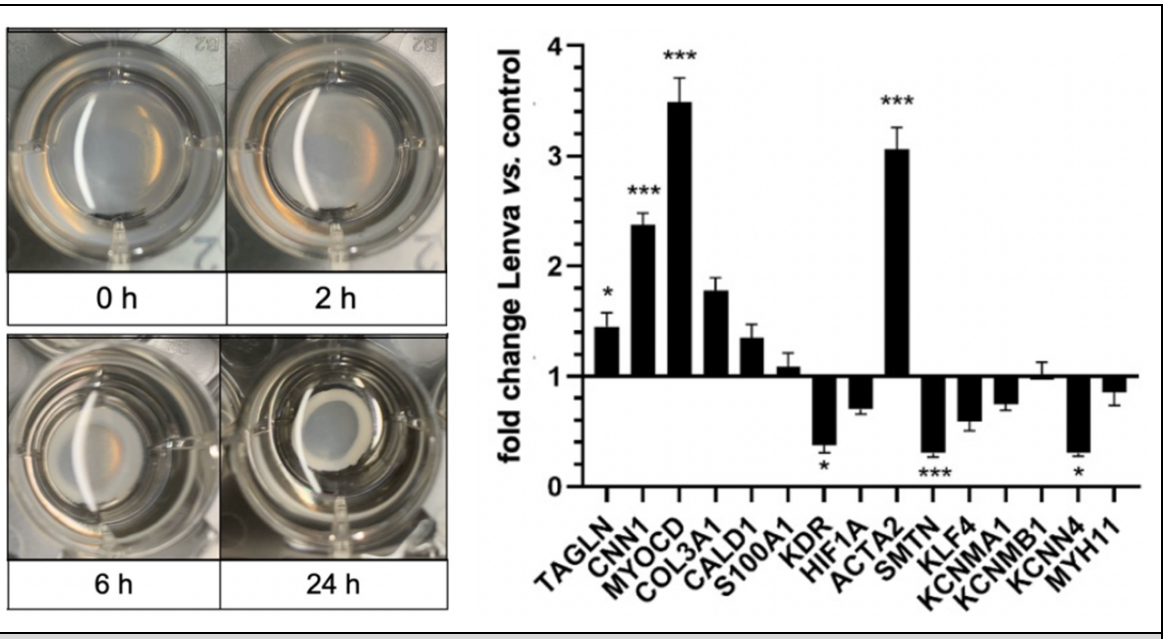

Figure 15. Left: Representative images of collagen matrices from the collagen contractility assay after treatment with lenvatinib in patient AAA derived VSMCs. **Right:** Gene expression at the 24h time-point of the contractility assay. *Figure S9C,D from study III.*

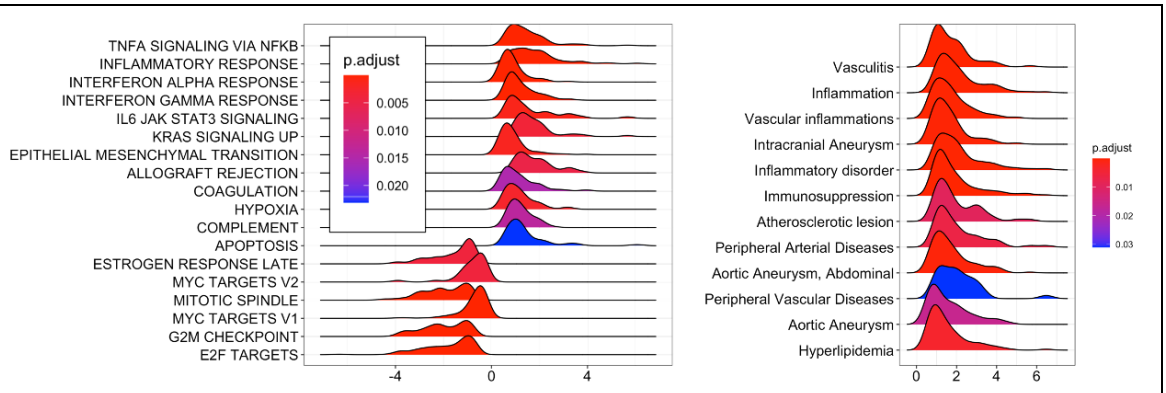

Figure 16. Left: Ridge plot of GSEA analysis results using MSigDB-H (hallmark) gene-sets. **Right:** Ridge plot of GSEA analysis results in manually selected DisGeNET gene-sets of relevant diseases. Both based on differential gene expression from RNA sequencing of human aortic smooth muscle cells transfected with miR-15a-mimic. *Figures 5A,E from study IV.*

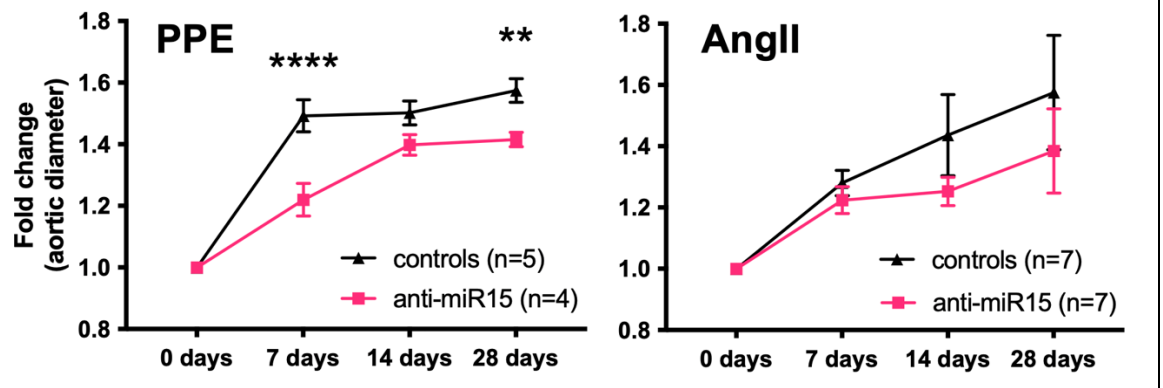

Figure 17. Left: Time-course of relative change in aortic diameter in PPE model mice treated with miR-15a inhibitor (anti-miR15; n=4) or scrambled control inhibitor (controls; n=5). **Right:** Time-course of relative change in aortic diameter in AngII model mice treated with miR-15a inhibitor (anti-miR15; n=7) or scrambled control inhibitor (controls; n=7). Mean ± SEM. *Figures 3A,B from study IV.*

There is no doubt that phenotypic polarization of VSMCs plays major roles in vascular pathologies. Our studies show that while maintenance of a certain (primarily contractile) phenotype can be beneficial, equally important is the ability of SMCs to de-differentiate into phenotypes that can stabilize late-stage plaques and AAAs. In the past decade, the regulatory roles of ncRNA on these processes have been greatly elucidated, contributing to a deeper understanding of VSMC phenotypic regulations in response to different conditions/stimuli.

4.3 TREATMENT OF VASCULAR DISEASE

Given the immense complexity of the cardiovascular system, in the era of personalized medicine, many are starting to realize that *one-fit-for-all* approaches are associated with limitations. In the past decades, we have learned that vascular diseases such as atherosclerosis and AAA more resemble cascades of interconnected pathological events rather than one-dimensional pathologies. With this comes the realization that regulation of specific disease-relevant processes might be beneficial at a certain point of the pathological process but highly detrimental at later stages.

In **study I**, we discovered that *MIAT* regulates KLF4-signaling and the ERK1-ELK1-EGR1-axis to promote a more proliferative, de-differentiated VSMC phenotype characterized by lower expression of contractile markers and drive them towards more macrophage-like phenotypes. It also affects NF-κB signaling, promoting the activation of pro-inflammatory macrophages. *In vivo*, in early disease stages, beneficial effects of *Miat*-inhibition on atherosclerotic plaque development were observed in mice (**Figure 18**). Given the similar dynamics of *MIAT* expression during porcine plaque development, it appears likely that a similar mechanism was present in our *LDLR*$^{-/-}$ Yucatan mini-pig model of atherosclerosis. However, when established, plaques of *Miat*-deficient mice were more likely to rupture (**Figure 18**). This further underlines the fact that vastly different therapeutic approaches might be required at various stages of vascular disease.

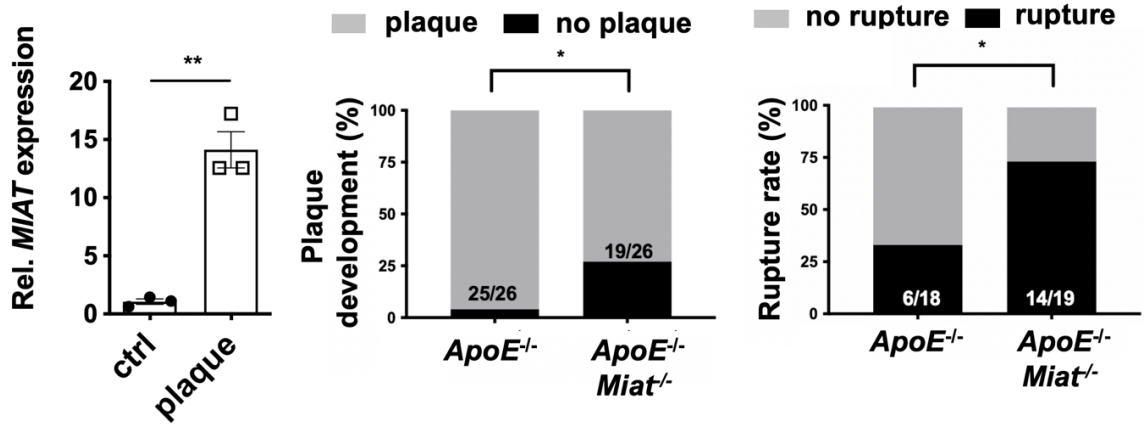

Figure 18. Left: Expression of *Miat* in the mouse inducible plaque rupture model. **Middle:** Plaque development (%) the inducible plaque rupture model in *Apoe*$^{-/-}$*Miat*$^{-/-}$ versus *Apoe*$^{-/-}$*Miat*$^{+/+}$ mice. **Right:** Rupture rate (%) in the inducible plaque rupture model comparing *Apoe*$^{-/-}$*Miat*$^{-/-}$ versus *Apoe*$^{-/-}$ (*Miat* wildtype) controls. *Figures 2B, 7F,G from study I.*

In **study II**, we studied the role of NAT *NUDT6*, an anti-sense partner of *FGF2*. We observed up-regulation of *FGF2* by the way of *NUDT6* inhibition *in vivo* and *in vitro*. *FGF2* possesses pro-mitogenic properties, and mice receiving *NUDT6*-inhibitors had reduced rupture plaque rupture rates and smaller AAA diameters (**Figure 12**). *FGF2* is also able to induce the MEK-ERK1/2 cascade and inhibit TGF-β. Both of these effects could be expected to promote a more de-differentiated, less contractile VSMC phenotype.

In **study III**, VEGFR2-inhibition, through treatment with lenvatinib, augmented aneurysm development in both murine and porcine AAA models (**Figure 19**) and contributed to the rescue of contractile gene expression otherwise down-regulated in these models (**Figures 14,19**). The treatment also reduced the proliferation of VSMCs, also characteristic for a more differentiated, quiescent phenotype.

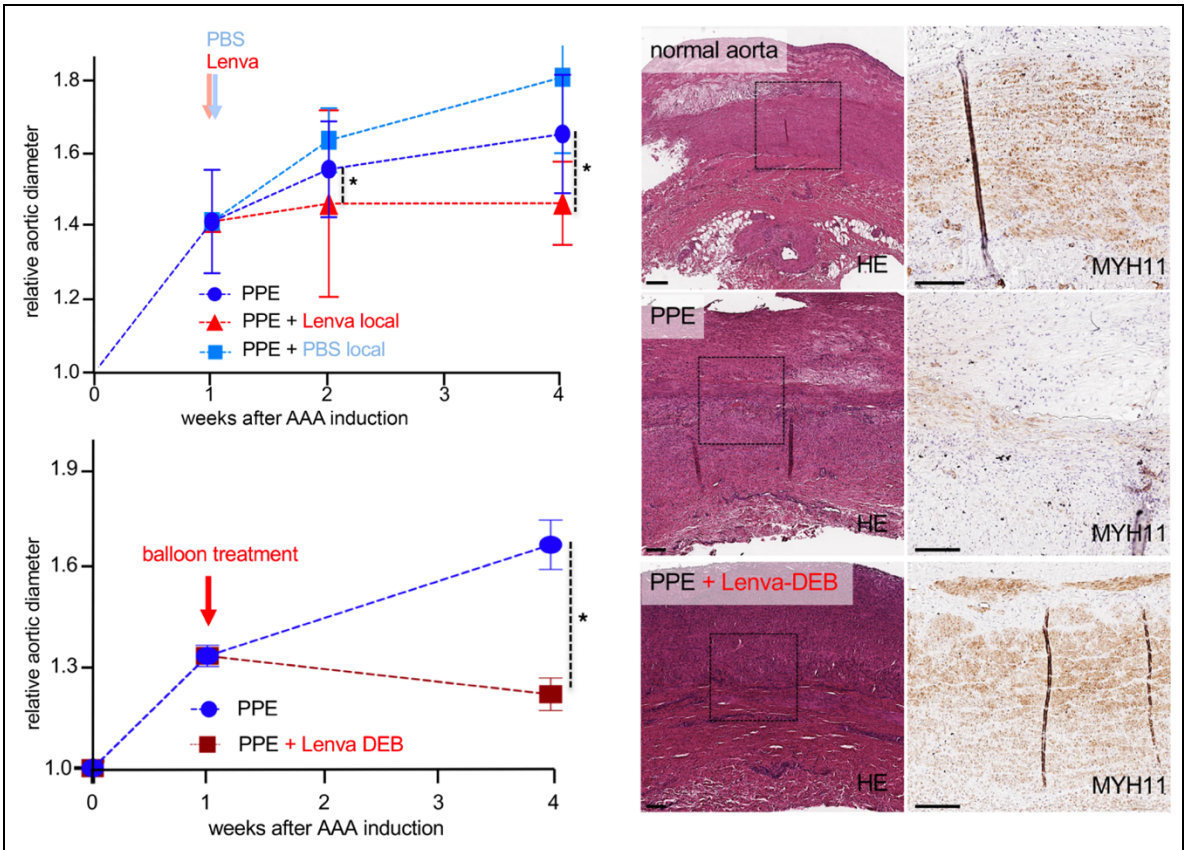

Figure 19. Top-left: Ultrasound measurements of aortic diameter of PPE mice locally treated with PBS (n=3), lenvatinib (n=5) or untreated/sham-reoperated (n=11). **Bottom-left:** Ultrasound measurements of aortic diameter of PPE *LDLR*$^{-/-}$ Yucatan mini-pigs treated with lenvatinib-coated drug-eluting balloon (DEB; n=4) or untreated (n=7). **Right:** Histological stains (hematoxylin and eosin, anti-MYH11 immunostain) of the PPE *LDLR*$^{-/-}$ Yucatan mini-pig aortas treated as above. Substantial MYH11 restoration can be observed upon local lenvatinib treatment. *Figures 2B, 5B,C from study III.*

Having slightly opposing roles on VSMC phenotypic polarization, the experimental treatment approaches in **studies I, II, and III** could therefore perhaps be perceived as contradictory, and the fact that all could achieve experimental success somewhat perplexing. However, given that we could successfully reproduce these approaches across several different animal models (and species), we believe them to be complementary. We argue that they are likely to be beneficial to the studied diseases at various stages and could be useful depending on the underlying disease phenotype and/or at different time points.

Given the fact that the experimental treatments are unlikely to be universally beneficial, and very likely to be harmful to other organs than the vasculature, methods of precise and tailored delivery are essential. For example, the mitogenic activity of *FGF2*, while protective in experimental late-stage atherosclerotic plaques and AAAs, is clearly detrimental in many other tissues. It has been described to promote cardiac hypertrophy and fibrosis [324–326] and several different cancers [327, 328]. The same can be said for lenvatinib, which would not constitute a plausible treatment for AAA through systemic delivery, due to associated severe adverse effects [329]. In **studies II** and **III**, we successfully employed different strategies for localized delivery of both traditional therapeutics (lenvatinib), but also anti-sense oligonucleotides (anti-*NUDT6* GapmeR). The fact that we could do so in more human-like models (Yucatan *LDLR*$^{-/-}$ mini-pigs) lends the approaches further translational credibility.

Non-coding RNA-based therapeutics, especially NATs and miRNAs, possess certain key advantages over other therapeutic mechanisms. Beneficial/harmful effects of different proteins (e.g. growth factors, receptors) on a plethora of diseases and conditions have been described. Inhibition of protein targets can be achieved through a variety of drugs. Monoclonal antibodies possess a highly selective affinity for a specific target. However, one drawback is that their target needs to be on the cell surface, and they can therefore not target intracellular proteins. Delivery of ncRNA therapeutics can be delivered into target cells through multiple mechanisms, some of which are explored in **studies II and III**. In addition, compared to protein inhibition, achieving up-regulation of target proteins is more complex, and many such targets have long been considered 'undruggable'. However, given that ncRNAs can be targeted and inhibited by ASOs, their mRNA targets can in this way be de-repressed. An example of such an approach is presented through **study II**, where expression of FGF2 is increased in the tissue of choice.

4.4 DIAGNOSIS AND MANAGEMENT OF AAA

While potentially lethal, AAAs develop and progress silently, a significant challenge in diagnosing them [330]. This makes it a disease suitable for screening, and several studies have shown one-time ultrasound screening to be beneficial for men aged 65 years or older [331, 332]. However, the evidence for screening is most substantial for patients who are, or have been, cigarette smokers, and the screening benefit of non-smokers is not as clear-cut. According to a recent WHO report [333], globally, tobacco use has decreased by roughly a third during the past 20 years, a trend that is expected to continue. Already today, the prevalence of smoking in Sweden is much lower, especially in the younger population [334]. This is likely one of the reasons why AAA prevalence in Sweden is lower than the reported prevalence worldwide. Nonetheless, the Swedish screening program of all men at the time they turn 65-years has so far been shown to be a cost-effective measure [130]. However, given the changing demographics and tobacco smoking patterns, this may well not be the case in the future. Screening for female AAA, less prevalent [122–124] but also more lethal, is currently not recommended [208, 332]. Rupture risk of female AAAs has also been shown to be harder to predict, and a larger percentage of small AAA rupture than in men [335]. Currently, most of

the risk-stratification and decision on which patients benefit from AAA surgery (either open or endovascular), aside from patients' overall health status, is solely based on AAA diameter. The development of novel tools to better approximate risks involved in managing each individual patient, as well as novel tools to diagnose AAAs, would undoubtedly be of value.

In **study IV**, we performed a discovery study looking for miRNA biomarkers in plasma from a large AAA patient cohort, identifying miR-15a as a potential disease biomarker. We were not only able to confirm the findings in other patient cohorts but saw that they also correlated to aneurysm diameter (**Figure 20**). Through treatment with miR-15a-inhibitors *in vivo*, we could limit experimental AAA growth in the murine PPE model (**Figure 17**). Previous studies on the role of miRNAs as potential AAA disease biomarkers have been performed, but thus far these studies have been largely underpowered and correlative. By showing that miR-15a is causally involved in AAA pathology, it is not only a potential biomarker but also a tool to better understand factors involved in AAA disease development and/or progression.

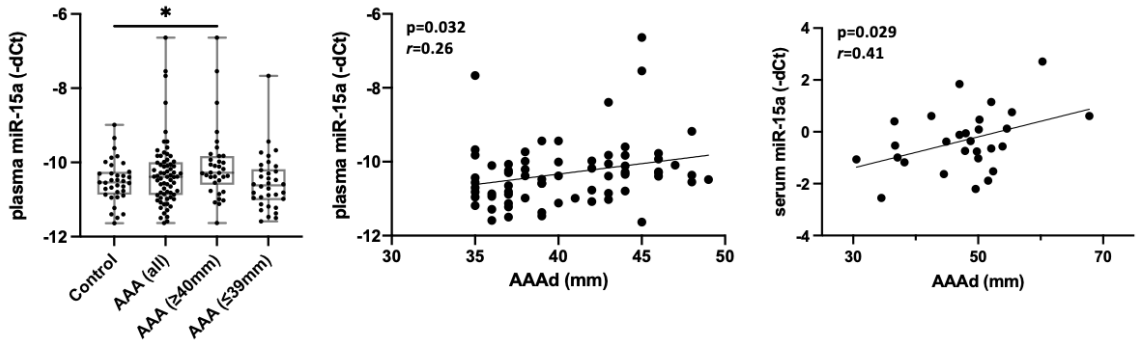

Figure 20. Left: Expression of miR-15a in plasma of AAA patients (Stockholm AAA screening cohort; n=68 for AAA of which n=34 for AAA ≥40mm and n=34 for AAA ≤39mm) and matched controls (n=35). **Middle:** Correlation of plasma miR-15a in AAA patients (Stockholm AAA screening cohort; n=68) and their AAA diameter (AAAd). **Right:** Correlation of serum miR-15a in AAA patients (Vienna AAA cohort; n=28) and their AAAd. *Figures 2D,E,G from study IV.*

4.5 LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

4.5.1 Patient material

Hypotheses of **studies I, II and IV** have been constructed on analyses of collected patient material. For studies of atherosclerotic lesions, samples from patients undergoing CEA were collected. During the procedure, the vessel is opened, and the intimal plaque is dissected from the surrounding tissue, after which the remainder of the vessel wall is left intact. Therefore, only the innermost parts (if any) of the medial layer will be extracted and available for analysis. In addition, control tissues often stem from non-atherosclerotic arteries in other regions (in our studies, iliac arteries), collected during other vascular procedures or from organ donors. On top of this, even if a healthy control specimen were to be collected, it would not be possible to divide the layers in a CEA-like fashion, other than perhaps by removal of the adventitial layer. When comparing patient CEA samples to control tissues, it is therefore important to be mindful of the differences in overall tissue composition. For example, due to the extensive presence of VSMCs and VSMC-like cells in the intima-located atherosclerotic lesion, it is feasible to assume that these cells will constitute a significant fraction of all VSMCs in patient tissues and a much smaller fraction in control tissues. However, if one aims to understand the mechanism driving the activation of these *'activated'* lesion VSMCs, then this approach is still likely to point in the right direction.

On the other hand, one would also expect some of these phenotypic changes to be important to the vascular homeostasis and VSMCs' ability to correctly respond to different stimuli. Some of the observed transcriptomic/proteomic changes are therefore likely to be beneficial. This question can hopefully better be answered by comparing stable and unstable plaques, and even more so if looking specifically at certain regions of interest, such as the fibrous cap.

Many of the above points can also be extrapolated onto AAA samples. Control samples most commonly stem from the aortas of e.g. organ donors. For understandable reasons, the organ donor population is often healthier and, most importantly, much younger on average. As age is one of the biggest risk factors of AAA, this is certainly a problem to be aware of. To aid this, through the MVB, we have recently started collecting aortic tissue from non-dilated and dilated parts of aortas from the same patient, whenever possible. This type of paired material has been used for analyses in studies **II and IV**.

The same problem applies to commercially available cell cultures created from aortic tissues of organ donors. Manufacturers either do not discriminate between cells from abdominal or thoracic aorta, or more commonly, only provide cells collected from the thoracic regions. During our studies, we have not been able to purchase explicitly abdominal primary cell cultures commercially. Given that these two regions of the aorta have different embryonic origins, care must be taken when drawing conclusions from *in vitro* studies on AAA-related mechanisms performed on thoracic primary SMC cultures. In **study III**, in which primary patient cells were isolated, we did often observe different responses to pathological stimuli than in commercially purchased primary cell cultures.

4.5.2 Animal models

The advantages and disadvantages of the different animal models are briefly discussed in **section 1.5.2**. While all aspects of the human disease are hard to mimic, translational research would not be possible without the use of animals. Our group has developed a modified plaque rupture model, which we believe gives us a closer understanding of mechanisms governing plaque stability and rupture. In studies of AAA pathogenesis, we believe that the AngII and PPE models, while both carrying significant limitations, can answer different questions about the effects of different mechanisms or treatments on disease phenotypes. The described transgenic mini-pig model (*LDLR*$^{-/-}$ Yucatan mini-pigs), with more human-like physiology, adds an additional dimension to these studies. A further limitation of animal models is that the disease time course is profoundly accelerated, especially as both atherosclerosis and AAA develop slowly over time in humans. In contrast to human disease, the model atherosclerotic plaques do not become as advanced and complex. There are also apparent differences in, especially murine, coagulation and immune systems, preventing observations of disease-specific outcomes.

Nonetheless, it needs to be acknowledged that a lot of knowledge regarding novel concepts of vascular disease has been built based on animal (primarily mouse) studies. Additional efforts are required to ensure that these knowledge gains can be extrapolated onto human disease.

4.5.3 Use of ncRNA therapies

Much evidence points towards that ncRNA-therapies are indeed possible and could break ground for therapeutic approaches that have not been possible or feasible before (further discussed in the following section). While not explicitly ncRNA-based, the mRNA-based COVID-19 vaccines have certainly accelerated interest in nucleotide-based therapies.

Yet, while the actions of mRNA are known and more or less straightforward, we are still far from a complete understanding of all the roles that ncRNAs play in human biology. It is still common for new mechanisms of function to be described for already characterized lncRNAs. The relative novelty of the field and consequently our still limited understanding of lncRNA action mechanisms raises the potential for unexpected adverse effects. Here, we believe that targeted local delivery of potential lncRNA therapeutics might be an important tool to overcome these obstacles. In addition, many ncRNAs tend to be expressed in relatively low concentrations during physiological states. In contrast, when supplying additional exogenous ncRNAs as a treatment strategy, these are often delivered in supraphysiological concentrations, many orders of magnitude higher than what could be achieved through the intracellular processes of their synthesis. Perhaps for this reason, the majority of RNA therapeutics that are approved by the FDA/EMA or are undergoing phase II/III trials, are ASO based, and function through inhibition of target genes [336].

5 POINTS OF PERSPECTIVE

A lot of resources in the past decades have been dedicated to gaining a better understanding of the critical processes that drive vascular disease [337]. While the studies in this thesis have looked at two correlated but seemingly different [338] vascular pathologies – atherosclerosis and AAA, the critical role that VSMCs play in these diseases has undoubtedly emerged as a common theme. The recent studies of diseased-state VSMC origins, aided by the advancements of *in vivo* cellular lineage-tracing technologies, have reaffirmed the long-existing suspicion that these cells heavily contribute to disease phenotypes [57, 59, 68, 71, 80, 92, 94, 339, 340].

Looking at atherosclerosis, successful therapeutic approaches have been focused on regulating lipid metabolism and/or reducing inflammation [341, 342]. Meanwhile, no pharmacological treatment is available for patients with AAA [343]. This is especially problematic for patients with small, asymptomatic AAAs or patients who for other reasons are not candidates for surgical treatment. Effective medical therapies could also help avoid risks associated with surgical AAA repair. Given the vast importance VSMC dynamics seem to play in these pathologies, treatment strategies targeting VSMC phenotypic modulation seem promising. In the cancer field, the leader of experimental therapeutic efforts, many treatments have been focused on inhibiting protein interactions through antibody-based therapies or small molecules. Transcription factors, enzymes, and signaling pathways, involved in vascular disease are feasible targets for novel treatment approaches. An example of such a treatment is evolocumab, a monoclonal antibody that targets PCSK9, approved for clinical use in 2015 [344]. Inhibition of PCSK9 increases the expression of LDL-receptors in the liver, resulting in a striking decrease of circulating LDL-particles. However, there are also many feasible targets with potential protective functions. An example of such a target is FGF2 (evaluated in **study II**), known to positively affect VSMC proliferation and survival [345, 346]. Induction of increased expression of these targets is not possible through the aforementioned approaches.

RNA-based therapeutics provide a potential solution to this problem, with a number of RNA-interference-based therapeutics currently being evaluated in phase II and III clinical trials [336]. By targeting lncRNAs such as NATs (e.g. *NUDT6* in **study II**), expression of mRNA partners can be de-repressed and increased protein expression induced. No clinical trials have yet been initiated for lncRNAs, but several newly discovered lncRNAs with the ability to regulate the phenotype of VSMCs certainly seem like promising targets.

Systemic treatments can only be developed against targets that are either: a) specific to a particular tissue, b) have actions that are uniformly beneficial or detrimental in all types of tissues, or c) where the significant adverse effects can be tolerated – e.g. anti-cancer drugs. A potential solution to this problem is targeted drug delivery, demonstrated in **studies II and III** through DEBs or ultrasound targeted microbubble destruction (UTMD). While certain VSMC-specific lncRNAs (e.g. *CARMN*) exist, several CVD-relevant lncRNAs are expressed in other tissues [261, 263]. Without targeted drug delivery, many treatments are likely to fail due to undesirable adverse effects.

The academic community is currently suffering from a *'reproducibility crisis'* [347]. This term pertains mainly to the inability to reproduce research findings using identical or similar methods. However, there is also a translational aspect to this problem. The development of potential therapeutics is highly dependent on the ability to bridge results of *in vitro* and *in vivo* studies into clinical practice. Today, most researchers are hopefully aware that findings in murine models do not necessarily translate into human pathologies [348]. Nonetheless, many of these results are often taken for granted when describing the state of knowledge within the field. To truly understand the pathologies in question, better disease models are required. Translational animal models (such as the *LDLR*$^{-/-}$ Yucatan mini-pig model of atherosclerosis and AAA used in **studies I, II, and III**) are only one aspect of this. More relevant *in vitro* approaches and techniques are just as important. This can be performed through the use of patient cells (as in **studies II and III**), through better matching of patient and control tissues (**studies II and IV**), but also in many other ways – e.g. organs-on-chips, organoids, and other advanced cell co-culture approaches [349].

Not only medical therapeutics can be targeted better. Currently, in many cases, it is difficult to know ahead of time how much a patient will benefit from the surgical treatment and whether this exceeds the dangers associated with surgery. This is true for AAA repair (small aneurysms, women patients, older/fragile patients) as well as for CEA (asymptomatic carotid stenosis, low-moderate symptomatic carotid stenosis). The positive outcomes are also measured for a whole group of patients. What this means is that many (if not the majority) of these patients would not have adverse effects without surgical intervention. However, it is currently impossible to risk-stratify them closer than on a group level. Therefore, much can be gained from developing novel tools of risk stratification. A better selection of patients to undergo intervention would lead to fewer adverse events and likely to significant cost-savings – resources that can be repurposed for the care of patients who need it most. This is especially true for countries with underdeveloped healthcare systems or without universal healthcare, where the ability to treat certain patient groups is limited by economic factors.

Ultrasound-based screening for AAA is a cost-efficient way of preventing AAA-rupture-related deaths. However, its effectiveness diminishes in the absence of risk factors such as smoking or male sex [125, 129, 331]. Much effort has been put into studying potential protein biomarkers [194]. Although several seem promising, none are yet close to being used in clinical practice [194]. The fact that ncRNAs are involved in AAA disease and abundant in circulation makes them interesting and promising targets. The constant development of novel genomic technologies is likely to eventually allow for the screening of many different ncRNAs, an approach that may turn out more powerful and sensitive than existing proteomic approaches. miRNAs seem like promising disease biomarkers, given their ability to regulate whole networks of genes (as is the case for miR-15a in **study IV**) and their ease of detection in circulation. Many potential ncRNA biomarkers have been implicated in cancer [350, 351], and there is no reason to believe this will not be the case in CVD.

6 CONCLUSIONS

The overall aim of this thesis was to evaluate the role and therapeutic potential of novel ncRNAs associated with AAA disease development and progression. Through the constituent studies we were able to conclude the following:

Study I. Long non-coding RNA *MIAT* has is able to regulate proliferation and transdifferentiation of arterial smooth muscle cells and inflammatory activity in macrophages during atherosclerotic plaque development and progression.

Study II. Natural antisense transcript *NUDT6* is able to regulate SMC survival *in vitro*, through its inhibition of growth factor *FGF2*. *NUDT6*-inhibition is a successful treatment approach in advanced pre-clinical models of plaque rupture and AAA.

Study III. Local treatment with lenvatinib is able to inhibit AAA progression *in vivo* in both murine and translational porcine animal models, through the restoration of VSMCs contractility.

Study IV. miR-15a is a disease-relevant circulating biomarker of AAA, and its increased expression in AAA patient aortas has a pro-inflammatory effect on VSMCs. Inhibition of miR-15a in the murine PPE model of AAA augments diameter growth.

An additional conclusion of **studies II and III** is that local delivery of ncRNA-therapeutics through either DEBs or UTMD-delivery is a feasible therapeutic approach for the treatment of AAA and likely also feasible for the treatment of advanced atherosclerotic lesions.

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