The role of microRNA alterations in post-ischemic neovascularization

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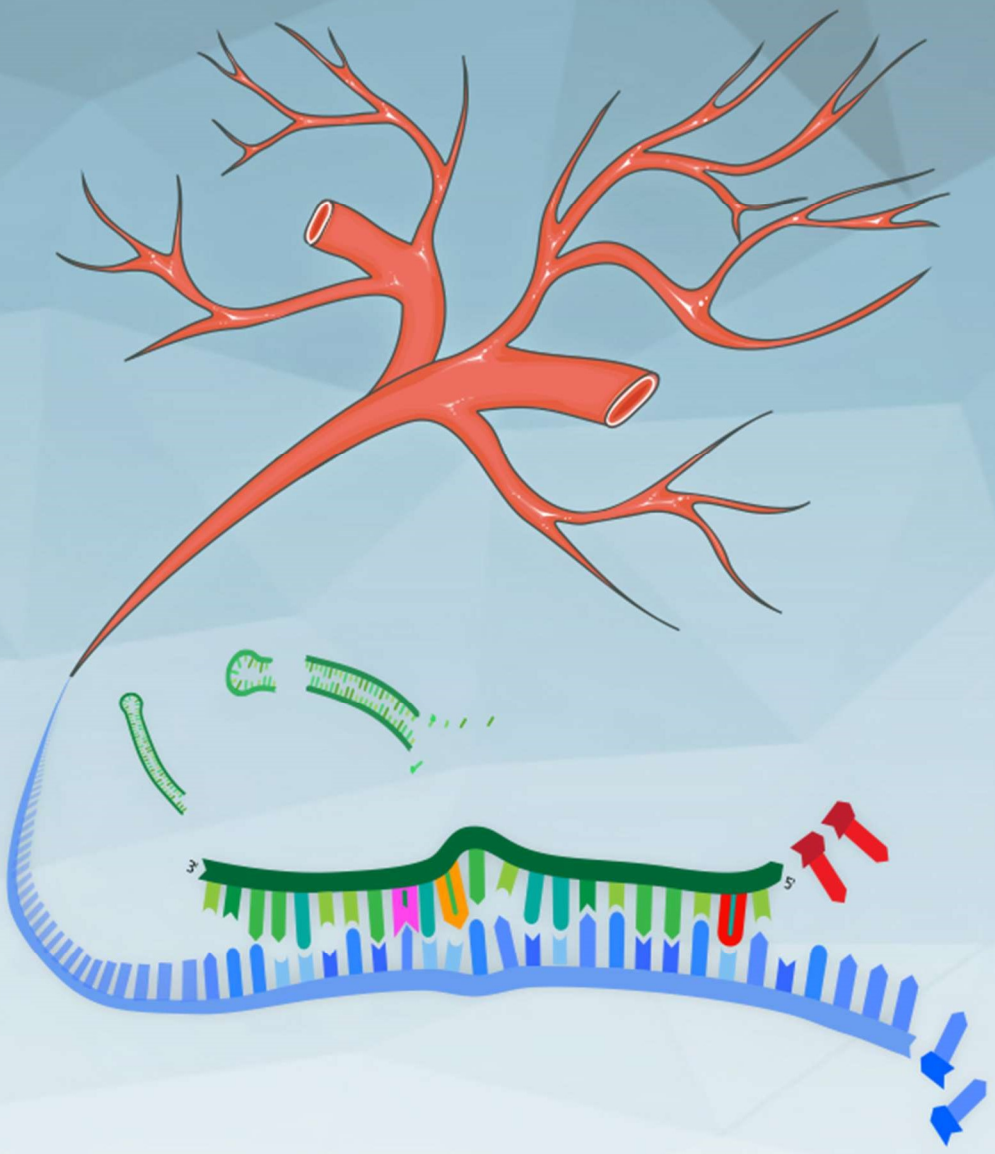
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CHAPTER 1

General Introduction

RATIONALE

The heart and blood vessels make up the cardiovascular system, which circulates blood throughout the body. This system provides every single organ access to oxygen and nutrients, as well as a highway to send chemical signals and relocate waste products. Cardiovascular disease (CVD) is the collective term used for diseases that affect the cardiovascular system. CVDs are the leading cause of death worldwide. In 2017, approximately 17.8 million deaths were caused by CVD, representing 31% of all global deaths[2,3]. 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[4,5]. Therefore, there remains 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. Ischemia causes the affected tissues to become starved of oxygen (hypoxic) and nutrients and unable to eliminate waste products, which lead to tissue death if left unresolved. These consequences of ischemia lead to most CVD-associated symptoms and deaths[2,3]. However, the body has an innate response mechanism that stimulates restoration of blood flow to ischemic tissues, known as neovascularization[6,7]. Neovascularization is comprised of angiogenesis, the growth of new vessels, and arteriogenesis, the maturation of pre-existing collateral arterioles. Both angiogenesis and arteriogenesis are highly multifactorial processes and are regulated by a number of different factors, including growth factors, haemodynamic forces and small, regulatory molecules, called microRNAs[8].

MicroRNAs are short non-coding RNAs of approximately 22 nucleotides that inhibit translation of messenger RNAs (mRNAs) into proteins. A single microRNA can target hundreds of mRNAs which allows them to potentially regulate an entire network or pathway simultaneously. During the last decade, microRNAs have also emerged as key regulators of cardiovascular biology and neovascularization[8-10].

MicroRNAs are typically defined as a single sequence of RNA nucleotides. However, recent findings suggest that this 'canonical' microRNA sequence can be altered, potentially leading to drastic changes in the microRNA's expression, its

silencing efficiency or even its targeting. As a result, these microRNA alterations represent a new layer for regulation of protein synthesis. Further understanding of this layer could potentially provide novel therapeutic options for ischemic CVD[11]. However, which cardiovascular microRNAs are altered and whether these microRNAs alterations can help modulate neovascularization is still unclear. Therefore, in this thesis, we examined the role of several different types of microRNA alterations in neovascularization after ischemia.

NEOVASCULARIZATION: ANGIOGENESIS & ARTERIOGENESIS

After an ischemic event, blood flow can be recovered by growth of new or existing vessels through two different types of neovascularization: angiogenesis and arteriogenesis (**Figure 1**). **Angiogenesis** is the sprouting of a new capillary from the existing vasculature. Angiogenesis is initiated when pro-angiogenic factors activate the vascular endothelial cells (ECs), which line the interior surface of blood vessels and form an interface between circulating blood in the lumen and the rest of the vessel wall[7]. The activated ECs begin to release proteases that degrade the extracellular matrix to allow vascular remodeling. Highly activated ECs become sprouting 'tip cells' that migrate towards the angiogenic stimulus while the neighbouring ECs become 'stalk cells' which proliferate and form the lumen of the new capillary sprout[12,13]. Next to ECs, other cell types are important regulators of angiogenesis. Vascular smooth muscle cells (SMCs), pericytes, fibroblasts and immune cells play key roles by supporting and modulating EC function and secreting the pro-angiogenic stimuli[14-16].

The pro-angiogenic signals required to start angiogenesis are produced by cells in response to ischemia. This cellular response is initiated when oxygen deprivation, or hypoxia, caused by ischemia stabilizes the transcription factor HIF1α (hypoxia inducible factor 1α), which is rapidly degraded in normoxic conditions[7,12]. HIF1α drives transcription of pro-angiogenic factors such as VEGF-A (vascular endothelial growth factor-A)[17]. VEGF is considered a key pro-angiogenic growth factor and is naturally secreted by most types of parenchymal cells in response to hypoxia[7,12]. Therefore, angiogenesis is driven directly by the cellular response to ischemia.

Arteriogenesis is the growth and maturation of collateral arteries from a pre-existing arteriole network, which connects all major arteries in the body[6]. Unlike

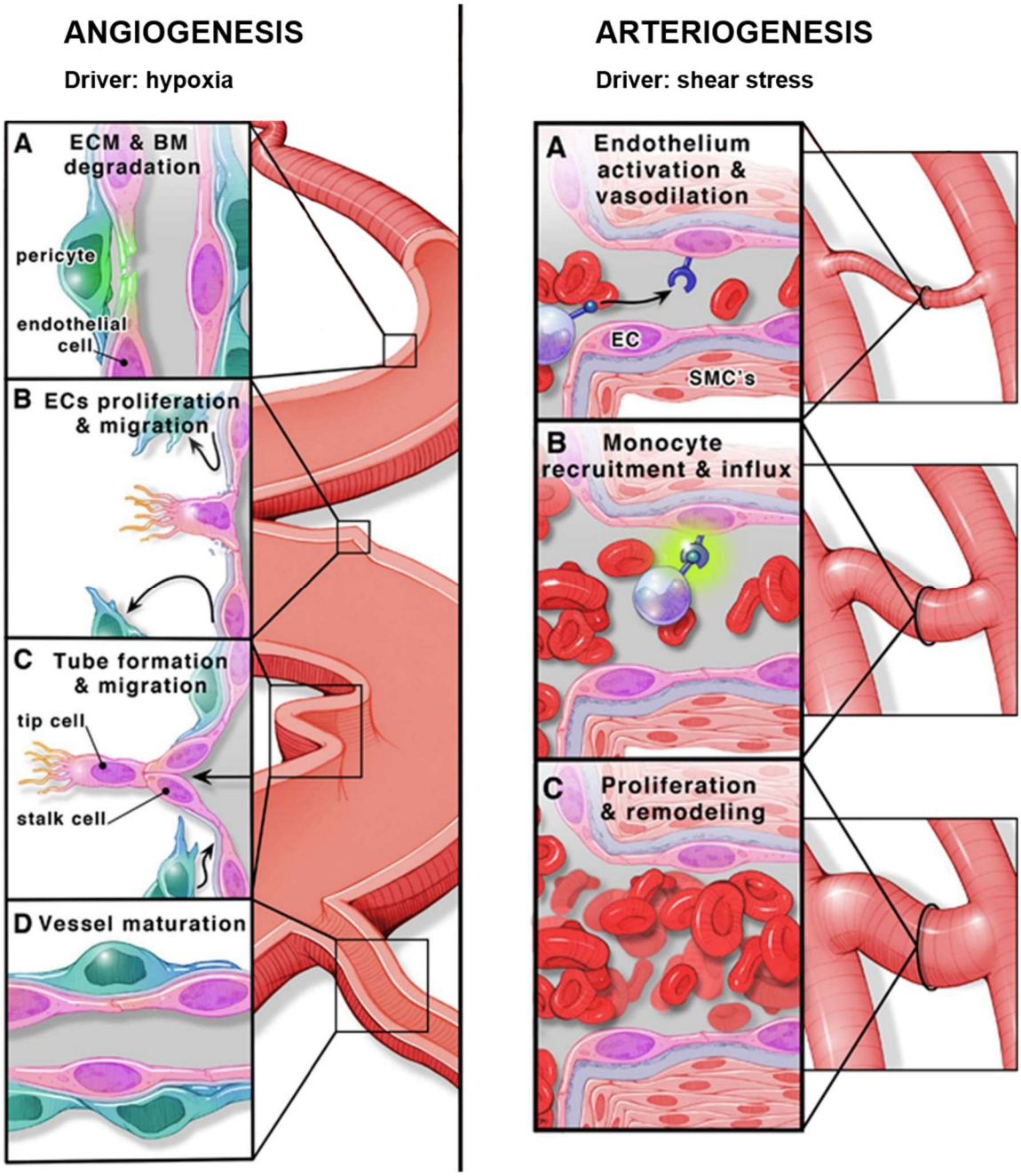

Figure 1: Neovascularization is comprised of angiogenesis, the growth of new vessels, and arteriogenesis, the maturation of pre-existing collateral arterioles. Both angiogenesis and arteriogenesis are highly multifactorial processes that help restoration of blood flow to ischemic tissues. Neovascularization is regulated by a number of different factors, including microRNAs. Figure is adapted from Ergul *et al*.[1]

angiogenesis, arteriogenesis is not driven by ischemia itself, but by increased shear stress in arterioles and the subsequent inflammatory processes[7,12]. This is initiated when an artery becomes occluded, which causes the blood flow to be redirected through the arterioles upstream of the occlusion. The increased shear stress stimulates ECs in the arteriole wall to express adhesion molecules and secrete cytokines, leading

to the attraction, adhesion and invasion of circulating monocytes and other immune cells[18-22]. These inflammatory cells produce growth factors and secrete cytokines and proteases that (partially) degrade the extracellular matrix to enable remodelling of the vessel[23-25]. The secreted factors induce migration and proliferation of both vascular ECs and SMCs, resulting in an increase in vessel diameter until fluid sheer stress decreases which halts the arteriogenic process. Finally, the vascular SMCs and fibroblasts secrete matrix components like collagen and elastin to reconstitute the vessel wall[12,26].

MICRORNAS REGULATE CARDIOVASCULAR BIOLOGY

For a long time, the central dogma in molecular biology was that DNA is transcribed into messenger RNA (mRNA), which is processed and then translated into proteins. Over the last two decades, however, we have learned that more than 80% of our genome is transcribed into RNA, of which less than 3% encodes proteins or peptides[27]. Many of these 'non-coding' RNAs are now known to be important regulators of protein expression. Especially the smallest class of RNAs, the microRNAs, have been shown to play an important role throughout life by regulating cell differentiation, development and homeostasis (**Figure 2**)[28]. These microRNAs are approximately 22 nucleotides long and their expression is tightly regulated and highly

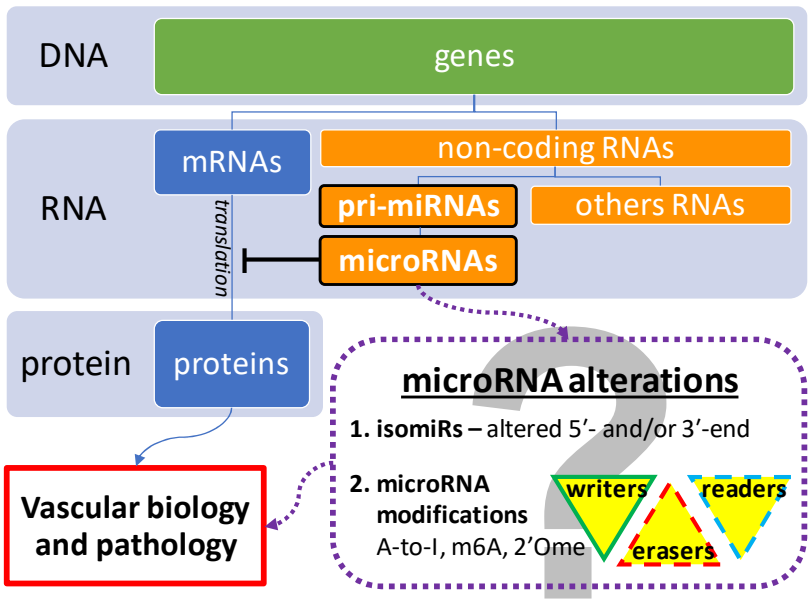

Figure 2: MicroRNAs regulate protein synthesis and can be altered in several ways. However, whether these microRNA alterations play a role in vascular biology and pathology is unknown.

tissue specific. Deregulation of microRNAs is associated with many human diseases, including ischemic CVD[28].

In 2007, the importance of microRNAs in neovascularization was demonstrated for the first time when several studies showed that microRNAs were required for angiogenesis[29-31]. Since then, microRNAs have been shown to play a functional role in all processes involved in neovascularization, including production and secretion of angiogenic stimuli, as well as EC, SMC, fibroblast and immune cell proliferation, migration and activation[10,32-34]. Several of these vasoactive microRNAs have also been well described to play an important role in vascular remodeling during ischemic cardiovascular diseases[10,35].

For example, miR-92a and miR-126 were shown to be highly expressed in human ECs and function as negative or positive regulator of angiogenesis, respectively. Inhibition of miR-92a increased angiogenesis in vivo and improved blood flow recovery after hindlimb ischemia in mice, a model for peripheral artery disease[36]. MiR-126, on the other hand, was shown to promote angiogenesis by stimulating EC proliferation and VEGF signaling and regulating leukocyte adhesion[37-40]. Inhibition of miR-126-3p decreased recovery after myocardial infarction and hindlimb ischemia in mice[38,41,42].

MicroRNA genes are often located within close proximity of one another within the genome, forming microRNA clusters. It is noteworthy that several microRNA clusters have been identified that are able to broadly regulate neovascularization in response to ischemia. MiR-92, for example, is part of the miR-17/92 gene cluster, located on chromosome 14 in mice and on human chromosome 13. Studies have shown that this cluster as a whole inhibits both angiogenesis and arteriogenesis[43,44]. MicroRNAs from the 14q32 microRNA cluster, located on human chromosome 14 and chromosome 12F1 in mice, also appear highly involved in regulating neovascularization. Inhibition of miR-329, miR-487b, miR-494 or miR-495, four 14q32 microRNAs, resulted in significantly improved blood flow recovery after hindlimb ischemia in each case, due to stimulation of angiogenesis and arteriogenesis[8]. Furthermore, miR-487b was also shown to play an important role in hypertension-induced remodelling of the aorta[45].

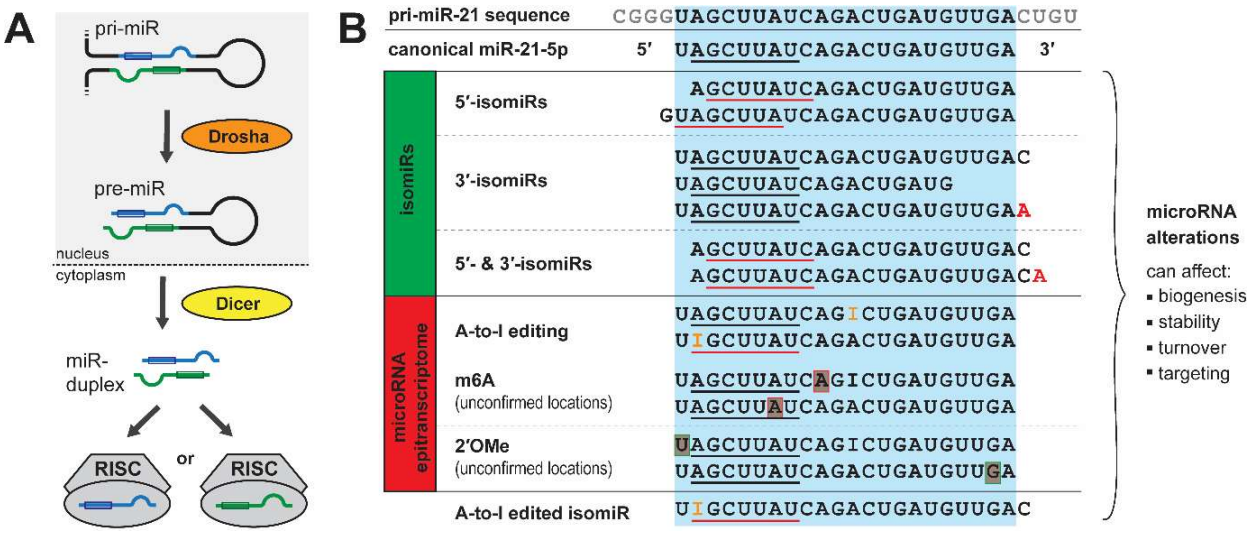

Figure 3: microRNA biogenesis (A) and alterations (B).

MicroRNA functioning and their biogenesis

MicroRNAs are able to exert their function by inhibiting translation of mRNAs to which they are complementary, allowing them to modulate protein synthesis[46]. Which mRNAs are targeted by a specific microRNA is largely dictated by nucleotides 2 to 8 from the microRNA's 5'-end, known as the microRNA's 'seed-sequence' [47,48]. Even though the inhibitory effect of a microRNA on an individual target mRNA is often subtle, a microRNA can have hundreds of mRNAs in its 'targetome'[49]. As a result, a microRNA can fine-tune protein expression levels of large sets of target genes, allowing it to regulate complex, multifactorial processes, including vascular remodelling[50].

MicroRNAs are initially transcribed as part of a longer, primary transcript known as the pri-miR. The pri-miR subsequently undergoes several maturation steps to ultimately yield a mature miR. During microRNA biogenesis, two distinct cleavage steps determine the 5'- and 3'-ends of a microRNA pri-miR (**Figure 3A**)[51,52]. First the pri-miR is cleaved in the nucleus by the ribonuclease DROSHA to generate a hairpin-shaped precursor miRNA (pre-miR)[53]. The pre-miR is exported to the cytoplasm where it is cleaved again by DICER into a microRNA duplex[54]. Finally, either strand of the microRNA duplex can be incorporated into the RNA-induced silencing complex (RISC) to become a functional mature microRNA[55].

MICRORNA ALTERATIONS

MicroRNAs have typically been defined as a single sequence of RNA nucleotides, and are listed as such in the principle public microRNA database, miRBase[56]. However, recent studies have shown that that this 'canonical' microRNA sequence can be altered. MicroRNA alterations can be separated into two types: isomiRs and RNA nucleotide modifications (**Figure 3B**).

IsomiRs

IsomiRs are microRNAs with one or more nucleotides added or deleted from the 5′- and/or 3′-ends compared to the canonical microRNA sequence. While originally dismissed as errors or artifacts, isomiRs have since been shown to associate with RISC and regulate mRNA translation, and thus function like canonical microRNAs[52,57-59]. IsomiRs are highly prevalent and generally account for approximately 50% of the total microRNAs detected during RNA sequencing studies[60,61]. The 5′ and 3′ heterogeneity that characterize isomiRs is primarily generated by cleavage variations of DROSHA or DICER during microRNA biogenesis[62-64]. IsomiRs with altered 3′-end sequences, 3′-isomiRs, often have altered microRNA stability and turnover[65-69]. 5'-IsomiRs on the other hand have a completely altered seed sequence, due to their altered 5'-end, which can have a major impact on the microRNA's functionality and targets definition[57,70-72]. Therefore, isomiRs could potentially have a different effect on neovascularization than their canonical microRNA versions.

RNA nucleotide modifications

RNA nucleotide modifications (RNMs) are biochemical modifications of the standard RNA nucleotides and can be found in all living organisms[73]. Similar to DNA nucleotide modifications in the field of epigenetics, RNMs are performed by naturally occurring enzymes, which have been termed modification 'writers'. In fact, more than 100 different RNMs have been identified, occurring in organisms ranging from archaea and bacteria, to eukaryotes[73]. Recent studies have demonstrated that these RNMs have a functional regulatory role and form what has been named the 'epitranscriptome'[73-75]. Furthermore, for a few specific modifications, proteins have been discovered which can recognize or remove this modification. These '*readers*' and '*erasers*' help to modulate

the functionality of these particular modifications and allow them to be even more dynamically regulated[76,77].

MicroRNA nucleotide modifications

Although our knowledge on RNMs and the epitranscriptome is slowly expanding, there is a strong focus on modifications in longer RNA species and therefore microRNAs are left understudied. Nevertheless, Lan *et al* recently used mass spectrometry to demonstrate that microRNAs in human HEK293T cells contain at least 24 distinct types of RNMs[78]. Moreover, whether RNMs of microRNAs play a role in cardiovascular disease was unknown. During our studies we focussed on 3 abundant RNMs: adenosine-to-inosine editing (A-to-I editing) and N6-adenosine methylation (m6A) and 2′-O-methylation (2′OMe), which are shown in **Figure 4**.

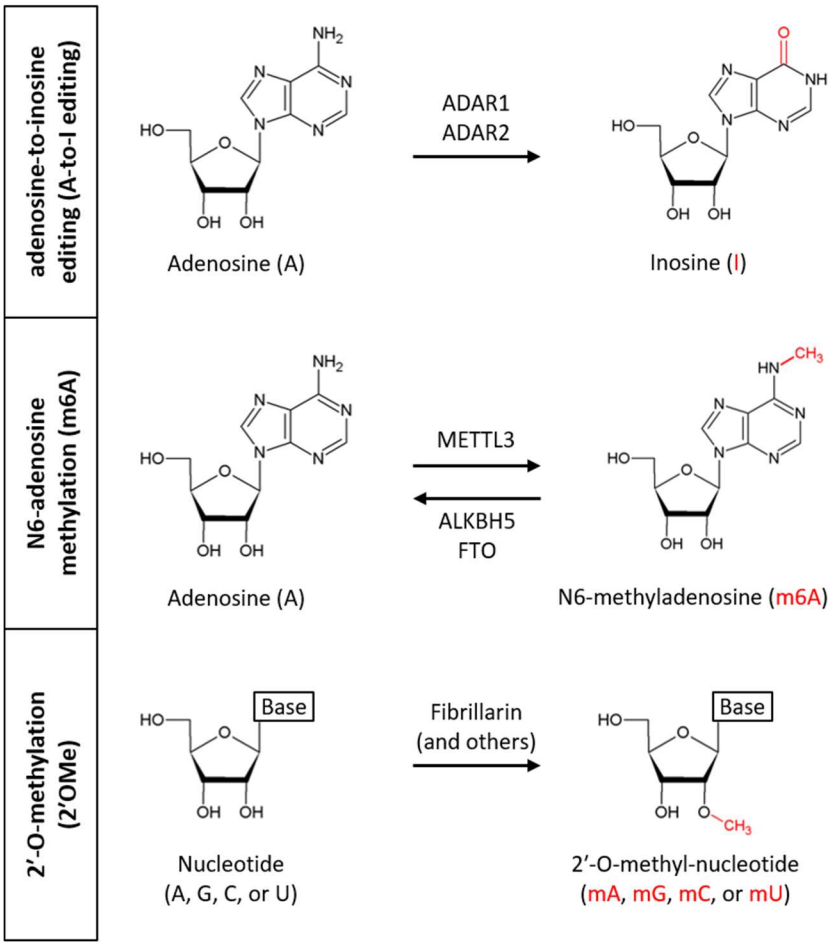

Figure 4: The RNMs we studied in microRNAs. The biochemical modifications of the standard RNA nucleotides are shown in red.

Adenosine-to-Inosine (A-to-I) Editing. In A-to-I editing, adenosines are deaminated to inosines by either ADAR1 and ADAR2 (adenosine deaminase acting on RNA 1 or 2) in mammals. Unlike adenosine (A), inosine preferentially binds to cytidine (C) and is therefore generally interpreted as guanosine (G) by the cellular machinery[79]. Therefore, A-to-I editing introduces specific changes in the genetic code of RNAs by causing certain adenosines to act like guanosines. This form of RNA editing can have a number of consequences on RNA functioning, ranging from destabilizing the RNA molecules' secondary structure to altering a protein amino acid sequence due to editing of the mRNA's coding sequence[80-82]. ADARs specifically target double stranded RNA structures, including those found in pri-miRs (**Figure 2**). The editing of a pri-miR can profoundly influence microRNA maturation, resulting in changes in mature microRNA expression[83-85]. However, when editing alters the microRNA's seed sequence, this can completely change the mature microRNA's target selection, resulting in the regulation of a different targetome[86]. Whether microRNA editing events could lead to functional consequences for neovascularization was unknown.

2'-O-Methylation (2'Ome). All four ribonucleotides that make up RNA can be subjected to 2′Ome. This common RNM is installed by methyltransferases like Fibrillarin[73]. 2′Ome stabilizes 'household' RNAs like ribosomal RNAs, small nuclear RNAs, and transfer RNAs and is likely to have a similar effect on microRNAs[87-91]. A few studies have suggested that 2′Ome may protect some adenosine residues from A-to-I editing, however, the precise location and function of many 2′Ome sites are currently unclear[92-94].

N6-Methyl-Adenosine (m6A). m6A is one of the most abundant RNMs in cells and tissues and is installed by m6A 'writer' METTL3[73]. However, this RNM can also be removed by m6A 'erasers' ALKBH5 and FTO, which allows for highly dynamic regulation of m6A levels. The biological function of m6A modifications is often mediated through a group of m6A 'readers'. For example, m6A in mRNAs can stimulate translation, direct alternative splicing or mark RNAs for decay, depending on which reader protein is involved. Regarding microRNAs, m6A within the pri-miR was shown to impact the subsequent microRNA maturation process and could thus play an active role in regulation of microRNA expression[95]. Additionally, studies have

suggested that microRNA m6A might even affect microRNA silencing efficiency by influencing mRNA-microRNA interaction strength[96-98]. However, whether microRNAs with a vascular function are often subject to m6A methylation is unknown.

OUTLINE OF THIS THESIS

In this thesis we have assessed whether microRNA alterations can indeed be functionally relevant in ischemic cardiovascular disease using a focussed strategy: we investigated whether the 4 described types of microRNA alterations 1) are present within specific microRNAs with a known cardiovascular function; 2) are actively regulated in response to ischemia; and 3) can indeed regulate the functioning of these vasoactive microRNAs.

In **Chapter 2** we review the formation and function of isomiRs and various forms of microRNA modifications and discuss recent findings that suggest that these microRNA alterations directly affect neovascularization. Additionally, we highlight how this newfound regulatory layer could potentially provide novel therapeutic options for ischemic CVD.

In **Chapter 3** we characterize the expression and function of the 5'-isomiR of miR-411, a microRNA from the 14q32 cluster, in vascular cells and tissue. To do so we examine if the expression of the 5'-isomiR is independently regulated from the canonical miR-411 in response to ischemia in a murine hindlimb ischemia model and in chronically ischemic human blood vessels. Additionally, we investigate whether miR-411's 5'-isomiR has a different effect on vascular cell functioning than miR-411 itself.

In **Chapter 4** we describe that vasoactive miR-487b is subject to A-to-I editing or 2′Ome during neovascularization in a murine model for hindlimb ischemia. Furthermore, we investigate whether there is a correlation between the levels of A-to-I editing and 2′Ome of this microRNA. Additionally, we examine if A-to-I editing affects miR-487b's target selection and its angiogenic properties.

In **Chapter 5** we investigate which other vasoactive microRNAs are A-to-I edited in different vascular cell types and examine how editing is regulated in response to ischemia. We then further characterize post-ischemic A-to-I editing of 4 abundant microRNA candidates in murine hindlimb tissues, cultured human veins and arteries and in lower limb veins from patients with CVD. Finally, we also examine whether the

vasoactive microRNA A-to-I editing events affect the microRNA's target selection and its angiogenic properties.

In **Chapter 6** we studied vasoactive microRNA m6A methylation in human fibroblasts and compared it to previous reports of microRNA m6A methylation in kidney cells. Furthermore, we examined the effect of hypoxia on microRNA methylation and whether proteins that regulate m6A methylation affect the expression of these vasoactive microRNAs.

The results described in this thesis and the future perspectives are discussed in **Chapter 7**.

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