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The role of microRNA alterations in post-ischemic neovascularization

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Citation
Kwast, R. V. C. T. van der. (2020, October 15). *The role of microRNA alterations in post-ischemic neovascularization*. Retrieved from https://hdl.handle.net/1887/137728

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Cover Page

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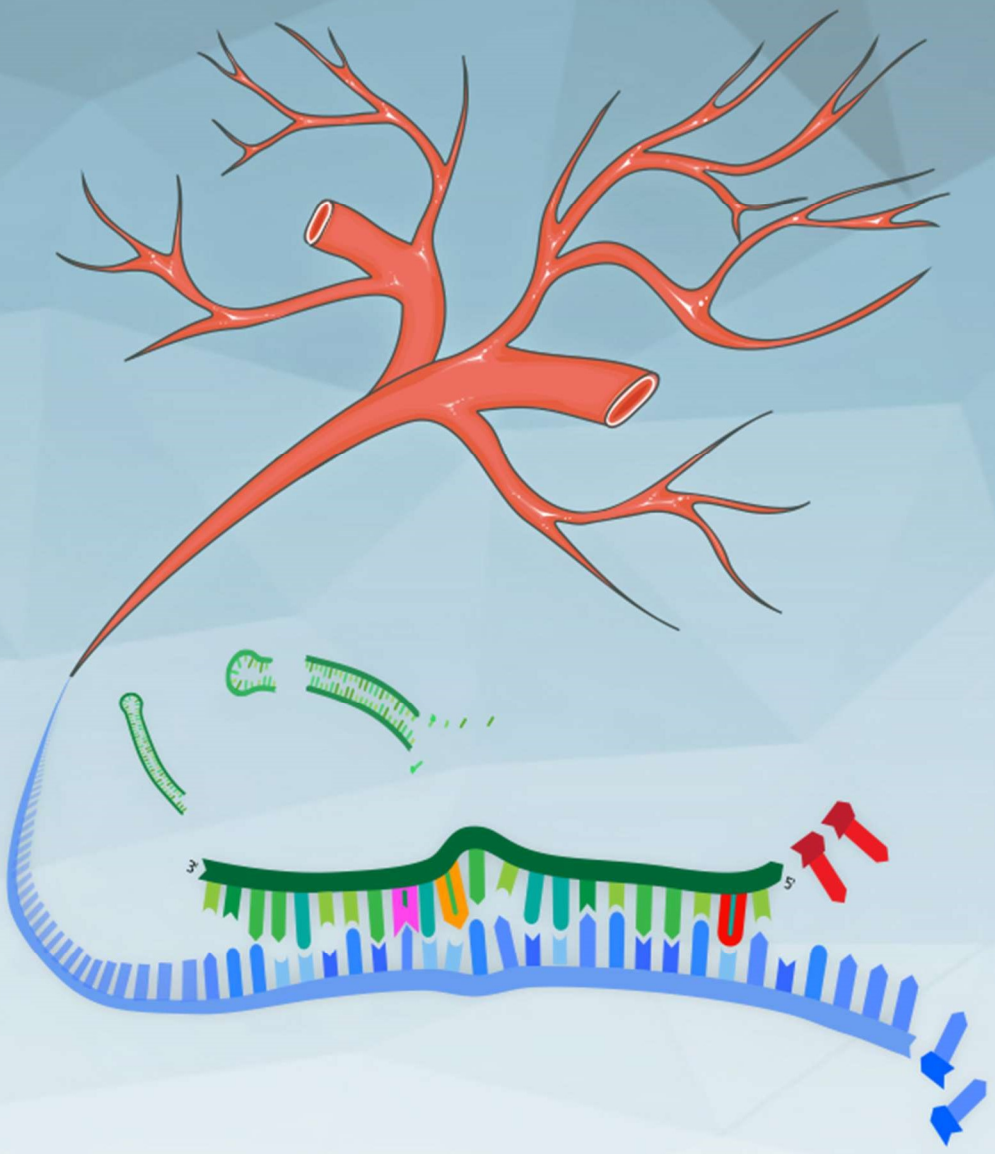
Title: The role of microRNA alterations in post-ischemic neovascularization
Issue Date: 2020-10-15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CHAPTER 7

Summary and Future perspectives

RATIONALE

The heart and blood vessels make up the cardiovascular system, which circulates blood throughout the body. Cardiovascular diseases (CVDs) are the leading cause of death in worldwide[1,2]. While current treatments have significantly improved the lifespan and wellbeing of patients, it is estimated that they are unsuitable or insufficient for 30% of patients[3,4]. Therefore, there is a critical need for new therapeutic treatments for CVD.

Although CVDs have diverse and complex pathologies, they generally result in local shortages in the blood supply, known as ischemia. The body has an innate response mechanism that stimulates restoration of blood flow to ischemic tissues, known as neovascularization[5,6]. Neovascularization is comprised of angiogenesis, the growth of new vessels, and arteriogenesis, the maturation of pre-existing collateral arterioles[7].

During the last decade, a specific type of small, regulatory molecules, called microRNAs, have emerged as key regulators of neovascularization, and can potentially serve as therapeutic target for ischemic CVD[7-9]. MicroRNAs are short RNA molecules that regulate protein expression by complementary binding to messenger RNAs (mRNA), which prevents the mRNA's translation into protein. Like other RNAs, microRNAs have a 5′-end and a 3′-end. However, the 5′-end of a microRNA is particularly important, because a microRNA's target selection is predominantly determined by nucleotides 2-8 at the 5′-end of a microRNA, known as the seed sequence[10,11]. A microRNA can have hundreds of mRNAs in its 'targetome', allowing them to regulate complex processes, including neovascularization[12].

The biogenesis of microRNAs starts with the transcription of the microRNA containing gene, yielding a primary microRNA (pri-miR). Pri-miRs are sequentially cleaved by ribonucleases DROSHA and DICER after which the mature microRNA is incorporated into the RNA induced silencing complex (RISC) to become functional (**Figure 1A**)[13].

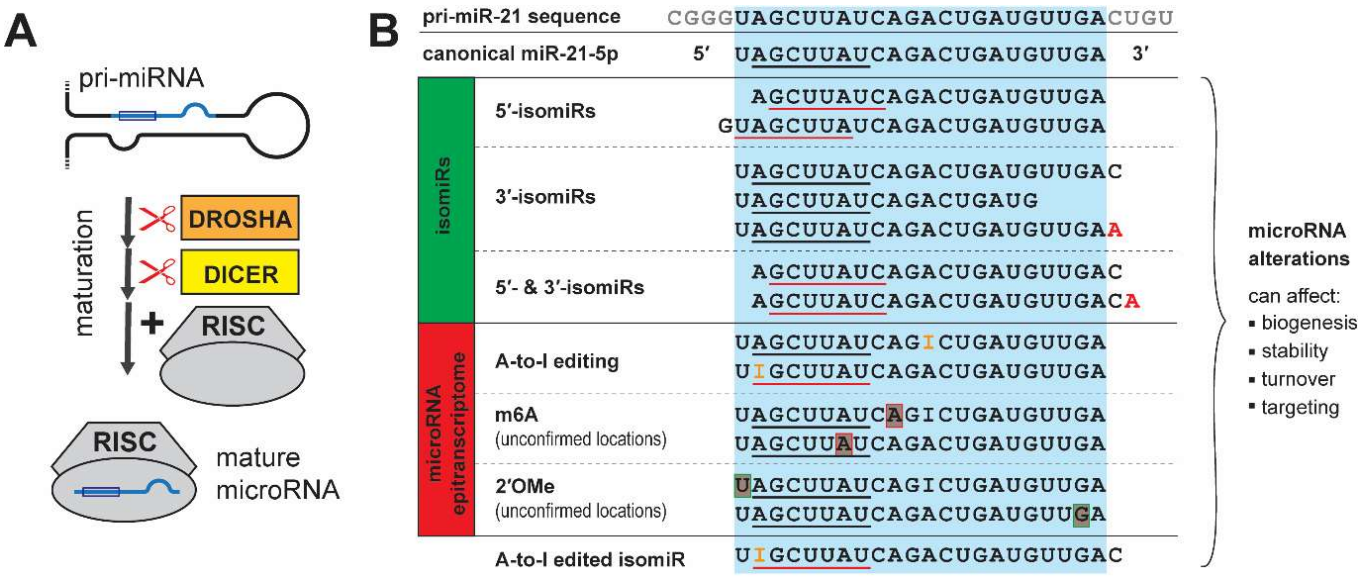

Figure 1: microRNA biogenesis (A) and alterations (B). Alterations of miR-21 are used to exemplify different types of microRNA alterations. The seed sequence is of each microRNA version consists of the underlined nucleotides.

MICRORNA ALTERATIONS

MicroRNAs have typically been defined as a single sequence of RNA nucleotides. However, recent studies have discovered that the 'canonical' microRNA sequence is often altered in a number of ways during their biogenesis. These microRNA alterations can change the expression and even functioning of the affected microRNA. However, whether microRNA alterations play a direct role in cardiovascular disease was unclear.

In this thesis we investigated whether alterations of microRNAs with a known vasoactive function can indeed regulate their expression, function and role in neovascularization. We studied a subset of abundant microRNA alterations and found they are indeed present in vascular microRNAs. Moreover, we demonstrated that the investigated microRNA alterations are dynamically regulated in response to ischemic conditions, often resulting in functional changes that impact angiogenesis.

In **Chapter 2** we review the current literature on microRNA alterations and discuss evidence that suggests microRNA alterations play a role in neovascularization. We highlight that microRNA alterations can be grouped into two overarching types: isomiRs and RNA nucleotide modifications (RNMs) (**Figure 1B**).

IsomiRs are microRNA sequence variants that have one or more nucleotides added or deleted at their 5′- and/or 3′-ends compared to the canonical microRNA sequence. IsomiRs are highly prevalent and generally account for approximately 50% of the total microRNA transcripts present in cells and tissue. Moreover, isomiR expression profiles can vary based on cell type and in response to biological stimuli. IsomiRs with altered 3′-end sequences, 3′-isomiRs are more abundant than 5′-isomiRs and often have altered microRNA stability and turnover. However, 5′-isomiRs were shown to be functionally different compared to the canonical microRNA sequence, since they have an altered seed sequence and thus an altered targetome. While the roles and functions of individual isomiRs remain largely unknown, their abundance, regulation and functional differences combined indicate that isomiRs are likely to play an important role in many processes, including neovascularization.

RNMs are biochemical modifications of the standard RNA nucleotides, which are performed by 'writer' enzymes present in all living organisms. Many different RNMs exist, each of which has its own unique properties. Studies have demonstrated that these RNA modifications have a functional regulatory role and form what has been named the 'epitranscriptome'. While recent studies have demonstrated the presence of numerous RNMs in microRNAs, only two are known to influence microRNA function: adenosine-to-inosine editing (A-to-I editing) and N6-adenosine methylation (m6A). However, we also discuss studies which indicate that at least 5 other RNMs found in microRNAs are likely to play a role in regulating microRNA function, most of which will be addressed later. Nevertheless, little is known about the direct influence of any RNMs on vasoactive microRNAs and whether microRNA RNMs affect neovascularization.

IsomiRs influence the adaptive responses to ischemia

In **Chapter 3** we assessed whether isomiRs can have a direct effect on vasoactive microRNA functioning by studying the 5'-isomiR of vasoactive miR-411 expression under ischemic conditions in different tissues. We found that the isomiR has tissue specific expression and is more abundant than the WT-miR-411 in vascular cells and tissue. Additionally, we discovered that isomiR expression decreased relative to canonical miR-411 expression after acute ischemia in both human vascular cells and in

a murine hindlimb ischemia model. Strikingly, relative isomiR expression was increased instead in chronically ischemic human blood vessels. To the best of our knowledge, these findings provide the first evidence that indicates that the expression of a microRNA and its isomiR can be independently regulated in response to pathophysiologic changes like ischemia.

By studying different the different maturation stages of isomiR and canonical miR-411 we demonstrate that the isomiR's 5'-end variation is the result of alternative DROSHA cleavage of the pri-miR-411 transcript. This results in 2 distinct pre-miR-411 versions with distinct 5'-ends: an isomiR pre-miR and a canonical pre-miR. Interestingly, we found that the observed changes in relative isomiR expression are not caused by altered alternative DROSHA cleavage rates, but are due to changes in subsequent version specific pre-miR maturation rates instead. Although the exact factors that govern the changes in relative isomiR biogenesis remain elusive, our findings reveal that changes in maturation rates can play an important role in isomiR expression.

Finally, we demonstrated that this 5'-isomiR and its regulation also has functional consequences. Using several assays to examine target regulation by microRNAs, we demonstrate that the isomiR has a significantly altered targetome compared to canonical miR-411, due to the isomiR's shifted seed. Furthermore, we validated that only canonical miR-411 decreases TGFB2 (transforming growth factor-beta 2) production, while only the isomiR decreases pro-angiogenic ANGPT1 (angiopoietin-1) secretion. In line with these findings, we demonstrated that the isomiR decreases the migration rate of cells compared to canonical miR-411 in an *in vitro* angiogenesis assay.

Combined, our findings provide novel insights into the role of miR-411 and its isomiR in the adaptive responses to ischemia. More importantly, however, our research demonstrates that isomiR formation in general is a functional pathway, which is actively regulated during ischemia, with direct implications for neovascularization. As mentioned in a commentary on our work by Dr. Hoefer[14], these insights may shed new light on previous work on miRNA expression profiles, predicted targets and miRNA silencing effects due to—unknowing—disregard of isomiR presence and/or expression. Therefore, further exploration of this new regulatory layer could provide novel therapeutic options for CVD and other pathologies.

A-to-I editing of microRNAs promotes angiogenesis after ischemia

A-to-I editing is the biochemical modification of adenosines into inosines. Unlike adenosine, inosine preferentially binds to cytidine and is therefore generally interpreted as guanosine by the cellular machinery[15]. In mammals, this form of RNA editing is of vital importance in mammals, and is performed by either ADAR1 or ADAR2 (adenosine deaminase acting on RNA 1 or 2)[16-18]. ADARs can target pri-miRs which can profoundly influence microRNA maturation or even result in a completely altered targetome when editing occurs in the microRNA's seed sequence[19-22]. However, it was unknown whether microRNA A-to-I editing plays an active role in cardiovascular biology and the adaptive responses to ischemia.

Previously, our group has demonstrated that 4 microRNAs from a microRNA gene cluster located human chromosome 14 (the 14q32 cluster), directly affect blood flow recovery after ischemia in mice[23]. Strikingly, one of these microRNAs, miR-487b, has very few conserved putative target genes, while the other microRNAs has more than a hundred[24]. Despite this, miR487b is still highly conserved in mammals and can simultaneously regulate both angiogenesis and arteriogenesis. Therefore, we hypothesized that the seed sequence of miR-487b undergoes A-to-I editing under ischemic conditions, which expands its targetome.

In **Chapter 4** we discover that the first nucleotide of miR-487b's seed-sequence is indeed A-to-I edited in mice and humans, resulting in a novel microRNA ED-miR-487b. The rate of miR-487b editing increased during in a murine model for successful neovascularization after hindlimb ischemia. Interestingly, we observed a different temporal regulation of miR-487b editing at the site of ischemia induced angiogenesis compared to that of shear stress induced arteriogenesis. To our knowledge, this study was the first report of dynamic microRNA editing during vascular remodeling and ischemia in general.

During mechanistic studies we uncovered that miR-487b editing can be performed by both ADAR1 and ADAR2. However, ADAR1 and ADAR2 expression does not appear to be directly correlated to miR-487b editing after ischemia, suggesting additional regulatory mechanisms help regulate editing of specific adenosine residues. This is in line with previous findings that have suggested that 2'-O-methylation (2'OMe), a different, stabilizing RNM, can protect adenosines from deamination by ADARs[25-27]. In

our study we demonstrate that the adenosine of miR-487b that undergoes A-to-I editing can in fact be subjected to 2'OMe instead. However, we found that, like editing, 2'OMe of miR-487b also increases after ischemia, suggesting a positive correlation between A-to-I editing and 2'OMe rather than the previously reported negative correlation. Furthermore, 2'OMe of miR-487b also did not appear to protect against A-to-I editing in additional *in vitro* studies. Instead, we uncovered an inverse correlation between the expression of the 'writer' enzymes responsible for miR-487b A-to-I editing and 2'OMe. This novel finding may help explain the previously reported inverse correlation between A-to-I editing and 2'OMe of RNAs.

The seed-sequence of ED-miR-487b is different from any known microRNA in humans and mice, meaning that because of A-to-I editing, an entirely new microRNA with a novel targetome is created. We confirmed that editing of miR-487b does indeed completely shift its target site selection using several assays to examine target regulation by microRNAs. Furthermore, we found that ED-miR-487b target genes are repressed stronger in murine muscle tissue after hindlimb ischemia, than canonical miR-487b target genes. The implications of miR-487b editing on neovascularization were assessed using several angiogenesis assays. In contrast to canonical miR-487b, ED-miR-487b promoted cell migration and sprouting of new blood vessels. These findings demonstrate that A-to-I editing can actively change microRNA-functionality during ischemia, which can promote neovascularization. However, whether vasoactive microRNAs besides miR-487b are also subject to A-to-I editing in the vasculature was still unknown.

In **Chapter 5** we aimed to identify which other vasoactive microRNAs are A-to-I edited in vascular cells and whether this editing is also regulated under ischemia and affects angiogenesis. We identify 35 mature vasoactive microRNAs that are A-to-I edited in a tissue-specific manner by analyzing public datasets of RNA-sequencing experiments on different human tissues. We found that 10 of these vasoactive microRNAs are indeed A-to-I edited at the pri-miR level in primary human vascular fibroblasts and endothelial cells. Knockdown experiments revealed that vasoactive pri-miRs editing is generally performed by ADAR1 or ADAR2, depending on the microRNA. Similar to miR-487b editing, pri-miR editing consistently increased under ischemic conditions, suggesting this is a widespread phenomenon.

Next we focused on the 4 most prevalent vasoactive microRNAs that undergo editing, miR-376a, miR-376c, miR-381 and also miR-411. All four mature microRNAs were edited in their seed-sequence and we demonstrated that microRNA editing consistently increases under ischemia in both human and murine models. More importantly, we found that vasoactive mature microRNA editing was also strongly increased in veins of patients with chronic ischemia due to peripheral artery disease, providing the first evidence that microRNA editing is actively regulated during CVD.

The seed-sequence of each of the edited vascular microRNAs is different from any validated microRNA, indicating that these A-to-I editing events result in an entirely new microRNA with a novel targetome. Using different assays, we confirm that editing of miR-376a, miR-376c, miR-381 and miR-411 does indeed completely shifts its target site selection. Moreover, we demonstrated the relevance of edited microRNAs *in vivo*, by showing that the increased expression of the edited microRNAs after ischemia goes hand-in-hand with a decreased expression of the targetome of the edited microRNAs but not of the targetome of canonical microRNAs. Finally, we found that, like for miR-487b, the edited microRNAs consistently have increased pro-angiogenic properties compared to the canonical microRNA versions using multiple functional assays.

Combined these studies show that vascular microRNA-editing is a wide-spread phenomenon, induced by ischemia. These editing event often result in a novel microRNA with a unique seed-sequence and thus a unique targetome, generally leading to pro-angiogenic functional changes. Therefore, microRNA editing can play a direct role in cardiovascular disease and increasing vasoactive microRNA editing appears to be an attractive therapeutic strategy to stimulate neovascularization after ischemia.

N6-methyladenosine methylation of microRNAs increases with hypoxia

N6-methyladenosine (m6A) is one of the most abundant RNMs in cells and tissues. This RNM is installed by the m6A 'writer' METTL3[28], which can be guided to specific methylation sites by several non-essential cofactors like WTAP (Wilms' tumor 1-associating protein)[29]. m6A sites can also be demethylated by 'erasers' ALKBH5 (alkB homolog 5) and FTO (fat mass and obesity-associated protein), which allows for more dynamic regulation of m6A compared to most other RNA modifications[30,31]. In fact,

hypoxia caused by ischemic CVDs has been associated with increased m6A in mRNAs[32-34].

m6A is of vital importance and can influence most processes related to RNA function and turnover[35-38]. Recently, studies demonstrated that m6A can also affect microRNA biogenesis and functioning. Pri-miR m6A was shown to be able to promote microRNA biogenesis, while m6A within the mature microRNA might result in altered silencing efficiency by affecting mRNA-microRNA interaction strength[39-41]. Interestingly, several microRNAs with a known vasoactive function were found to be methylated in HEK293 cells, a human embryonic kidney cell line, by Berulava *et al*[42]. However, whether these mature vasoactive microRNAs are also subject to m6A methylation in other cell types is unknown. Furthermore, it is also unknown if microRNA m6A methylation is dynamically regulated in response to hypoxic conditions, similar to mRNA m6A methylation.

In **Chapter 6** we investigated whether selected vasoactive microRNAs are methylated in primary vascular fibroblasts and in a fibroblast cell line, which are known to have high microRNA expression levels[43]. By specifically isolating microRNAs that contain m6A, we demonstrate that 16 of the 19 investigated vasoactive microRNAs are abundantly methylated in both types of fibroblasts. Interestingly, at least 7 of the 19 microRNAs had the opposite methylation status in fibroblasts when compared to a previous study using HEK293 cells, indicating that, like microRNA expression itself, microRNA m6A methylation can be cell-type specific. More importantly, we found that mature microRNA m6A increased upon subjecting cells to hypoxia. Therefore, our data demonstrates for the first time that, like mRNA m6A levels, microRNA m6A levels are also upregulated in response to hypoxia, and thus can be dynamically regulated in general.

Additional measurements revealed that the hypoxia-induced increase in microRNA m6A methylation is unlikely to be caused by changes in the expression of m6A-associated proteins METTL3, WTAP and ALKBH5. However, recent studies have revealed that changes in m6A levels can also be achieved without changes in m6A-writer complex abundance, by directly influencing methylation activity or by specifically promoting maturation of m6A containing[44-46]. Therefore, the mechanisms

that mediate dynamic regulation of m6A levels appear to be more complex than previously anticipated and remain to be defined.

Finally, we investigated whether ALKBH5, WTAP and METTL3 play a role in microRNA processing in fibroblasts. Surprisingly, individual knockdown of each factor only sporadically affected the expression of the microRNAs examined. While these findings need to be confirmed, they could suggest that m6A plays a more microRNA-specific role in microRNA biogenesis than previously thought.

When combined, our findings indicate that microRNA m6A methylation is dynamically regulated in a cell type specific manner. While the mechanism behind regulation of vasoactive microRNA m6A methylation remains elusive, we found that microRNA m6A increased upon hypoxia. Since m6A within microRNAs might result in altered microRNA silencing efficiency, dynamic regulation of microRNA m6A can potentially have a significant impact on microRNA mediated transcriptional regulation.

FUTURE PERSPECTIVES

Combined, the findings in this thesis demonstrate that multiple microRNA alterations are dynamically regulated in response to ischemia and can have a direct effect on the expression and function of microRNAs, often leading to changes in neovascularization-associated processes. Therefore, we show that microRNA alterations represent a novel regulatory layer and can provide new therapeutic opportunities to combat CVD, as well as other microRNA-associated pathologies.

The feasibility of altered microRNA-based therapies is highlighted by the success of the drug Patisiran, which became the first small RNA therapeutic to be FDA approved in 2018. Patisiran consists of small RNAs that enter the microRNA biogenesis pathway and subsequently target the root cause of a rare hereditary peripheral nerve disease. Moreover, several microRNA-oriented RNA therapeutics are currently being tested on large cohorts of patients during phase 2 clinical trials to see if they are effective[47]. A microRNA therapeutic to treat CVD patients, the pro-angiogenetic drug MRG-110, is also expected to enter phase 2 clinical trials soon, after successful completion of two phase 1 clinical trials[47].

A key issue encountered during the development of microRNA therapeutics is the need to maximizing the therapeutic effect on the diseased tissue, while minimizing the off-target binding and toxicity[48,49]. Our findings highlight that altered microRNAs could potentially provide more potent therapeutic targets or could be unappreciated off-targets that cause unintended side effects of a microRNA therapeutic. For example, in **Chapter 4 and 5** we demonstrated that therapeutic overexpression of several edited microRNAs can potentially be used to promote angiogenesis after ischemia, in contrast to their canonical versions. However, unintentional targeting of these edited microRNAs, by a microRNA inhibitor for example, could potentially lead to reduced wound healing as a side effect due to unintended inhibition of angiogenesis. Therefore, our findings reveal the importance to take microRNA alterations into account during design and development of microRNA therapeutics.

In **Chapters 4 and 5** we also found that A-to-I editing of vasoactive microRNAs generally promotes angiogenesis. These findings suggest that the factors that introduce or regulate microRNA alterations could also provide novel therapeutic options to influence the expression of altered microRNA on a broader scale.

Besides the need for novel therapies for CVD, biomarkers are also required to help facilitate earlier disease detection and identification of high risk patients, as highlighted in European guidelines[50,51]. Numerous studies have demonstrated that microRNAs can serve as potent circulating biomarkers which can help differentiate between CVD subtypes and improve clinical decision making[52,53]. For example, studies have shown that the expression of a set of microRNAs in whole blood can accurately predict acute myocardial infarction and allows for earlier detection of this disease compared to currently used biomarkers[54,55]. Furthermore, incorporating the patient's levels of circulating miR-26b-5p, miR-320a, and miR-660-5p levels into clinical risk prediction models could help identify patients likely to experience another major cardiovascular event more accurately[56].

In this thesis, we have demonstrated that the expression of several edited microRNAs is increased in both acute and chronically ischemic human veins (**Chapter 5**), while the relative expression of miR-411's 5'-isomiR is decreased after acute ischemia but increased after chronic ischemia (**Chapter 3**). These findings indicate that microRNA alterations can also be exploited to find new clinically relevant biomarkers

for CVDs. In fact, recent findings have demonstrated that isomiR expression profiles can be used to distinguish between cancer subtypes, while canonical microRNA expression profiles were insuffictient[57]. Therefore, identification of new microRNA based biomarkers can potentially be facilitated by including altered microRNAs next to canonical microRNAs during the assessment.

Another intriguing finding was that we observed that multiple different microRNA alterations within a single microRNA are dynamically regulated in response to ischemic conditions. As mentioned previously, we found that miR-411's 5'-isomiR formation and A-to-I editing were differentially regulated after acute and chronic ischemia, resulting in distinct effects on miR-411's targetome and angiogenic properties. The RNA sequencing data used in these studies suggest that miR-411's 5'-isomiR also undergoes A-to-I editing, which would in fact result in another new microRNA, with a new seed sequence and targetome associated with it. Therefore, effects of microRNA alterations on miR-411's functionality is expected to be even larger than we have shown so far.

Similarly, in **Chapter 6** we also discovered that miR-487b is subject to m6A methylation in addition to A-to-I editing and 2′OMe methylation (**Chapter 4**). While m6A and 2′OMe are unlikely to affect the microRNA's targetome, several studies have indicated that both m6A and 2′OMe could protect adenosine residues from A-to-I editing[25,27,58-60]. Although we have not confirmed these interactions, these findings indicate that microRNA modifications might affect one another and should be studied simultaneously whenever possible.

In this thesis, we examined several abundant microRNA alteration subtypes to demonstrate the regulatory potential of microRNA alterations in general. As described in **Chapter 2**, multiple other microRNA alteration subtypes are likely to affect microRNA functioning. 3′-isomiRs, for example, are highly abundant and often have altered stability and turnover. Therefore, 3′-isomiR formation might in fact play an important role in regulating the expression of specific microRNAs. Additionally, studies have suggested that certain 3′-isomiRs have altered targeting silencing activity[61,62]. Regarding RNMs, a study by Lan et al. found 20 distinct RNMs, in addition to inosine, m6A and 2′OMe, using mass spectrometry on an RNA fraction containing mainly microRNAs[63]. While little is known about the effect of these RNMs on the

functioning of microRNAs, several have been studied in other RNA types. The findings in these studies suggest that several other RNMs could potentially affect microRNA function: pseudouridine (Ψ), N1-methyladenosine (m1A) and N2-methylguanosine (m2G).

Pseudouridine (Ψ) is generated from isomerization of uridine and is one of the most abundant and conserved RNA modifications[64,65]. Studies have shown that pseudouridylation increases under stress conditions, including serum deprivation, a key component of ischemia[66]. Compared to an uracil, Ψ forms a stronger base pairing interaction with adenosine, which allows it to alter RNA secondary structures, suggesting that microRNA pseudouridylation could affect mRNA silencing[67,68].

N1-methyladenosine (m1A) is a different type of adenosine methylation which has been found in many different RNAs[69,70]. Similar to m6A, m1A is reversible and is dynamically regulated by various types of cellular stress[70,71]. Unlike a normal adenosine, m1A carries a positive charge. As a result, m1A methylation alters protein–RNA interactions and RNA secondary structures dramatically[72], which can potentially lead to disruption of microRNA biogenesis[13]. Furthermore, m1A does not appear to engage in RNA base-pairing, suggesting that m1A may also disrupt microRNA-target interactions[73,74].

N2-methylguanosine (m2G) is a type of guanosine methylation which is relatively common in microRNAs according to the study by Lan et al.[63]. However, little is known about this RNA modification due to a lack of high-throughput detection methods[75,76]. Nevertheless, studies have shown that m2G can form both canonical and non-canonical Watson–Crick base pairing interactions, allowing m2G to regulate the stability of tRNA tertiary structures and potentially influence microRNA silencing activity[75,77].

Besides their effect on endogenous microRNA modifications, the unique properties of certain RNMs might also be able to enhance the specificity of microRNA-therapeutics. For example, 2′OMe is often already used to enhance the stability and specificity RNA therapeutics and endogenous 2′OMe of microRNAs is thought to have similar effects[78-81]. Therefore, it might be fruitful to also study the less abundant RNMs. To this end, it is important to note that further advances in technology and methodology are required, especially to expand our knowledge of the microRNA

epitranscriptome[63,82]. However, given the surge of interest in this field, we expect many more clinically relevant microRNA alteration events to be discovered in the near future.

In conclusion, in this thesis we have shown that several distinct microRNA alterations are regulated in response to ischemia and can directly affect neovascularization. These findings suggest that microRNA alterations represent a novel regulatory layer that can provide new therapeutic opportunities to combat CVD and other microRNA-associated pathologies.

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