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## Small regulatory RNAs in vascular remodeling and atherosclerosis

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# Chapter 6

## General Discussion

## General discussion

Cardiovascular disease (CVD) is considered to be one of the most alarming health problems globally. CVD affects the quality of life and life expectancy of many patients<sup>1</sup>. Over the past few decades, major improvements have been made in both the prevention and treatment of CVD. Nevertheless, a rapidly ageing population, increasing prevalence of CVD, rising numbers of risk factors (e.g. obesity), and the great economic burden, emphasize the urgent need for novel therapies to improve prevention and treatment of CVD<sup>1,2</sup>.

Over the past few years, ncRNAs have emerged as potential therapeutical targets for different cardiovascular pathologies<sup>3</sup>. One type of small ncRNA that received much attention is the microRNA. MicroRNAs have the ability to fine-tune expression of multiple target genes simultaneously. They may act as master switches in complex diseases as CVD. Another type of small ncRNA is the snoRNA. C/D box snoRNAs have long been known to guide 2'-O-methylation (2'Ome) of ribosomal RNA (rRNA)<sup>4</sup>. However, half of the human C/D box snoRNAs lack a known target and their function is unknown<sup>5</sup>. Numerous C/D box snoRNAs have been associated with diseases, including CVD<sup>6,7</sup>. This implies they have a regulatory role in disease.

The 14q32 ncRNA cluster (12F1 in mice) transcribes the largest known human microRNA cluster, a cluster of 54 microRNA genes. MiR-494-3p and miR-329-3p, both 14q32 microRNAs, have been shown to be involved in different processes of vascular remodeling<sup>8-11</sup>. In a previous study, inhibition of miR-494-3p reduced lesion development in a mouse model with early atherosclerosis<sup>11</sup>. This study focused on early lesion development. Patients at risk of (recurrent) cardiovascular events however, generally present with advanced and symptomatic lesions. Patients often also receive plasma cholesterol lowering drugs (e.g. statins) in order to reduce the risk of a (recurrent) cardiovascular event<sup>12</sup>. In this thesis, we therefore aimed to investigate 14q32 microRNA inhibition in a mouse model that more closely resembles the human clinical setting. We used mice with advanced atherosclerosis and treated them with either 3<sup>rd</sup> Generation Antisense against miR-494-3p (3GA-494) or miR-329-3p (3GA-329). We simultaneously lowered plasma lipids by changing their diet from high-fat high-cholesterol to regular chow.

Macrophages have a key role in the onset and progression of atherosclerosis<sup>13</sup>. In a follow-up study, we investigated whether miR-494-3p directly influences macrophage activation and polarization. Whether this affects atherosclerotic plaque stability was unknown.

Adjacent to the cluster of microRNA genes lies a cluster of 41 C/D box snoRNA genes<sup>7</sup>. This cluster of C/D box snoRNAs is strongly associated with CVD. This association is both

independent of and stronger than that of the 14q32 microRNAs. Expression of 14q32 snoRNAs is regulated under ischemic conditions in PAD patients and during vascular remodeling<sup>7, 14</sup>. Compared to the 14q32 microRNAs however, much less is known about the 14q32 snoRNAs. The canonical function of C/D box snoRNAs is to guide 2'Ome of rRNA<sup>4</sup>. However, none of the 14q32 snoRNAs has a known RNA target. Direct binding to the methyltransferase fibrillarin does suggest a canonical function of 2'Ome, but possibly on noncanonical RNA targets<sup>7</sup>. We therefore aimed to elucidate both the function and RNA targets of 14q32 snoRNAs. We focused on one of the most abundantly expressed 14q32 snoRNAs, SNORD113-6 in humans and its equivalent AF357425 in mice. First, we investigated several mRNAs of the integrin pathway that AF357425/SNORD113-6 targets via two mechanisms, pre-mRNA processing and 2'Ome. As integrin signaling is important for cell-cell and cell-matrix interactions<sup>15</sup>, we also examined human arterial fibroblast function. Second, we aimed to determine whether AF357425/SNORD113-6 can also target small RNAs. We found that tRNAs were predominantly targeted by AF357425/SNORD113-6 and investigated whether 2'Ome by AF357425/SNORD113-6 affects fragmentation of tRNAs.

### **MicroRNA-494-3p and microRNA-329-3p in advanced atherosclerosis**

In **chapter 2**, LDLr<sup>-/-</sup> mice with advanced atherosclerosis were treated with 3GA-494, 3GA-329 or a scrambled sequence control (3GA-ctrl). A subset of mice (baseline) was sacrificed directly after 10 weeks of high-fat high-cholesterol diet. We show that inhibition of miR-494-3p and, in part, miR-329-3p halted plaque progression in the carotid artery and promoted plaque stability in the aortic root. Plasma cholesterol levels were strongly reduced after changing diet from high-fat high-cholesterol to regular chow. Although we combined plasma lipid lowering with 3GA treatment, plaque sizes from either 3GA-494 or 3GA-329 mice were not reduced compared to baseline mice. This indicates that even though plaque progression was reduced, plaque regression did not occur in this setup.

Unlike the carotid artery plaques, plaque sizes in the aortic root did not differ between groups. Plaque stability however, is just as, or even more important than plaque size in reducing the risk of cardiovascular events. Collagen provides structural support in the fibrotic cap. Inhibition of miR-494-3p increased intra-plaque collagen and reduced macrophage content in advanced aortic root plaques. The number of plaque macrophages was reduced by diet switch alone and was further reduced when mice were treated with miR-494-3p inhibitors.

We observed a reduction in circulating platelets in particular, but also in pro-atherogenic Ly6C<sup>hi</sup> monocytes and neutrophils in 3GA-494 treated mice. Platelets are highly involved in

proinflammatory responses and facilitate monocyte and neutrophil extravasation into the lesion<sup>16-18</sup>. Reducing their numbers may have contributed to the decrease in plaque macrophages and thus, is favorable in arresting plaque progression. Platelets are essential in blood clotting, which is often the direct cause of acute ischemic diseases like ischemic stroke and myocardial infarction<sup>19</sup>. Perhaps lowering their numbers would reduce the risk of an acute cardiovascular event as well. However, a dramatic decrease in platelets is likely accompanied by an increased bleeding risk, even though this was not (yet) observed in the 3GA-494 treated mice. Another concern is the enlarged spleen in these mice. We found a strong increase in number of splenic megakaryocytes, the progenitor cells of platelets. This is likely a compensatory mechanism to prevent severe thrombocytopenia. Treatment with 3GA-494 led to hyper-activation of human platelets *in vitro* and could be the underlying cause of rapid platelet clearance *in vivo*.

### MicroRNA-494-3p in macrophage polarization

Inhibition of miR-494-3p resulted in smaller lesions with increased stability, both in early<sup>11</sup> and advanced atherosclerosis (**chapter 2**). Aortic root lesions contained less macrophages and numbers of pro-atherogenic Ly6C<sup>hi</sup> monocytes were reduced in the circulation. Based on these findings, we hypothesized that miR-494-3p directly influences macrophage polarization in atherosclerosis. This was investigated in **chapter 3**.

First, we showed that endogenous miR-494-3p is being regulated during macrophage polarization. MiR-494-3p expression was decreased in proinflammatory M1 and increased in anti-inflammatory M2 polarization. Second, key polarization markers at mRNA and protein levels, were regulated by miR-494-3p. 3GA-494 treatment inhibited miR-494-3p expression in M1 macrophages and dampened M1 polarization. 3GA-494 treatment simultaneously enhanced M2 polarization, while miR-494-3p expression was increased in M2 macrophages. Both are favorable in reducing plaque formation and increasing stability. This is correspondingly shown in **chapter 2**. In **chapter 3**, we also showed that inhibition of miR-494-3p in atherosclerotic plaques *in vivo* led to an apparent reduction of the proinflammatory marker C-C motif chemokine receptor-2 (CCR2).

Pathway enrichment analysis predicted that miR-494-3p has more than 70 targets involved in macrophage polarization. The pathway containing most assigned genes was the Wnt signaling pathway. MiR-494-3p indeed regulates expression of multiple Wnt signaling components, including LRP6 and TBL1X. In canonical Wnt signaling, non-phosphorylated  $\beta$ -catenin is translocated into the nucleus, where it induces downstream transcription<sup>20</sup>. Wnt signaling

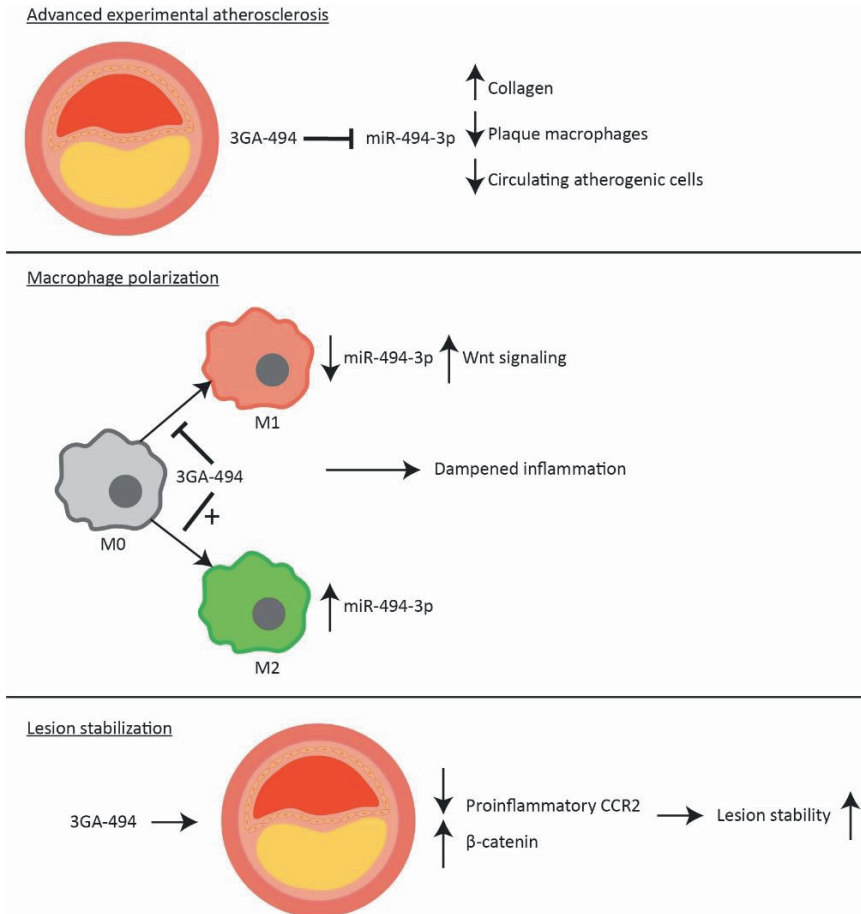
appeared activated through increased non-phosphorylated  $\beta$ -catenin, in both cultured M1 macrophages and in plaques of hypercholesterolemic mice treated with 3GA-494. The dampened M1 polarization is thus, at least in part, regulated via activated Wnt signaling.

Wnt signaling is mostly known from determining (stem) cell fates and involvement in diseases, such as cancer. Some studies, however, suggest a protective role of Wnt signaling against atherosclerosis<sup>21-23</sup>. Wnt signaling may have a role in limiting cholesterol accumulation in atherosclerotic plaques, for example<sup>22, 24</sup>. Findings from previous studies and this thesis indicate that miR-494-3p inhibition affects cholesterol metabolism. Efflux of high-density lipoprotein (HDL)<sup>11</sup> for instance, was increased in miR-494-3p inhibited macrophages<sup>11</sup>. MiR-494-3p inhibition reduced necrotic core sizes in early atherosclerotic plaques<sup>11</sup> and lowered plasma cholesterol levels *in vivo* (**chapter 2**). TREM2 is a marker for anti-inflammatory foamy lipid-laden macrophages involved in cholesterol metabolism<sup>13</sup>. Even though not a direct miR-494-3p target, TREM2 expression increased in M2 polarized macrophages treated with 3GA-494. 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1) is a putative miR-494-3p target and involved in cholesterol synthesis. Out of four human donors, three donors showed differential expression of HMGCS1 after 3GA-494 treatment, in both cultured M1 and M2 macrophages (**chapter 3**). MiR-494-3p is therefore likely involved in the regulation of cholesterol metabolism genes. Investigations on exact mechanisms remain to be performed in future research though, and also whether they involve the Wnt signaling pathway or other relevant pathways.

Polarization of macrophages towards M2 induced miR-494-3p expression. MiR-494-3p expression was even further induced in response to 3GA-494 treatment (**chapter 3**). This phenomenon was also observed in both platelets and the whole spleen after treatment with 3GA-494 (**chapter 2**). Perhaps miR-494-3p regulates expression of RNA binding proteins and RNA binding proteins in turn regulate miR-494-3p processing. RNA binding protein Mef2A, for example, directly binds to pri-miR-494-3p<sup>10</sup>. Which precise mechanism underlies this subset-, cell-, and tissue-specific autoregulation, however, remains to be determined.

Summarizing, the first part of this thesis shows that miR-494-3p contributes to the progression of atherosclerosis. Its inhibition halts plaque progression and increases stability of advanced lesions. MiR-329-3p inhibition has a less profound effect on the progression of atherosclerosis. Furthermore, miR-494-3p directly modulates macrophage activation and polarization. Inhibition of miR-494-3p reduces M1 polarization, while M2 polarization is

enhanced. A graphical representation of the findings from the first part of this thesis is shown in **Figure 1**.



### AF357425/SNORD113-6 targets mRNAs via pre-mRNA processing and 2'Ome

**Figure 1. Graphical representation of miR-494-3p in advanced experimental atherosclerosis and macrophage polarization.** 3<sup>rd</sup> Generation Antisense against miR-494-3p (3GA-494) was used to inhibit microRNA expression. (Top) 3GA-494 treatment in advanced atherosclerosis. (Middle) 3GA-494 dampens M1 polarization, while enhancing M2 polarization. (Bottom) Treatment with 3GA-494 reduces proinflammatory M1 marker C-C motif chemokine receptor-2 (CCR2) and increases non-phosphorylated β-catenin, via activated Wnt signaling, in atherosclerotic plaques. This likely contributed to increased lesion stability.

C/D box snoRNAs that have no known targets are called orphan snoRNAs. Since they lack known targets, their function is also unknown. Orphan C/D box snoRNAs have been described to guide fibrillarin-dependent 2'Ome on noncanonical RNA targets like mRNAs, microRNAs and tRNAs<sup>25-27</sup>. Completely different functions, e.g. directing alternative splicing and regulating gene expression in a microRNA-like manner, have also been described for orphan C/D box snoRNAs<sup>28, 29</sup>.

In **chapter 4**, we aimed to lift the orphan status of one of the 14q32 snoRNAs, SNORD113-6 and its mouse equivalent AF357425. The D' box seed of this snoRNA is fully conserved between mice and humans. SNORD113-6/AF357425 and 14q32 snoRNAs in general, are highly expressed in fibroblasts. We aimed for AF357425 knockout fibroblasts. Unfortunately, those could not be obtained due to the lack of any viable AF357425-knockout clones. This either suggests that our CRISPR/Cas9 strategy was not successful or, more likely, that AF357425 is essential for cell survival. We therefore used antisense technology to knockdown or overexpress AF357425 in murine fibroblasts, followed by two different RNA sequencing (RNA-seq) strategies. In one strategy, RNA-seq was performed on whole cell lysates to examine alternative splicing and processing. In the other strategy, RNA from a fibrillarin pulldown and depleted from rRNA, was sequenced to identify fibrillarin-associated targets.

We found an enrichment of AF357425 binding sites (i.e. the reversed complement of the D' antisense box) in the last exon and the 3'UTR of (pre)mRNAs. We identified 46 genes with putative snoRNA binding sites that showed differential expression of splice- or processing-variants between AF357425 overexpression and knockdown cells. Of these genes, 20 genes had a conserved putative binding site in humans. We selected three genes, DUSP7, JAG1 and EBPL, and confirmed altered processing under SNORD113-6/AF357425 knockdown. It appeared that the location of the snoRNA binding site determines whether pre-mRNA processing is affected by SNORD113-6/AF357425 knockdown or not. A binding site in the last exon and 3'UTR, but not in introns, resulted in increased expression of the dominant variant (defined by the variant with a binding site in exon/3'UTR, the protein coding variant or the variant with most binding sites) over the alternative variant under SNORD113-6/AF357425 knockdown.

We identified 7 genes from the integrin pathway, MAP2K1, ITGB3, ITGA7, FLNB, NTN4, PARVB and COL4A4, that are fibrillarin-dependent 2'Ome mRNA targets of AF357425/SNORD113-6. The integrin pathway is important for cell-cell and cell-matrix interactions<sup>15</sup>. Binding sites of these genes were conserved both in mouse and human, albeit for some on different locations

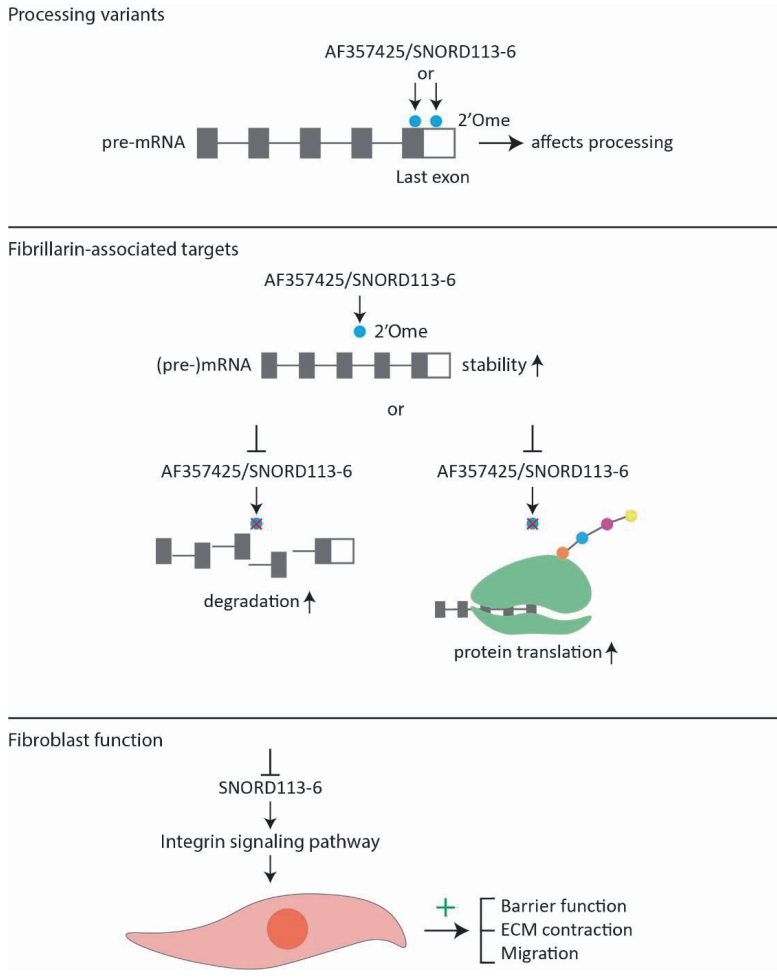


(last exon/3'UTR in mouse versus introns in human). We focused on the integrin pathway since it had the strongest enrichment of targeted genes. However, we also found putative targets involved in other cardiovascular relevant pathways. After blocking novel transcription, mRNA degradation rates increased under knockdown of AF357425/SNORD113-6. 2'Ome by AF357425/SNORD113-6 is therefore important for stability of these mRNA targets. The effects on protein levels were ambiguous though and most likely depend on several factors. The location of the binding site and whether binding of AF357425/SNORD113-6 leads to 2'Ome or not, could affect protein translation. On the one hand, we show that 2'Ome protects the mRNA from degradation. More mRNA is thus present to be translated into protein. On the other hand, 2'Ome placed on the mRNA may hamper binding of the ribosome, which results in less protein<sup>25</sup>. When we examined fibroblast function, we found an increased barrier function, increased ability to contract extracellular matrix and increased migration in SNORD113-6 knockdown fibroblasts. Both the integrin pathway and fibroblast function are important in cardiovascular remodeling and disease<sup>15</sup>. How fibroblast function regulated by SNORD113-6 affects cardiovascular remodeling and disease exactly, remains to be determined in future research. A graphical representation of the findings from **chapter 4** is shown in **Figure 2**.

#### **AF357425/SNORD113-6 directs fragmentation of tRNAs via 2'Ome**

In **chapter 4**, we show that AF357425/SNORD113-6 targets a broad range of mRNAs and influences their expression through two mechanisms, pre-mRNA processing and 2'Ome. Recent studies have shown that post-transcriptional modifications guided by snoRNAs, 2'Ome and pseudouridylation  $\Psi$ , on tRNAs can direct their cleavage into smaller tRNA-derived fragments (tRFs)<sup>27, 30</sup>. In **chapter 5**, we aimed to determine whether AF357425/SNORD113-6 can also target small RNAs.

Small RNA sequencing (sRNA-seq) was performed on cell lysates from either AF357425 inhibited or overexpressed murine fibroblasts. The small RNAs that changed most in expression were tRFs. The overall formation of tRFs was reduced in AF357425 knockdown fibroblasts. When stratified by fragment length, the smaller sized tRFs (18-30 nucleotides) were enriched and the longer sized tRFs (30-45 nucleotides) depleted in these cells.



**Figure 2. Graphical representation of AF357425/SNORD113-6 in 3'end processing/splicing, 2'-O-methylation (2'Ome) of mRNA targets and fibroblast function.** RNA sequencing was performed on whole cell lysates to examine alternative splicing and processing of putative targets. RNA from fibrillarlin pulldown was sequenced to identify fibrillarlin-associated targets. (Top) An enrichment of SNORD113-6/AF357425 binding sites was found in the last exon and 3'UTR of (pre)mRNAs. It appears that snoRNA binding sites in the last exon and 3'UTR, but not in introns, affects pre-mRNA processing. (Middle) 2'Ome by SNORD113-6/AF357425 is important for stability of mRNAs. Inhibition of SNORD113-6/AF357425 reduces 2'Ome on its mRNA targets. This could lead to either decreased or increased protein translation via two proposed mechanisms. 2'Ome protects the mRNA from degradation. Fewer mRNAs are thus present to be translated into protein. Alternatively, 2'Ome placed on the mRNA may hamper binding of the protein. Fewer 2'Ome could therefore lead to increased protein. (Bottom) SNORD113-6 targets mRNAs of the integrin signaling pathway and affects fibroblast function.

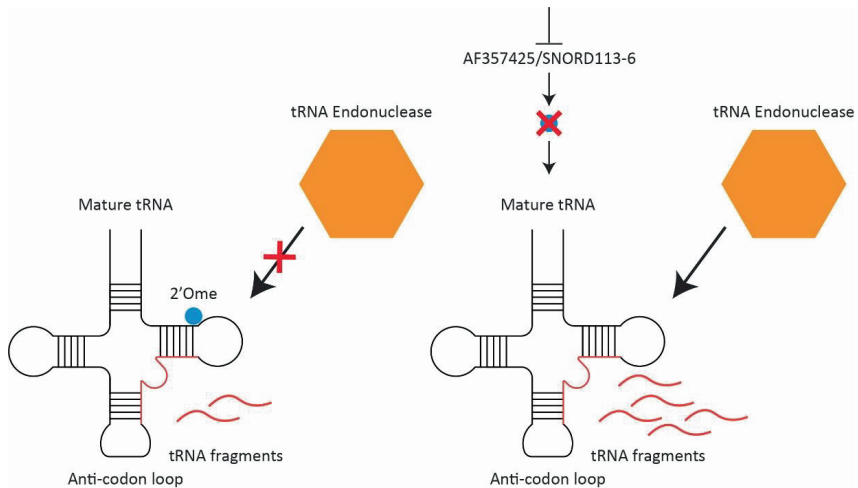
We focused on one of the tRNAs, tRNA Leucine anti-codon TAA (tRNA<sup>Leu</sup>(TAA)), in order to investigate the underlying mechanism-of-action. tRNA<sup>Leu</sup>(TAA) has a predicted binding site for AF357425/SNORD113-6, which was indeed methylated by AF357425/SNORD113-6. AF357425/SNORD113-6 knockdown did not affect degradation rates of tRNA<sup>Leu</sup>(TAA). This single 2'Ome site is therefore not essential for the overall stability of the tRNA. The dominant tRF of tRNA<sup>Leu</sup>(TAA), tRF<sup>Leu 47-64</sup>, is formed adjacent to the 2'Ome site. Knockdown of AF357425/SNORD113-6 increased the ratio of tRF<sup>Leu 47-64</sup> to tRNA<sup>Leu</sup>(TAA). This suggests that 2'Ome by AF357425/SNORD113-6 acts in preventing site-specific fragmentation of tRNAs into small fragments (~18 nucleotides in length).

It has been shown that fragmentation of tRNAs increases under cellular stress, including hypoxia and oxidative stress. Plasma levels of 14q32 snoRNAs are also regulated under ischemic conditions in PAD patients<sup>7</sup>. As expected, exposing cells to cellular stress increased expression of AF357425/SNORD113-6, but also tRNA<sup>Leu</sup>(TAA) and tRF<sup>Leu 47-64</sup>. However, inducing cellular stress did not increase the ratio of tRF<sup>Leu 47-64</sup> relative to mature tRNA<sup>Leu</sup>(TAA) even further in AF357425/SNORD113-6 knockdown cells. In fact, the strongest increase in this ratio was in cells cultured under physiological conditions, without induction of cellular stress. An explanation could be that other endonucleases are being activated during cellular stress and produce alternative tRF species from tRNA<sup>Leu</sup>(TAA). It could also be that the upregulation of AF357425/SNORD113-6 under cellular stress, counters the effects of snoRNA knockdown.

This particular tRF<sup>Leu 47-64</sup> may have an important role in cell physiology as it is produced under physiological conditions and not exclusively during cellular stress. All different sorts of regulatory functions have been described for tRFs. They regulate protein translation, perform microRNA-like functions and interact with RNA-binding proteins<sup>31, 32</sup>. tRFs can also regulate cell phenotypes, including cardiac muscle cells, skeletal muscle cells and endothelial cells, and complex vascular processes like angiogenesis<sup>31-34</sup>. The exact function of this AF357425/SNORD113-6-dependent tRF<sup>Leu 47-64</sup> and its role in vascular remodeling is not known yet and therefore remains to be determined in future research. A graphical representation of the findings from **chapter 5** is shown in **Figure 3**.

Summarizing, the second part of this thesis shows that SNORD113-6/AF357425 targets a broad range of mRNAs and affects their expression via two mechanisms, pre-mRNA processing and 2'Ome. Both mechanisms stabilize mRNAs and affect fibroblast phenotype. Furthermore, SNORD113-6/AF357425 guides 2'Ome and thereby affects fragmentation of tRNAs in general and of tRNA<sup>Leu</sup>(TAA) in particular. These insights in the function of

SNORD113-6/AF357425 and how this snoRNA affects fibroblast function, may provide novel therapeutical opportunities in the treatment of CVD.



**Figure 3. Graphical representation of 2'-O-methylation (2'Ome) by AF357425/SNORD113-6 in preventing site-specific fragmentation of tRNAs.** TRNA endonucleases can cleave mature tRNAs into smaller tRNA fragments (tRFs). TRNA Leucine anti-codon TAA ( $\text{tRNA}^{\text{Leu}}(\text{TAA})$ ) has a conserved binding site for AF357425/SNORD113-6. The dominant tRF of  $\text{tRNA}^{\text{Leu}}(\text{TAA})$ ,  $\text{tRF}^{\text{Leu} 47-64}$ , is formed adjacent to the 2'Ome site. 2'Ome by AF357425/SNORD113-6 prevents formation of  $\text{tRF}^{\text{Leu} 47-64}$ . Inhibition of AF357425/SNORD113-6 reduces 2'Ome and leads to increased formation of  $\text{tRF}^{\text{Leu} 47-64}$ .

## Future perspectives

This thesis has uncovered (part of) the contribution of 14q32 microRNAs and snoRNAs to the progression of CVD. We have also explored their therapeutical potential. In the first part of the thesis we have demonstrated that targeting miR-494-3p has potential in reducing atherosclerosis. In the second part, we investigated both the function and RNA targets of one 14q32 snoRNA, human SNORD113-6 and its mouse equivalent AF357425. This provided novel insights in human arterial fibroblast function. There are still numerous hurdles to overcome for this fundamental knowledge to be developed into medicine. However, a better understanding of the underlying pathogenesis of the disease, to which this thesis contributed, does create new therapeutic opportunities in CVD.

A key challenge in the development of RNA therapeutics, and drug development in general, is to prevent or at least minimize adverse off-target effects. In principle, sequence-specific RNA inhibitors can only be biologically active in those cells that express the targeted RNA. A target microRNA or snoRNA is therefore ideally only expressed in the diseased vessel and not in other cells and tissues (e.g. healthy vessels). Both 14q32 microRNAs and snoRNAs have location-specific expression patterns throughout the human vasculature<sup>35</sup>. In complex diseases as CVD however, many different cell types of both the cardiovascular and immune system are involved<sup>36</sup>. The ideal therapeutical agent would therefore target multiple aspects of the disease, in order to maximize the therapeutic effect, without affecting physiological processes.

MicroRNAs facilitate a modest downregulation of their target genes rather than a full knockdown. They are promising candidates in that respect. In **chapter 2**, we explored miR-494-3p as a therapeutical target for reducing advanced experimental atherosclerosis. MiR-494-3p is an abundantly expressed microRNA in many different cell types. We indeed found effects of 3GA-494 treatment at a multicellular level, e.g. in plaque macrophages and circulating pro-atherogenic Ly6C<sup>hi</sup> monocytes, neutrophils and platelets. 3GA-494 even had distinct anti-atherogenic effects in different subsets of macrophages (**chapter 3**). All of these effects together most likely contribute to maximize the therapeutic effect of 3GA-494 and reduced atherosclerosis.

The dramatic decline in circulating platelets in 3GA-494 treated mice was an unexpected off-target effect. Reducing platelet numbers may be favorable in arresting plaque progression and reducing the risk of an acute cardiovascular event. However, a strong decline in platelets is likely accompanied with an increased risk of bleeding. In previous studies, 3GA-494 treated

mice neither had reduced numbers of circulating platelets nor enlarged spleens<sup>8-11</sup>. These mice received 3GA-injections less frequently compared to the study in **chapter 2**, indicating that 3GA-494-induced depletion of platelets is a time- and dose-dependent response. Platelets have no nucleus and lack novel transcription. We therefore investigated whether 3GA-494 could directly target miR-494-3p expression in these anucleate cells. MiR-494-3p expression was initially downregulated by 3GA-494 treatment, followed by an upregulation. The primary miR-494-3p transcript, pri-miR-494-3p, was rapidly depleted in response to the miR-494-3p inhibition.

3GA-494 thus directly targets miR-494-3p expression in platelets and triggered hyperactivation compared to 3GA-ctrl treated platelets (**chapter 2**). The question remains whether altered miR-494-3p expression directly led to hyperactivation or not. It could also be that the hyperactivation of the platelets is triggered by the composition of the 3GAs. Antisense oligonucleotides have been reported to trigger hypersensitive platelets and thrombocytopenia<sup>37-39</sup>. These effects have been linked to several types of nucleic acids and the phosphorothioate (PS) backbone of antisense oligonucleotides<sup>38, 39</sup>. We did not observe a decline in circulating platelets in our 3GA-ctrl treated group. 3GA-ctrl is similar as 3GA-494 in chemical composition and structure, except for the targeted sequence. This suggests that 3GA-494-induced hyperactivation of platelets is triggered by the specific sequence rather than the chemical composition and structure of the 3GAs themselves.

The question remains how exactly 3GA-494, and antisense oligonucleotides in general, affect hypersensitivity of platelets and more importantly, how this could be avoided to prevent severe thrombocytopenia. More research into the exact mechanisms is therefore needed to overcome this current limitation for therapies using antisense oligonucleotides.

Another surprising observation in both **chapter 2** and **chapter 3** is the upregulated miR-494-3p expression in specific tissues, cell types and even in cell subsets, after treatment with 3GA-494. We found an increased expression of miR-494-3p *in vivo* in the spleen and platelets in **chapter 2**, but also in cultured human M2 polarized macrophages in **chapter 3**, after 3GA-494 treatment. MicroRNAs can regulate expression of RNA-binding proteins, which in turn can regulate processing of microRNAs. RNA-binding protein Mef2A for example, directly binds to pri-miR-494-3p<sup>10</sup>. Whether Mef2A or other RNA-binding proteins are responsible for the cell type- and subset-specific autoregulation of miR-494-3p, is yet to be determined.

MicroRNAs have cell type-specific target genes<sup>40</sup>. We showed in **chapter 3** that even subsets of the same cell type, the macrophages, have distinct Wnt pathway genes that were targeted by miR-494-3p. 3GA-494 treatment dampened M1 polarization, while M2 polarization was enhanced. The effects in both subsets are favorable in reducing atherosclerosis. However, distinct effects of miR-494-3p, and microRNAs in general, in different tissues, cells and cell subsets, are important to take into account when developing (micro)RNA therapeutics. It remains a technical challenge to specifically target cells or even their subsets, but could avoid unwanted side effects in future studies. More research is therefore needed to overcome this technical restriction that has important implications for the potential future use of (micro)RNA therapeutics.

The cluster of 14q32 C/D box snoRNAs is strongly associated with vascular remodeling and human CVD<sup>7, 14, 41</sup>. Plasma levels of 14q32 snoRNAs were associated with disease outcome in peripheral arterial disease (PAD)<sup>7, 14</sup>. These findings indicate a regulatory role of 14q32 snoRNAs in CVD. In the second part of the thesis, in **chapter 4** and **chapter 5**, we aimed to unravel the function of SNORD113-6/AF357425. Even though we succeeded to identify mRNA targets as well as tRNA targets of this snoRNA, the full function remains to be elucidated. We focused in **chapter 4** on mRNA targets from the integrin signaling pathway, but found also enrichments of mRNA targets in other cardiovascular relevant pathways, including blood coagulation and interleukin signaling. We showed that 2'Ome by AF357425/SNORD113-6 acts in stabilization of mRNAs, but effects on protein level were quite ambiguous. More research is therefore needed to fully explore the function of 2'Ome by AF357425/SNORD113-6. Furthermore, SNORD113-6/AF357425 has two antisense boxes and we now focused on just one, the D' antisense box, for target prediction. The question whether the other antisense box is active or not, still needs to be answered.

What complicates the research in (orphan) C/D box snoRNAs is that it can be challenging to reliably knockdown these snoRNAs<sup>42</sup>. We aimed to overcome this issue by generating an AF357425 knockout model. Our knockout strategy however, proved unsuccessful. This suggests nevertheless that AF357425 is essential for cell survival. AF357425 likely also acts in cell proliferation. Prior treatment with KN93 in order to synchronize the cell cycle, was needed to reproducibly knockdown AF357425 using oligonucleotides. How and by which exact mechanism SNORD113-6/AF357425 expression is being regulated in cell survival and proliferation, remains elusive for now.

Of course, the technical challenges in snoRNA knockdown also complicate investigating the function of AF357425/SNORD113-6 *in vivo*. Targeting AF357425 in, for example, a hind limb ischemia model (a model for PAD) would explore the therapeutical potential of AF357425. Knockdown of SNORD113-6 affected human arterial fibroblast phenotype and increased barrier function, extracellular contraction and cell migration. In **chapter 5**, we showed that exposing both human and murine fibroblasts to hypoxia and oxidative stress upregulated SNORD113-6/AF357425 expression. Both are important triggers of vascular remodeling processes. Perhaps modulating AF357425 expression *in vivo* would consequently also affect entire processes like angiogenesis.

In **chapter 5** it was demonstrated that 2'Ome by SNORD113-6/AF357425 regulates tRNA fragmentation. We focused on the formation of tRF<sup>Leu 47-64</sup> in particular. The question remains what the function of this AF357425/SNORD113-6-dependent tRF could be. TRFs have been shown to be functionally active in cardiac and skeletal muscle cells, but also in inhibition of processes like angiogenesis<sup>33, 34</sup>. The function and targets of tRF<sup>Leu 47-64</sup> as well as its role in vascular remodeling remains to be identified in future studies.

Even though many questions still need to be answered, we managed to lift the orphan status of AF357425/SNORD113-6. Like a microRNA, AF357425/SNORD113-6 facilitates a modest regulation of its targets, but affects many target genes simultaneously. Modulation of AF357425/SNORD113-6 can thus be powerful in complex diseases as CVD. SnoRNAs or tRNA fragments that act like microRNAs or that perform functions completely different from their canonical function, increases the complexity of ncRNAs. It also provides a new regulatory layer to their function that may yield novel therapeutical targets. We explored the function of one of the 14q32 snoRNAs in humans. Still, more research into the remaining 40 14q32 snoRNAs remains to be done.

Research over the past few years has led to more understanding of CVD. Atherosclerosis, the underlying cause of most CVDs, is no longer considered to be just a lipid-driven disease. The inflammatory component of atherosclerosis is now being recognized as a major contributor to the progression of the disease. The CANTOS trial is a prominent example proving the role of the immune system in CVD, independent of lipid lowering<sup>36, 43</sup>. Advances in sequencing techniques created new angles for therapeutic strategies to combat CVD. A large part of the noncoding human genome is no longer considered non-functional. In fact, the role of ncRNAs in regulating gene expression and in different cardiovascular pathologies is now increasingly being recognized<sup>3</sup>. The use of mRNA vaccines in the current COVID-19 pandemic may also



pave the way for RNA therapeutics in clinical practice<sup>44</sup>. Many steps still have to be taken before ncRNA therapies will be used to treat CVD. However, this thesis showed that 14q32 microRNAs and snoRNAs may be promising therapeutical targets to treat and prevent CVD.

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