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## Small regulatory RNAs in vascular remodeling and atherosclerosis

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# Chapter 1

## General Introduction

## General introduction

### Cardiovascular disease

Cardiovascular disease (CVD) is a major cause of death worldwide, representing 31% of all global deaths<sup>1</sup>. The heart and blood vessels, called the cardiovascular system, circulate blood through the body in order to deliver oxygen and nutrients and to remove waste products. CVD is a collective term for multiple diseases affecting the cardiovascular system, including myocardial infarction, ischemic stroke and peripheral arterial disease (PAD).

#### *Atherosclerosis*

The underlying cause of most CVD is atherosclerosis. Atherosclerosis is a chronic inflammatory disease, characterized by progressive plaque build-up in the arterial wall. Plaques are composed of lipids and inflammatory cells, and develop in the subendothelial intimal layer in large- and medium-sized arteries<sup>2, 3</sup>. Most plaques that develop during a person's life remain clinically silent. Patients generally only start developing symptoms when the artery becomes severely narrowed (stenosis of over 70%) or when a plaque ruptures. The latter causes acute occlusion of the artery and patients often present with more severe symptoms that can even be fatal, i.e. a myocardial infarction and ischemic stroke. Full rupture of a plaque or plaque erosion (this is when a superficial piece of the plaque breaks off) triggers thrombus formation in the artery. The thrombus partially or completely occludes the artery, blocks blood flow downstream and causes acute lack of oxygen and nutrients in the affected area, called an infarction<sup>4-6</sup>. Oxygen and nutrients are crucial for tissue survival and function<sup>3, 8</sup>. Immediate action is therefore needed to restore blood flow and to limit adverse consequences, including cell death and permanent damage in the ischemic tissues<sup>10, 11</sup>.

#### *Risk factors and current therapies*

Risk factors for atherosclerosis-induced CVD are hypercholesterolemia, diabetes, obesity, genetic predisposition, hypertension and age<sup>11</sup>. Many of these risk factors can be the result of an unhealthy lifestyle, such as high fat, cholesterol and salt intake, smoking and lack of physical activity. Healthy lifestyle changes can therefore help in the prevention of CVD. Plasma lipid lowering drugs (e.g. statins), platelet inhibitors and antihypertensives are examples of widely used medications to lower the risk of a (recurrent) cardiovascular event<sup>11-13</sup>. Once a cardiovascular event has occurred, therapies are aimed at restoring blood flow in order to prevent recurrence and support the affected organ, such as the heart.

Thrombus dissolving drugs (thrombolytics) and surgical interventions are current therapies to remove the arterial blockage<sup>4, 10, 14</sup>.

Balloon angioplasty with or without stent placement is often used to treat a myocardial infarction<sup>10</sup>. These endovascular interventions can restore blood flow quickly. A disadvantage of these endovascular interventions however, is the high risk of recurrent occlusions. An alternative surgical procedure is bypass placement. Bypass surgery is less favorable in the acute situation, but can be performed when endovascular interventions are not successful (e.g. recurrent occlusions) or possible. Often the saphenous vein from the patient's own leg is used as a bypass graft. Bypasses made from venous vessels are more easily available than arterial vessels. A disadvantage of venous bypasses however, is the high risk of reocclusion of the vein graft. In this case a second intervention is required, although this is not always possible<sup>15-17</sup>.

An endarterectomy of the carotid artery in the neck is performed to prevent an ischemic stroke. An endarterectomy is a surgical procedure to remove the plaque from the arterial wall. This is a risky surgical procedure and therefore only performed if the potential benefit of the intervention outweighs the potential adverse peri-operative risks. Criteria are when the plaque is causing significant stenosis (>70%) or when the plaque is symptomatic and causes transient ischemic attacks (TIAs). In many cases, however, symptoms occur during an ischemic stroke, making prevention no longer possible. Once a patient is diagnosed with ischemic stroke, often thrombolytics are used to dissolve the thrombus that blocks blood supply to part of the brain<sup>4</sup>.

The presentation of PAD varies and includes acute and chronic presentations. In PAD patients, symptoms are caused by acute blockage or gradual narrowing (i.e. chronic limb ischemia) of a peripheral artery. Blockage of the artery leads to distal ischemia with symptoms such as severe pain during exercise or even in rest, coldness and numbness in the affected limb and non-healing ulcers. Neovascularization is the formation of new blood vessels and the body's own capacity to restore blood flow. In the more severe PAD patients, however, this capacity is not sufficient to restore blood flow. Surgical interventions are required to restore blood flow in these patients. Prolonged lack of blood supply may eventually lead to cell death, non-healing wounds and gangrene in the affected limb. Amputation of the affected limb is then needed to release a patient from pain and to prevent life-threatening sepsis<sup>14, 18</sup>.

Even though current therapeutic strategies succeed in restoring blood flow and contribute to reduce the risk of (recurrent) cardiovascular events, a need remains to improve clinical outcome.

### **Pathogenesis of atherosclerosis**

Traditionally, atherosclerosis was seen as a cholesterol storage disease in the intimal layer of arteries. Nowadays, it is increasingly recognized that atherosclerosis is largely driven by inflammation too<sup>2, 3, 19</sup>. In the initial phase of plaque development low-density lipoprotein (LDL) particles enter the intima and start to accumulate in the arterial wall, where they become oxidized (oxLDL). Endothelial cells lining the inner layer of the vessel wall undergo inflammatory activation. Circulating monocytes and other leukocytes bind to the endothelium and extravasate into the lesion. Once in the lesion, these inflammatory cells start secreting chemokines in order to recruit more inflammatory cells. Monocytes that entered the lesion from the blood stream differentiate into tissue macrophages, which then start to internalize oxLDL and eventually can become lipid-laden foam cells. Foam cells are a hallmark of an atherosclerotic plaque. Dying foam cells form the necrotic core, which is often located in the center of the plaque. In the growing plaque, smooth muscle cells (SMCs) start to proliferate and migrate towards the outer layer of the plaque to form a fibrous cap. The fibrous cap is the structural support of the plaque and is predominantly composed of SMCs and extracellular matrix proteins produced by these SMCs, such as collagen and elastin. Local intra-plaque inflammation however, triggers secretion of matrix metalloproteinases (MMPs). MMPs degrade the matrix in the fibrous cap, thus reducing the structural barrier of the plaque and increasing the risk of plaque rupture. A growing necrotic core, a thinning fibrous cap and persistent inflammation contribute to a vulnerable plaque phenotype<sup>20, 21</sup>.

Inhibiting plaque progression and increasing stability of existing atherosclerotic plaques are potential therapeutic strategies to reduce the risk of rupture and its clinical consequences. Reducing intra-plaque inflammation and presence of a thick fibrous cap makes a plaque less vulnerable and thus, less prone to rupture<sup>6</sup>. Since atherosclerosis is a complex, multifactorial disease, most likely therapeutic strategies targeting multiple aspects of the disease are needed rather than single-factor therapeutics to reduce atherosclerosis and thereby the risk of a (recurrent) cardiovascular event.

Many cell types of both the innate and adaptive immune system are involved in the development and progression of atherosclerosis<sup>3</sup>. In this thesis, we focused on two different cell types in atherosclerosis, namely macrophages and blood platelets.

### *Macrophages*

Macrophages play a key role in the onset and progression of atherosclerosis. After differentiation from monocytes, macrophages can polarize into different subtypes within the plaque. Macrophages continuously adapt their functional phenotype in response to environmental stimuli. At the broadest sense, macrophage subsets are classified into proinflammatory M1 or anti-inflammatory M2 macrophages. However, macrophage phenotypes *in vivo* are rather a continuum than either M1 or M2, and many different intermediate subtypes exist<sup>22-24</sup>. Macrophages expressing M1 markers are the predominant subtype in atherosclerotic plaques and promote plaque progression. M2 macrophages counteract inflammation and are associated with an anti-atherogenic response<sup>25</sup>.

Because of their dynamic plasticity and key role in atherosclerosis, macrophages are an attractive therapeutic target to potentially resolve atherosclerosis. Several cellular pathways have been shown to either promote or inhibit inflammatory phenotypes of macrophages<sup>26, 27</sup>. For instance, activation of Toll-like receptor 4 (TLR4) induces the nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway and promotes proinflammatory M1 polarization<sup>28</sup>. On the other hand, the interleukin-4 (IL-4)/ signal transducer and activator of transcription (STAT) 6 pathway regulates the expression of pro-resolving genes (i.e. the mannose receptor CD206) and promotes M2 polarization<sup>29, 30</sup>. Inhibiting M1 polarization and enhancing M2 polarization, e.g. via macrophage activation and polarization pathways, is both favorable in reducing plaque development and increasing stability.

### *Blood platelets*

Blood platelets are well-known from their function in hemostasis and blood coagulation, but their function goes beyond that. In fact, platelets are highly involved in proinflammatory responses<sup>31</sup>. Platelets are cells without a nucleus. They derive from a large precursor cell, the megakaryocyte. Anuclear platelets lack novel gene transcription, but are capable of splicing and protein synthesis with their repertoire of (pre)mRNAs derived from the megakaryocyte<sup>32</sup>. The lifespan of a human platelet is between 8 and 12 days. Senescent platelets are removed from the circulation and mostly degraded by the liver and spleen<sup>33</sup>. In atherogenesis, platelets can bind to the activated endothelium, secret chemokines to boost inflammation and facilitate extravasation of immune cells, like monocytes and

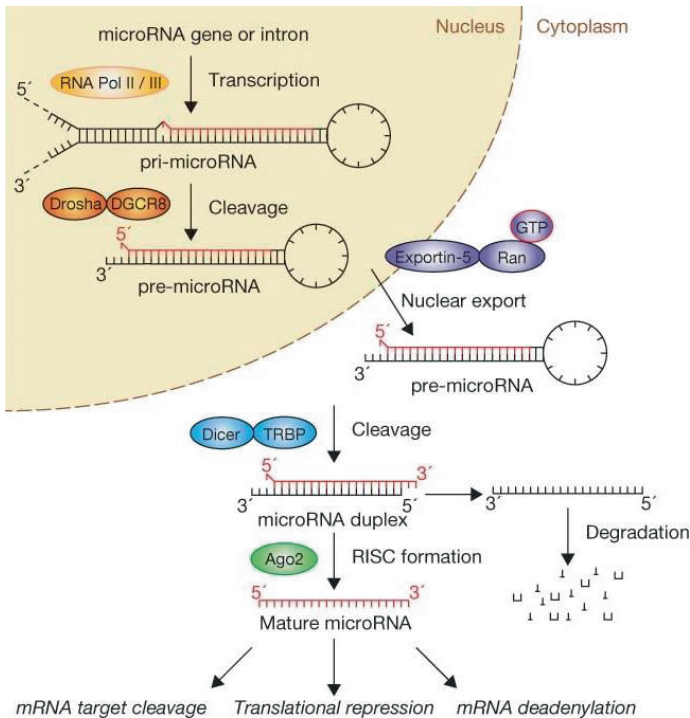
neutrophils, into the lesion<sup>34-36</sup>. These insights from basic science indicate that treatment with antiplatelet drugs would be beneficial in reducing the risk of a cardiovascular event. However, the use of antiplatelet drugs is limited by the increased risk of bleeding<sup>31</sup>. Yet, no recommendation for the use of antiplatelet drugs in primary prevention of CVD exists, though the identification of novel targets to reduce platelet inflammation without increasing bleeding risk, may be promising for future treatments.

### **Noncoding RNAs**

Noncoding RNAs (ncRNAs) are RNAs that are not translated into protein. Over 97% of the human transcriptome consists of ncRNAs. For years, parts of the human genome that do not encode proteins were considered junk DNA. However, over the past 2 decades there has been increasing evidence that ncRNAs are crucial in regulating gene expression and fulfill functions in different pathologies, including CVD<sup>37, 38</sup>. ncRNAs target and regulate expression of other RNAs. Based on their size, ncRNA species are classified in either small or long ncRNAs (either shorter or longer than 200 nucleotides in length, respectively)<sup>39</sup>. There are many different types of ncRNAs, which perform various functions. This thesis will focus on microRNAs, small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs).

#### *MicroRNAs*

A type of ncRNA that received much attention over the past few years in CVD are microRNAs. MicroRNAs regulate expression of their target genes at the posttranscriptional level. MicroRNAs are small molecules of about 22 nucleotides in length<sup>40</sup>. An overview of the microRNA biogenesis is shown in **Figure 1**. The biogenesis of microRNAs starts with transcription of a microRNA gene by RNA polymerase II or III. The resulting transcript, a primary microRNA, is processed into precursor microRNA. The precursor microRNA is exported out of the nucleus into the cytoplasm, where it is further processed into a microRNA duplex with two mature microRNA strands. One strand of the mature microRNA is incorporated in the RNA-induced silencing complex (RISC). Once in RISC, microRNAs hybridize with their seed sequence to complementary sequences in the 3' untranslated region (3'UTR) of their target messenger RNAs (mRNAs). This binding inhibits translation of target mRNAs into protein<sup>41, 42</sup>. MicroRNAs down tune expression of their target genes rather than completely silencing them. However, one microRNA has multiple target genes. Therefore, changing expression of one microRNA affects expression levels of multiple target genes simultaneously<sup>40</sup>. Modulating microRNA expression can thereby have major impact on complex cellular pathways and multifactorial diseases, including atherosclerosis.



**Figure 1. Schematic overview of microRNA processing.** The biogenesis of microRNAs starts with transcription of a microRNA gene by RNA polymerase II or III, forming a primary microRNA transcript (pri-microRNA). Pri-microRNA is cleaved into precursor microRNA (pre-microRNA) by the microprocessor complex Drosha-DGCR8 in the nucleus. The pre-microRNA is exported out of the nucleus by Exportin-5-RAN-GTP. Dicer-TRBP complex processes the pre-microRNA to its mature length by cleaving the hairpin structure. The functional strand (in red) is loaded into the RNA-induced silencing complex (RISC) together with Argonaute 2 (Ago2), where it performs its function through mRNA cleavage, translational repression and mRNA deadenylation. The other strand (in black) is degraded. Figure adapted from Winter et al<sup>9</sup>.

### Small nucleolar RNAs

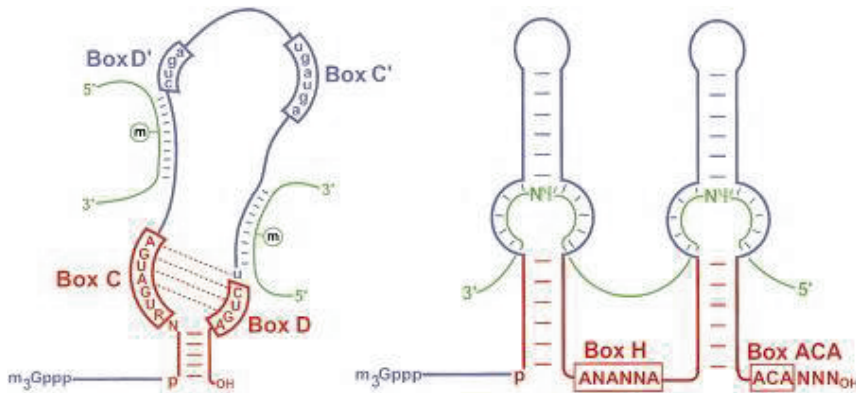
SnRNAs are a relatively unexplored type of small ncRNA in the cardiovascular field. Their presence in both eukaryotes and archaea indicate a common ancestor, implying that snRNAs are one of the most evolutionarily ancient types of RNA. Furthermore, the canonical function of snRNAs and the set of proteins they associate with, is the same in archaea and all eukaryotic lineages<sup>43-45</sup>. SnRNAs mediate site-specific RNA modifications of their target RNAs. SnRNAs are 60-300 nucleotides in length and are classified as either C/D box or H/ACA box snRNAs. A schematic overview of both snRNA structures is shown in **Figure 2**. Most snRNAs contain two sets of conserved C/D or two sets of conserved H/ACA boxes, and two sets of antisense boxes. Both snRNA species have a specific topology and



form a complex with ribonucleoproteins to mediate RNA modifications. C/D box snoRNAs guide 2'-O-ribose methylation (2'Ome) and H/ACA snoRNAs guide RNA pseudouridylation ( $\Psi$ ). Both types of RNA modifications are abundantly present on target RNAs<sup>44, 46, 47</sup>.

C/D box snoRNAs associate with ribonucleoproteins NHP2L1, NOP56, NOP58 and Fibrillarin (FBL). FBL is a methyltransferase that catalyzes 2'Ome. SnoRNAs hybridize to target RNA via Watson-Crick base-pairing with their antisense box. The 5<sup>th</sup> nucleotide upstream of D or D' box is positioned for 2'Ome by FBL. A canonical C/D box snoRNA target is ribosomal RNA (rRNA), but many C/D box snoRNAs antisense sequences do not match with known rRNA 2'Ome sites. These snoRNAs have no known targets and thus are considered orphan<sup>47, 48</sup>.

The canonical function of snoRNAs is well-known, however, other non-canonical targets and functions are currently being discovered. Some suggest that orphan snoRNAs can perform 2'Ome on other, non-canonical targets, like mRNA<sup>49</sup>. Others demonstrate that orphan snoRNAs can perform entirely different functions than guiding 2'Ome, such as mediating alternative splicing and 3'UTR processing<sup>50, 51</sup>. Some C/D box snoRNAs are processed into smaller fragments and exert microRNA-like functions by binding to mRNA 3'UTRs and inhibiting translation<sup>52</sup>. Dysregulation of snoRNAs is associated with clinically relevant events, including CVD<sup>53</sup>, which implies that they have a regulatory role in diseases. In-depth research into their mechanism of action is needed to investigate whether orphan snoRNAs could serve as novel therapeutical targets.



**Figure 2. Schematic structure of C/D box and H/ACA box small nucleolar RNAs.** (Left) C/D box snoRNA containing box C and D motifs. The C' and D' boxes represent internal copies of the C and D boxes. The two antisense boxes are located upstream of the D and D' box. The 2'-O-methylation site is marked with a circled m. Nucleotides interacting in the C and D boxes are indicated with a dashed line. (Right) H/ACA snoRNA containing H and ACA boxes. The unpaired uridine of the target RNA is located about 15 nucleotides from the H or ACA box of the snoRNA. The pseudouridylation site is marked with  $\Psi$ . Target RNA is shown in green. Regions essential for snoRNA location and processing are highlighted in red. Figure adapted from Kiss<sup>7</sup>.

### Transfer RNAs

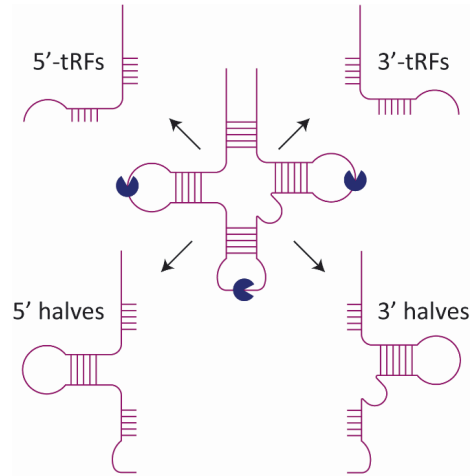
The tRNA plays a fundamental role in protein translation by delivering amino acids to the growing peptide chain. However, advances in RNA sequencing enabled the identification of a new class of small RNAs, namely tRNA derived small fragments (tRFs), suggesting that tRNAs can also perform functions other than carrying amino acids. Fragments of tRNAs were first disregarded as degradation products, but are now recognized to have a biologically active function in regulating gene expression. These tRFs originate from different parts of their parental mature tRNA and range from ~18-50 nucleotides in size. An overview of tRNA-derived fragments is shown in **Figure 3**. The production of tRFs is conserved throughout evolution<sup>54, 55</sup>. Angiogenin is an enzyme

belonging to the RNase A family and is a known tRNA-processing endonuclease. Cellular stress-induced activation of Angiogenin results in increased formation of tRFs<sup>56, 57</sup>.

The mature tRNA is heavily decorated with modifications. These modifications may both protect against and direct tRNA cleavage. Some modifications may be guided by orphan snoRNAs. For example, orphan SNORD97 has a 2'Ome site on the wobble cytidine C34 of tRNA<sup>Met</sup>(CAT), which is protected against Angiogenin-induced fragmentation<sup>58, 59</sup>. However, little is still known about tRF biogenesis and, in particular, their role in cardiovascular disease.

### The 14q32 locus

MicroRNA and snoRNA genes are often located in clusters in the human genome. Advances in bioinformatics have led to prediction algorithms for targets of microRNAs, snoRNAs and tRFs<sup>60-62</sup>. In previous work from our group, reversed target prediction (RTP) identified microRNAs that were predicted to regulate expression of genes involved in neovascularization and atherosclerosis<sup>63, 64</sup>. Remarkably, RTP identified 27 microRNAs with



**Figure 3. Schematic overview of tRNA-derived fragments.** The mature tRNA can be cleaved into different sized fragments. Shorter fragments (<26 nucleotides) are called tRFs. Fragments derived from cleavage at the anticodon site are called tRNA halves. tRFs and tRNA halves are called 5' or 3', depending on the location they were generated from.

enrichment of putative binding sites, which were all encoded by a single microRNA cluster located on the long arm of human chromosome 14 (14q32). The 14q32 locus, also named DLK1-DIO3 locus by its protein coding genes DLK1 and DIO3, is an imprinted locus containing numerous maternally expressed ncRNAs. The 14q32 cluster is highly conserved in human and mice. The equivalent in mice is located on chromosome 12 (12F1). The 14q32 cluster encodes besides its 3 protein coding genes and 54 microRNAs, a cluster of 41 C/D box snoRNAs, 3 long noncoding RNAs (lncRNAs), MEG3, MEG8 and MEG9, and Piwi-interacting RNAs (piRNAs)<sup>63, 65, 66</sup>. Genetic association analyses demonstrated that the 14q32 microRNAs, snoRNAs and lncRNAs are, independently of each other, strongly linked to CVD<sup>53</sup>.

#### *MicroRNA-494-3p and microRNA-329-3p*

One of the 14q32 microRNAs that is highly involved in different processes of vascular remodeling is miR-494-3p<sup>63, 64, 67, 68</sup>. In a murine model for early atherosclerosis, inhibition of miR-494-3p resulted in reduced initial lesion development, increased plaque stability and decreased plasma cholesterol levels<sup>64</sup>. These findings demonstrate the potential of miR-494-3p as a therapeutic target to reduce atherosclerosis and subsequently, the risk of a cardiovascular event. However, this first study focused on initial lesion development, while patients at risk of atherosclerotic complications generally present in the clinic with advanced and unstable lesions. Also, most patients at risk of a (recurrent) cardiovascular event receive plasma cholesterol lowering drugs<sup>11, 69</sup>. Therefore, a second study using mice with advanced lesions and including plasma cholesterol lowering in addition to treatment with miR-494-3p inhibitors, would more closely translate these findings to a human clinical setting.

Another 14q32 microRNA that is highly involved in vascular remodeling is miR-329-3p<sup>63, 67, 68</sup>. Just as miR-494-3p, miR-329-3p was predicted to regulate expression of genes involved in neovascularization and inhibition of miR-329-3p indeed improved neovascularization and blood flow recovery in a murine model for PAD<sup>63</sup>. However, its role in the development and progression of atherosclerosis is unknown.

The increased plaque stability that was observed in mice treated with miR-494-3p inhibitors, may indicate a shift in macrophage subsets from proinflammatory M1 towards an anti-inflammatory M2 phenotype. Downregulation of miR-494-3p resulted in upregulated expression of miR-494-3p targets metalloproteinase inhibitor 3 (TIMP3), interleukin 33 (IL-33) and transforming growth factor beta 2 (TGFB2) in the carotid artery<sup>64</sup>. Expression of these genes contributes to a decrease in local plaque inflammation. Furthermore, in a

murine model for intimal hyperplasia, miR-494-3p inhibition reduced macrophage influx in the intima<sup>67</sup>. Investigations into whether miR-494-3p directly influences macrophage activation and polarization and if so, via which specific activation pathways, are needed to answer these research questions.

### *Orphan 14q32 snoRNAs*

The 14q32 locus encodes a cluster of 41 C/D box snoRNAs, named SNORD112, SNORD113 1-9 and SNORD114 1-31. Compared to 14q32 microRNAs, much less is known about the 14q32 snoRNAs. However, previous findings do suggest a regulatory role in CVD. Genetic association analysis demonstrated that the 14q32 snoRNAs associate stronger to CVD than 14q32 microRNAs and lncRNAs<sup>53</sup>. All seven measured 14q32 snoRNAs could be detected in both plasma of end-stage PAD patients and cycling athletes, and four of them, SNORD112, SNORD113.2, SNORD113.6 and SNORD114.1, were highly expressed in end-stage PAD patients in particular<sup>70</sup>. In a second study, PAD patients suffering from intermittent claudication but without critical limb ischemia were included. SNORD112, SNORD113.2, SNORD113.6 and SNORD114.1 were highly expressed, demonstrating that these snoRNAs are elevated in all PAD patients and not just the severe cases. Also, SNORD113.2 and SNORD114.1 appeared strongly linked to platelet activation, which is an important determinant of long-term outcome in PAD<sup>71</sup>.

Bioinformatic tools predict that all 14q32 snoRNAs lack a rRNA binding site, making all 14q32 C/D box snoRNAs 'orphan', as their targets are unknown<sup>47, 48</sup>. However, binding to FBL indicates that they do perform canonical functions, but likely on non-canonical targets, such as mRNAs and tRNAs<sup>53</sup>. In-depth research is needed to lift the orphan status of 14q32 snoRNAs and to investigate their therapeutic potential in treatment of CVD.

### **Emerging therapeutic potentials**

The importance of the inflammatory component in atherosclerosis is increasingly being recognized. The canakinumab anti-inflammatory thrombosis outcome study (CANTOS) trial showed for the first time that targeting inflammation reduced cardiovascular events, independent of lowering plasma cholesterol. Canakinumab is a monoclonal antibody that targets proinflammatory IL-1 $\beta$ <sup>19</sup>. Since then, more clinical trials are focusing on the inflammatory aspect of the disease. Colchicine is well-established anti-inflammatory medication for the treatment of gout and pericarditis. Several clinical trials have investigated the use of colchicine to reduce CVD, including the Low Dose Colchicine for Secondary Prevention of Cardiovascular Disease (LoDoCo) and a larger second trial

LoDoCo2, Colchicine Cardiovascular Outcomes Trial (COLCOT) and Colchicine in Patients With Acute Coronary Syndromes (COPS)<sup>72-75</sup>. Outcomes of these trials indicate that using colchicine in addition to standard therapies is promising to prevent a recurrent cardiovascular event in patients with coronary artery disease and hence emphasize the importance of reducing inflammation in CVD.

#### *Noncoding RNAs as therapeutic targets*

NcRNAs are emerging as novel therapeutic targets for potential CVD treatments. MicroRNAs in particular are being widely investigated in preclinical studies of CVD<sup>37</sup>. As microRNAs regulate expression of numerous target genes, they have the potential to simultaneously target multiple aspects of the disease, including inflammation<sup>40</sup>. MicroRNAs are expressed and thus active, in various cell types in the cardiovascular system, such as endothelial cells, smooth muscle cells, fibroblasts and leukocytes<sup>40</sup>. Changes in gene expression profiles, which can be facilitated by manipulation of one microRNA, can direct fate of cellular pathways and ultimately change cellular behavior. Therefore, one microRNA can act as a master switch on a multicellular level and has the potential to target all different aspects of CVD, including inflammation and cholesterol regulation, which subsequently may affect the course of CVD.

Expression of microRNAs can be modulated by using antisense oligonucleotides (ASOs), small interfering RNA (siRNA), mimics or viral vectors. To investigate its therapeutic potential in atherosclerosis, mostly mice with *LDLr<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* background on a high fat diet, with surgical interventions, are used to induce atherosclerosis<sup>76</sup>. Several studies show encouraging results in reducing atherosclerosis via targeting ncRNAs<sup>37</sup>. For example, systemic delivery of miR-181b mimics inhibited activation of the NF- $\kappa$ B pathway and reduced vascular inflammation and atherosclerotic lesion formation<sup>77</sup>. Deficiency of miR-155 in *ApoE<sup>-/-</sup>* mice resulted in decreased atherogenesis via reduced inflammatory responses of macrophages<sup>78</sup>. As yet, modulation of microRNAs and other ncRNAs in CVD have not been studied in clinical trials, except for miR-132-3p in heart failure patients. MiR-132-3p has been shown to affect signaling pathways involved in cardiomyocyte growth, autophagy, calcium handling and contractility. One target of miR-132-3p is Forkhead box O3 (FOXO3), a pro-autophagic transcription factor<sup>79</sup>. CDR132L is a synthetic locked nucleic acid ASO inhibitor, with a fully phosphorylated backbone, against miR-132-3p. A first-in-human Phase 1b study showed that CDR132L is safe and well-tolerated, had linear plasma pharmacokinetics and suggests improvements in cardiac function<sup>80</sup>. This clinical trial is

limited by the small number of patients, but results are promising for future follow-up clinical studies of miR-132-3p and other ncRNAs.

## Thesis outline

The aim of this thesis is to elucidate the molecular mechanism of action of 14q32 microRNAs and snoRNAs, and to evaluate the therapeutic potential of targeting 14q32 microRNAs and snoRNAs in CVD.

The first part of the thesis focuses on 14q32 microRNAs in atherosclerosis.

**Chapter 2** demonstrates the therapeutic potential of single 14q32 microRNA inhibition, miR-494-3p and miR-329-3p, in a murine model for atherosclerosis. We used a clinically relevant murine model with advanced, established atherosclerotic lesions, induced by a 10-week high fat diet and collar placement around both carotid arteries. After 10 weeks of high fat diet, diet was switched to regular chow to mimic plasma cholesterol lowering treatments. Simultaneously, mice received third-generation antisense treatment against miR-494-3p (3GA-494), miR-329-3p (3GA-329) or an antisense control (3GA-ctrl), and at week 12 and 14. We show that treatment with 3GA-494 and, in part, 3GA-329 halted plaque progression. Furthermore, plaque stability was increased in 3GA-494 treated mice compared to 3GA-ctrl. Pro-atherogenic cells in the circulation, including Ly6C<sup>hi</sup> monocytes, neutrophils and blood platelets were decreased upon miR-494-3p and, in part, miR-329-3p inhibition.

Based on results from chapter 2, we hypothesized that miR-494-3p directly influences macrophage polarization and activation.

**Chapter 3** shows that 3GA-494 treatment dampens proinflammatory M1 polarization, while enhancing anti-inflammatory M2 polarization. Proinflammatory marker CCR2 was reduced in plaques of 3GA-494 treated hypercholesterolemic mice. Furthermore, pathway analysis predicted that miR-494-3p targets genes involved in Wnt signaling. 3GA-494 treatment indeed activated Wnt signaling both in cultured M1 macrophages and in plaques of hypercholesterolemic mice, which at least in part, dampened M1 polarization. 3GA-494 could therefore be a potential therapeutic agent for stabilizing vulnerable lesions and reducing the risk of a cardiovascular event.

The second part of the thesis focuses on the function of human 14q32 snoRNA SNORD113-6 and its equivalent AF357425 in mice.

**Chapter 4** shows that formerly orphan SNORD113-6/AF357425 targets mRNAs and acts via two mechanisms, namely pre-mRNA processing and 2'O-ribose methylation (2'Ome).

Several pre-mRNAs with conserved AF357425/SNORD113-6 D'-seed binding sites in the last exon/3'UTR were identified, which directed pre-mRNA processing and splice-variant-specific protein expression. Identified genes from pulldown of methyltransferase fibrillarin, were enriched for genes in the integrin pathway. 2'Ome of 6 integrin pathway mRNAs was confirmed, which appeared important for mRNA stability. Furthermore, primary human umbilical arterial fibroblasts (HUAFs) barrier function was altered under SNORD113-6 inhibition, indicating that SNORD113-6 is important for vascular function. SNORD113-6 could therefore be a novel therapeutic target for treating CVD. However, more research into remaining target RNAs, its second seed sequence and other 14q32 snoRNAs remains to be done.

Based on findings in chapter 4, we aimed to investigate whether AF357425/SNORD113-6 can also target small RNAs.

**Chapter 5** demonstrates that AF357425/SNORD113-6 targets tRNAs via 2'Ome and thereby protects the tRNA from cleavage into small fragments. Small RNA sequencing of murine fibroblasts in which AF357425 was overexpressed or inhibited, showed that expression of tRNA fragments (tRFs) was predominantly regulated. We focused on tRNA Leucine anticodon TAA (tRNA<sup>Leu</sup>(TAA)) that has a conserved predicted binding site for AF357425/SNORD113-6. Adjacent to this site, tRNA<sup>Leu</sup>(TAA) is cleaved and its dominant fragment, tRF<sup>Leu 47-64</sup>, is formed. 2'Ome by AF357425/SNORD113-6 prevented formation of tRF<sup>Leu 47-64</sup>. Exposing fibroblasts to oxidative stress or hypoxic stress induced tRNA<sup>Leu</sup>(TAA) and AF357425/SNORD113-6 expression, but AF357425/SNORD113-6 knockdown did not increase tRF<sup>Leu 47-64</sup> formation under stress even further. Thus, independent of cellular stress, AF357425/SNORD113-6 directs fragmentation of tRNA<sup>Leu</sup>(TAA) via 2'Ome.

**Chapter 6** describes a summary of all results shown in this thesis and discusses future perspectives of this research.



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