Small, non-coding RNAs: their modifications, and their detection in cardiovascular diseases

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Chapter 1

General introduction

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Cardiovascular disease

Cardiovascular diseases (CVD) are the leading cause of death globally, accounting for 32% of all global deaths in 2019 [1]. CVD encompasses a range of conditions that affect the cardiovascular system, including myocardial infarction, heart failure, stroke and coronary artery disease. The cardiovascular system is made up of the heart and a complex network of blood vessels, which ensure the circulation of blood throughout the body. This system plays a vital role in delivering oxygen and nutrients to tissues and organs, while efficiently removing waste products such as carbon dioxide. Any disruption in the structure or function of this system can lead to severe health consequences, highlighting the importance of understanding and addressing CVD.

Heart failure

Heart failure is a prevalent CVD, characterized as a heterogenous condition that affects over 64 million people worldwide [2]. It is a chronic condition in which the heart is unable to pump sufficient blood to meet de body's metabolic demands, resulting in symptoms such as breathlessness, fatigue and fluid retentions. Heart failure is classified into two main types based on ejection fraction: heart failure with reduced ejection fraction (HF-REF) and heart failure with preserved ejection fraction (HF-PEF). In HF-REF, the left ventricle becomes enlarged and weak, typically due to coronary artery disease or valve disorders. In HF-PEF, the heart muscle becomes stiff, making it difficult for the ventricle to relax and fill properly, and is often caused by high blood pressure [3, 4]. A hallmark of both types is cardiac remodeling, where the heart changes its size and structure in response to stress or injury. This can involve cell enlargement, increased fibrosis, and changes in heart shape [5].

Treatment of heart failure varies depending on its type and severity, but common strategies include lifestyle changes, such as increasing physical activity and dietary adjustments, alongside pharmacological interventions. These typically involve beta-blockers to reduce heart rate and blood pressure, and diuretics to alleviate fluid retention [6]. However, these therapies primarily focus on symptom management, and none of the treatments are curative. Understanding the molecular mechanisms behind cardiac remodeling, including gene expression changes, is crucial for developing more effective treatments for heart failure.

Atherosclerosis

Atherosclerosis is the underlying cause of most CVDs, making it a leading contributor to morbidity and mortality worldwide [7, 8]. It is characterized by the progressive thickening

of the arterial wall due to the accumulation of lipids, inflammatory cells, and fibrous tissue [9, 10]. Over time, this process can lead to vessel narrowing (stenosis), restricting blood flow, or the formation of plaques that may rupture, causing acute vascular events such as myocardial infarction or stroke [11-13]. When atherosclerotic lesions disrupt normal blood flow, oxygen and nutrient delivery to downstream tissues become compromised, while the removal of metabolic waste is impaired. This imbalance can ultimately lead to tissue ischemia and cell death. To counteract these effects, the body initiates a compensatory process called neovascularization, which aims to restore perfusion. Neovascularization occurs through two distinct mechanisms: arteriogenesis and angiogenesis. Arteriogenesis refers to the remodeling and enlargement of pre-existing collateral arteries to enhance blood flow [14, 15], while angiogenesis involves the formation of new capillaries form existing blood vessels [15, 16]. Although these adaptive processes help mitigate ischemic damage, they are often insufficient to fully restore function in advanced atherosclerosis. Therefore, therapeutic interventions are needed to overcome these limitations and improve tissue perfusion.

Coronary and peripheral artery disease

Coronary artery disease (CAD) and peripheral artery disease (PAD) are both clinical manifestations of atherosclerosis. CAD specifically refers to the narrowing or blockage of the coronary arteries, which supply blood to the heart muscle. This reduction in blood flow can lead to myocardial infarction and other heart-related complications. PAD, on the other hand, involves the narrowing or occlusion of arteries that supply blood to the limbs, often resulting in symptoms like leg pain (claudication), muscle weakness, and in severe cases, limb amputation due to critical ischemia. In both CAD and PAD, therapeutic interventions are often necessary to restore adequate blood flow. For CAD, endovascular procedures such as balloon angioplasty and stent placement are common options, but when these are insufficient, bypass surgery is performed to create an alternative route for blood flow to the heart [17]. This typically involves diverting a local artery, such as the left internal mammary artery, or grafting a vein, most commonly the saphenous vein, to bypass the blocked coronary artery. Similarly, in PAD, bypass surgery is employed to create a new pathway for blood supply to peripheral tissue [18]. However, the use of venous grafts in both CAD and PAD has limitations, as veins are less resilient to the higher blood pressures in arterial circulation, and are therefore prone to reocclusion and graft failure [19].

Stroke and intracranial atherosclerosis

Atherosclerosis also plays a central role in the pathogenesis of stroke, which can be broadly categorized into ischemic and hemorrhagic subtypes. Ischemic stroke, the most

common form, can result from both intracranial atherosclerosis (ICAS) and lesions in the carotid arteries [20]. These lesions can lead to thrombus formation when plaques rupture and release thrombi into the bloodstream, potentially blocking smaller cerebral vessels, depriving brain tissue of essential oxygen and nutrients. Hemorrhagic stroke accounts for approximately 10-20% of all stroke cases and is caused by the rupture of a weakened blood vessel . This weakening can result from chronic hypertension, aneurysms, or vessel wall fragility due to ongoing vascular inflammation. Hemorrhagic strokes are often associated with higher early mortality and morbidity, highlighting the need for distinct diagnostic and therapeutic strategies. Treatment of hemorrhagic stroke primarily focuses on reducing intracranial pressure through blood pressure management, osmotic therapy, or ventricular drainage, to minimize secondary brain damage. In contrast, ischemic stroke treatment aims to restore blood flow as quickly as possible. Thrombolytic therapy is commonly used to dissolve the obstructing clot , a strategy that would be harmful in the context of hemorrhagic stroke. Alternatively, endovascular interventions enable physical removal of the thrombus [21, 22]. To reduce the risk of ischemic stroke caused by carotid artery stenosis, carotid endarterectomy is employed, where the plaque is surgically removed from the carotid artery. However, this procedure carries risks and is only recommended for patients with a high-grade stenosis (50% or more) and those who have exhibited symptoms such as transient ischemic attacks (TIAs) [23]. In addition to extracranial atherosclerosis, ICAS is an important contributor to stroke as well. ICAS differs from its extracranial counterpart primarily due to difference in the vasculature and the presence of the blood-brain barrier (BBB) [24]. This specialized barrier limits the infiltration of immune cells and inflammatory mediators into the brain, influencing the pathophysiology of ICAS. ICAS often develops later in life and lesions tend to be less complex, with fewer calcifications and reduced monocyte infiltration compared to lesions in extracranial arteries [25-28]. However, despite these differences, much remains unknown about the pathophysiology and treatment of ICAS.

Small non-coding RNAs

The progression of many cardiovascular diseases is closely linked to alterations in gene expression, with small non-coding RNAs playing a critical regulatory role in these processes [29]. While protein-coding messenger RNAs (mRNAs) have traditionally been the focus of genetic research, they constitute only 3% of the human genome. A significant portion of the remaining genome consists of non-coding RNA, which was historically considered transcriptional “junk” but is now recognized as functionally significant [30]. Non-coding RNAs can be classified according to their length as small non-coding RNAs (sncRNAs) and longer transcripts. Well-characterized examples of non-coding RNAs include ribosomal RNA (rRNA), which forms the structural and enzymatic core of ribosomes, and transfer RNA (tRNA), which facilitates the translation of mRNA into proteins by delivering amino acids to the ribosome. sncRNAs are a heterogenous class of

RNA molecules, typically less than 200 nucleotides in length, which do not encode proteins but exert regulatory functions at multiple levels of gene expression. They modulate transcription, splicing, RNA stability, and translation, thereby influencing fundamental cellular processes. Given their role in post-transcriptional regulation, small non-coding RNAs have emerged as key contributors to cardiovascular homeostasis and disease pathogenesis. This thesis will focus on microRNAs and tRNA derived fragments (tRFs).

MicroRNAs

MicroRNAs have gained significant attention as essential regulators of cardiovascular function and disease, influencing key pathological processes in various CVDs [29, 31]. These small, non-coding RNA molecules, approximately 22 nucleotides in length, function as post-transcriptional regulators of gene expression by inhibiting the translation of mRNAs into proteins. Notably, microRNAs exhibit tissue- and disease-specific expression

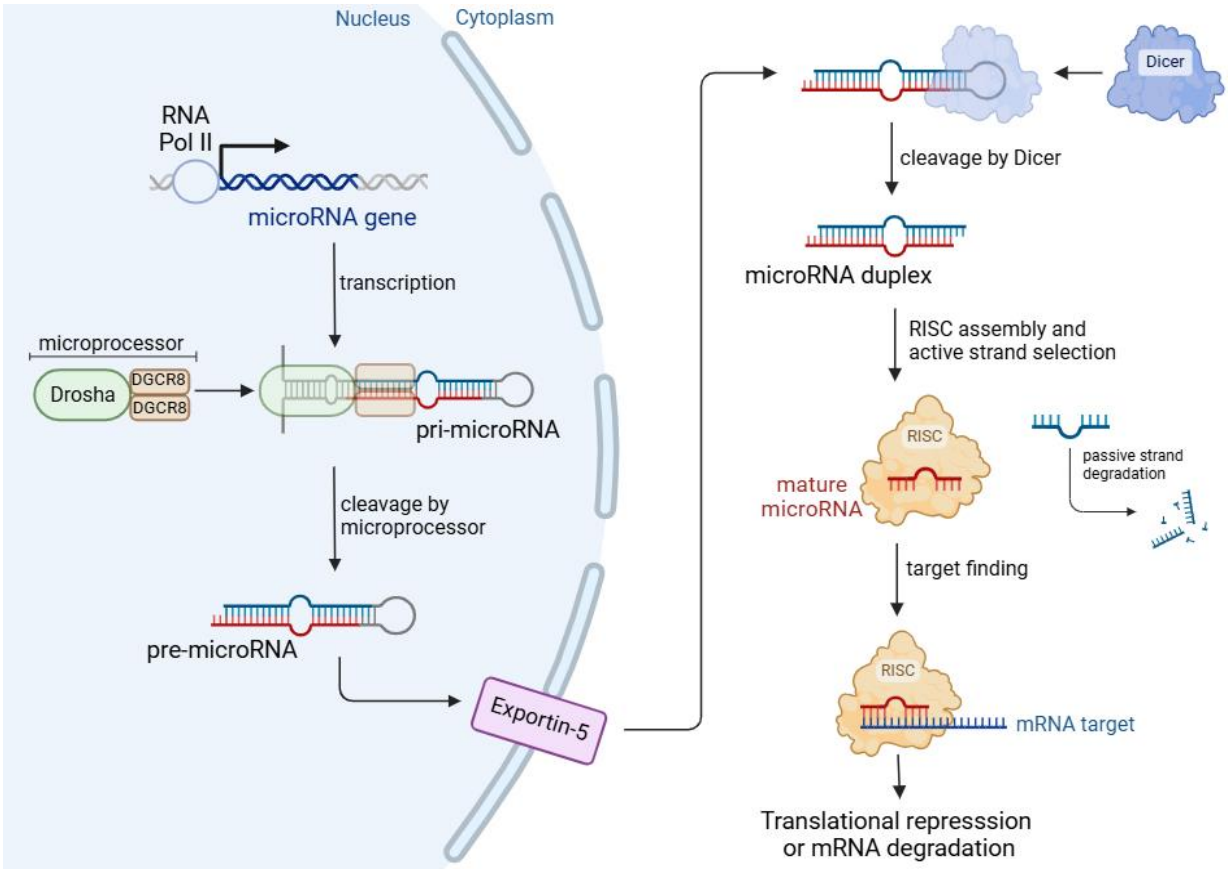

Figure 1: Schematic overview of microRNA biogenesis and processing. MicroRNA genes are transcribed in the nucleus by RNA polymerase II, producing a primary microRNA transcript (pri-miRNA). The microprocessor complex, consisting of Drosha and DGCR8, cleaves the pri-miRNA, generating a precursor microRNA (pre-miRNA) with a characteristic hairpin structure. This pre-miRNA is then exported to the cytoplasm via Exportin-5. In the cytoplasm, Dicer cleaves the hairpin loop, yielding a mircoRNA duplex. Either strand of this duplex can be incorporated into the RNA-induced silencing complex (RISC) as the functional, mature microRNA. Unincorporated strands are degraded. The mature microRNA guides RISC to its target mRNA, leading to translational repression or mRNA degradation.

patterns, allowing them to fine-tune gene regulation in a highly context-dependent manner [32]. MicroRNA biogenesis is a multistep process that starts in the nucleus, where primary microRNAs (pri-miRNAs) are transcribed by RNA polymerase II. These pri-miRNAs undergo cleavage by the Drosha-DGCR8 microprocessor complex to form precursor microRNAs (pre-miRNAs), which are then exported to the cytoplasm via Exportin-5. In the cytoplasm, the RNase III enzyme Dicer further processes pre-miRNAs into mature, double-stranded microRNAs [33]. One or both strands of this duplex can be incorporated into the RNA-induced silencing complex (RISC), where the guide strand directs the complex to target mRNAs through sequence complementarity [34]. MicroRNAs exert their function as post-transcriptional repressors primarily through binding to the 3′ untranslated region (3′ UTR) of target mRNAs [35, 36] (**Figure 1**). This interaction is mediated by a conserved seed sequence, typically located at nucleotides 2-8 of the microRNA, which is critical for target recognition [37]. A single microRNA can regulate multiple mRNAs, enabling the fine-tuning of complex cellular pathways. Consequently, dysregulation of microRNA expression can have profound effects on multifactorial diseases such as atherosclerosis, where altered microRNA profiles contribute to endothelial dysfunction, inflammation, and plaque formation [31, 38-40]. Given their broad regulatory capacity and context-dependent expression, therapeutic strategies aimed at modulating microRNA expression hold promise for treatment of CVDs [41] .

Transfer RNA-derived fragments

tRFs are small RNA molecules generated from tRNAs, which are traditionally known for their role in protein translation. Initially considered mere degradation products of tRNAs, tRFs are now recognized as biologically active molecules involved in regulation of gene expression [42]. These fragments can originate from different regions of the tRNA, including the 5′ end, 3′ end, and anticodon loop, and vary in size from approximately 18 to 50 nucleotides (**Figure 2**). Notably, tRF expression exhibits disease specificity, suggesting a regulating and potentially functional role across different biological contexts [43, 44]. Moreover, the production of tRFs is evolutionarily conserved, further supporting their significance in cellular processes [45]. Both mature tRNAs and their derived fragments are extensively modified post-transcriptionally. While it is known that stress-induced activation of Angiogenin, a RNAse A family member, results in increased formation of tRFs [46], the precise mechanisms underlying the tRF biogenesis remain largely unknown.

Small RNA alterations

sncRNAs are subject to various modifications and alterations that can influence their stability, function, and target recognition [47]. These modifications can be broadly

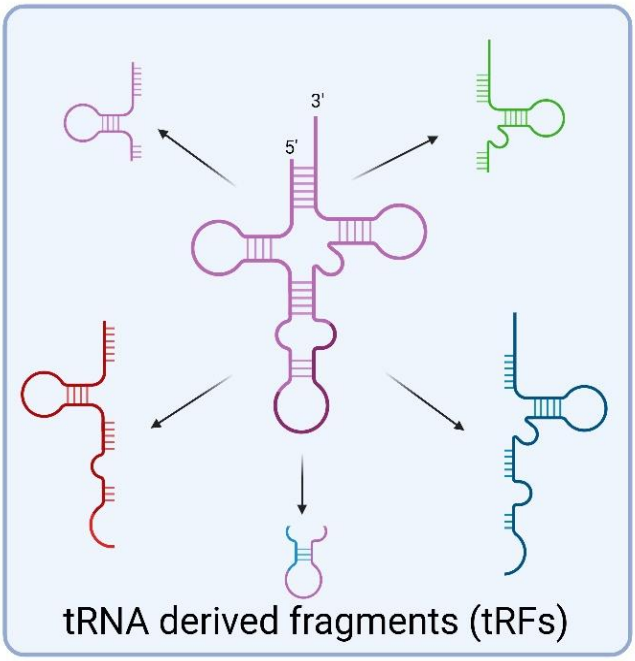

Figure 2: Schematic overview of tRNA derived fragments. Fragments can originate from different regions of the tRNA, including the 5′ end, 3′ end, and anticodon loop, and vary in size from approximately 18-50 nucleotides.

categorized into two types: chemical modifications of RNA nucleotides and alternative cleavage events that generate isomer-microRNAs (isomiRs) or other RNA isomers. Chemical modifications of ribonucleotides play essential roles in RNA stability, localization, and interactions with other molecules. These modifications can occur on the bases of the nucleotides or the ribose moiety. In Adenosine-to-Inosine (A-to-I) editing, adenosine is deaminated to inosine, which alters RNA base-pairing properties as inosine preferentially binds cytidine [48]. A-to-I editing can furthermore affect transcript stability or protein coding potential [49]. Another well-known example of RNA base modification is N6-methyl-adenosine (m6A), where the N6 position of adenosine is methylated, which regulates RNA processing, affects stability and translation repression efficiency [50, 51]. Additionally, sncRNAs, in particular microRNAs, can undergo alternative cleavage, leading to the formation of isomiRs – variations of microRNAs that differ from their canonical sequences due to nucleotide additions or deletions at the 5′ or 3′ end. These variants arise from alternative cleavage-site selection by the microprocessor complex or Dicer during microRNA biogenesis [52, 53]. IsomiRs are highly abundant, and can sometimes surpass their canonical counterparts in prevalence, constituting a significant portion of the total microRNAs detected in RNA sequencing studies [54-56]. Importantly, they can associate with the RISC and therefore regulate mRNA translation [53, 57]. However, because isomiRs and RNA nucleotide modifications can alter the seed sequence or overall RNA structure, they have the potential to influence target recognition and biological function, adding an additional layer of gene expression regulation. This additional layer of post-transcriptional gene expression regulation is collectively referred to as the “epitranscriptome”.

Small non-coding RNAs as biomarkers

sncRNAs have emerged as promising biomarkers for various diseases, including cardiovascular conditions, due to their unique biological properties. One key advantage is their remarkable stability in bodily fluids, as they are often encapsulated in extracellular vesicles, bound to proteins, or chemically modified, preventing rapid degradation by ribonucleases [58-60]. The majority of circulating sncRNAs are associated with Argonaute (AGO) proteins, forming stable protein complexes in the bloodstream [59, 61]. Only a small fraction, approximately 10%, is encapsulated within extracellular vesicles such as exosomes, apoptotic bodies, or bound to high density lipoproteins [60, 62, 63] (**Figure 3**). This stability allows for their reliable detection in the blood samples with minimal invasive techniques. As mentioned before, sncRNAs exhibit tissue- and disease-specific expression patterns [32, 64, 65], allowing for precise discrimination between pathological and physiological states. In the context of chronic cellular stress, sncRNAs can be actively released into the bloodstream within extracellular vesicles, whereas during acute tissue injury, they can also be passively released in an free form [66]. This further enhances their potential as dynamic biomarkers that reflect acute injury, disease progression, or response to treatment. These characteristics make sncRNAs highly suitable for minimally invasive diagnostic and prognostic applications.

Common detection methods

Several techniques are available to detect and quantify sncRNAs, each with its own advantages and limitations. Small RNA sequencing (small RNA-seq) is a high throughput approach that allows for the comprehensive profiling of sncRNA expression. This method provides detailed insights into both known and novel sncRNAs, including isomiRs and sequence-altering RNA modifications, but requires complex bioinformatics analysis and can be costly. Reverse transcription quantitative PCR (RT-qPCR) is a widely used method for targeted sncRNA quantification. It offers high sensitivity and specificity but is limited to detecting known sequences and requires carefully designed primers to account for RNA modifications and sequence variations. Northern blotting, though less commonly used, enables the visualization of specific sncRNAs by hybridization with labeled probes. This method provides information on RNA size and expression levels but has relatively low sensitivity and requires large amounts of RNA. Microarrays allow for the parallel detection of multiple sncRNAs using sequence-specific probes. While they are cost-effective for profiling known sncRNAs, they lack the ability to identify novel sequences and may have lower dynamic range compared to RNA sequencing. However, none of these methods allow for the rapid and accurate measurement of sncRNAs in limited amount of biofluids in acute clinical settings, and often need specialized, expensive equipment. Developing faster and more accessible detection platforms is essential to fully harness the potential of sncRNAs as diagnostic and prognostic biomarkers.

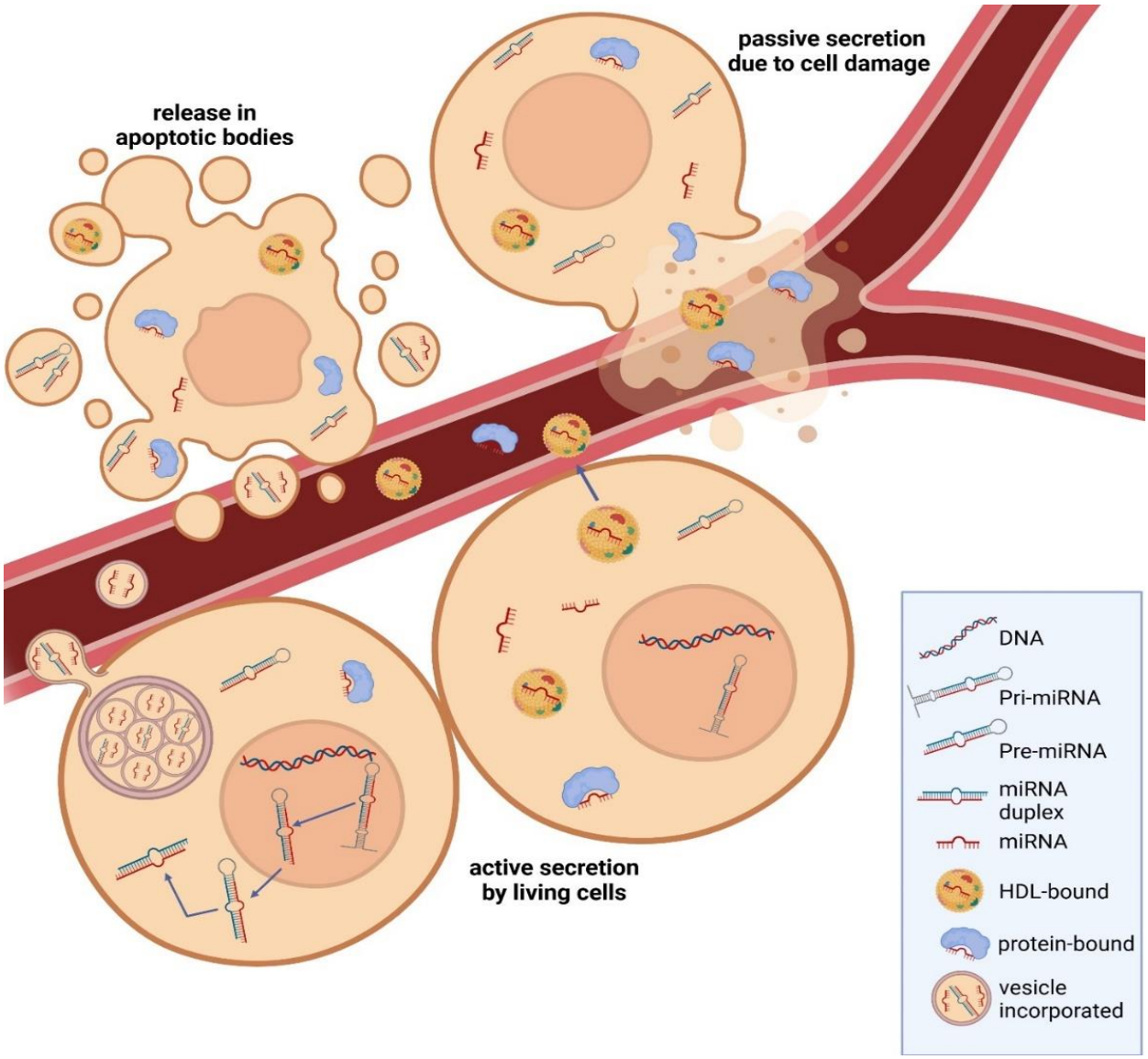

Figure 3: Schematic overview of microRNA secretion into the blood circulation. MicroRNAs are produced as primary transcripts (pri-miRNA) in the nucleus, cleaved into precursor microRNAs (pre-miRNA) and exported to the cytoplasm. Here a microRNA duplex is formed from the precursor. The majority of microRNAs are associated with proteins, the rest is encapsulated in vesicles, apoptotic bodies or bound to high density lipoproteins (HDL). MicroRNAs can be actively secreted, or passively released upon cell death or cell damage. Figure adapted from Foessl *et al* (58).

Detection in acute settings

Recent advancements in sncRNA detection technologies have focused on developing rapid and accurate detection techniques suitable for point-of-care diagnostics. One such approach involving isothermal strategy enables microRNA detection within 30 minutes [67]. However, while this method significantly reduces processing time compared to regular RT-qPCR, it still relies on expensive equipment such as a qPCR machine and requires labor-intensive sample preparation, including RNA isolation. Another strategy explored is a streptavidin-biotin-based microarray for microRNA profiling [68]. This method offers a broad profiling capability but requires a substantial microRNA input of 2.5 µg, has relatively low sensitivity, and is susceptible to cross-hybridization, limiting its

applicability in detecting low-abundance RNAs. In an effort to improve sensitivity, nanotechnology-based approaches have been investigated, such as the use of quantum dots and nanogold particles [69]. These methods achieve detection limits in the femtomolar range, offering high sensitivity. However, their reliance on specialized imaging systems, such as laser scanning microscope or CCD-equipped microscopes, restricts their feasibilities for bedside applications. Another promising technique involves molecular beacons. These engineered stem-loop oligonucleotides contain a donor and quencher fluorophore at their 5′ and 3′ ends. Upon hybridization with a target microRNA, the stem-loop structure unfolds, leading to fluorescence activation and a detectable signal [70]. Despite its simplicity and specificity, this approach suffers from low sensitivity due to limited intrinsic signal amplification in the system. While these approaches represent significant progress in small non-coding RNA detection, their translation into rapid, bedside-compatible diagnostics remains challenging. DNA-embedded hydrogels offer a potential solution for developing a truly point-of-care detection platform, as they can provide real-time, sensitive, and amplification-free detection in a user-friendly format, paving the way for future bedside applications.

Hydrogels

Hydrogels are three-dimensional networks of hydrophilic polymers capable of absorbing and retaining substantial amounts of water or biological fluids, making them highly versatile materials in biomedical applications [71]. These polymer networks are formed through crosslinking, which can occur via chemical crosslinking, where covalent bonds between monomers establish a stable network, or through physical crosslinking, which relies on non-covalent interactions such as hydrogen bonding, ionic interactions, and hydrophobic forces [72]. Their tunable nature allows for precise control over mechanical properties, permeability, and biochemical functionality, making hydrogels particularly suitable for biomedical engineering applications, including tissue engineering, drug delivery, and biosensing. One particularly promising approach in hydrogel-based biosensing is the incorporation of functional DNA motifs into the hydrogel matrix. Due to their programmable assembly, high specificity, and precise molecular recognition, DNA-based structures, including aptamers, DNAzymes, and hybridization probes, can be integrated into hydrogels to enable highly sensitive and selective detection of biomolecules [73]. These DNA-functionalized hydrogels can serve as molecular detection platforms for a variety of analytes, including small non-coding RNAs, by leveraging complementary base pairing or enzymatic signal amplification. Beyond their recognition properties, DNA motifs can also act as structural components, influencing the hydrogel's responsiveness to environmental stimuli, such as pH, temperature, or the presence of target biomolecules [74]. This responsiveness can be exploited for smart biosensing applications, where target detection triggers a measurable change in the hydrogel's

physical state (e.g., swelling, fluorescence activation, or degradation), enabling rapid and highly specific point-of-care diagnostics. Given their adaptability and biocompatibility, DNA-embedded hydrogels offer a promising strategy for advancing biosensing technologies, with potential applications in cardiovascular disease diagnostics, personalized medicine, and real-time monitoring of disease biomarkers.

Thesis outline

The aim of this thesis is to investigate the role of small non-coding RNA alterations in CVD, explore the potential of tRFs as diagnostic biomarkers for stroke in acute settings, and evaluate the feasibility of using sequence complementarity for the sensitive detection of small non-coding RNAs in such scenarios.

The first part of the thesis focuses on the implication of small non-coding RNA modifications in cardiovascular diseases.

Chapter 2 provides an overview of small non-coding RNAs, their post-transcriptional modifications, and their potential implications in the pathophysiology of heart failure. It emphasizes the emerging field of the small RNA epitranscriptome, highlighting recent advancements and the growing evidence that RNA modifications play a critical role in gene regulation associated with heart failure.

Chapter 3 explores the regulatory role of 5'- isomiRs, specifically ISO-miR-411, in cardiovascular disease, with a focus on post-ischemic neovascularization. This chapter highlights the active regulation of ISO-miR-411 formation in response to ischemia, both in vitro and in vivo. Additionally, it demonstrates that ISO-miR-411 has a distinct targetome compared to its canonical counterpart, WT-miR-411, due to a shift in its seed sequence. This distinct targetome allows ISO-miR-411 to negatively influence vascular cell migration, a function not observed with WT-miR-411. By identifying isomiR formation as an actively regulated process and revealing its functional implications in ischemia, this chapter highlights that isomiRs represent an additional layer of gene regulation with potential implications for future microRNA-based therapeutic strategies.

In **Chapter 4** we investigate the impact of m6A on intracranial atherosclerosis, a leading cause of ischemic stroke. This chapter highlights how m6A potentially influences endothelial integrity by affecting TJP1 repression efficiency by the pro-atherogenic microRNA miR-494-3p. By examining postmortem intracranial arteries at various stages of atherogenesis, we show that m6A levels are upregulated in atherosclerosis-affected vessels, accompanied by reduced TJP1 and PECAM1 expression. Importantly, specific m6A-methylation of miR-494-3p specifically represses TJP1 in vitro at brain microvascular endothelial cell-cell junctions, likely increasing blood-brain barrier permeability, while unmodified miR-494-3p or alternative m6A-miR-494-3p has no effect. By uncovering this novel regulatory mechanism in brain arteries, this chapter provides insights into the pathophysiology of intracranial atherosclerosis and potential therapeutic targets.

The second part of the thesis examines the role of tRFs as potential biomarkers for diagnosing stroke in acute situations, while also exploring the feasibility of designing a sequence complementarity-based system to accurately detect small non-coding RNAs in these high-stakes scenarios.

Chapter 5 investigates the potential of tRFs as diagnostic biomarker for acute stroke, with a particular focus on distinguishing ischemic stroke, intracerebral hemorrhage, and stroke mimics. Despite promising results from a small RNA-sequencing pilot study, where specific tRFs showed diagnostic potential, we were unable to replicate these findings in a larger cohort using RT-qPCR, the gold standard for RNA-sequencing validation. We were able to detect and quantify all selected tRFs, but only the fragments Arg-TCG$^{53-67}$ and Tyr-GTA$^{1-19}$ showed weak trends in differential expression between stroke subtypes, with insufficient predictive value for clinical use. However, technical challenges in accurate tRF quantification, stemming from limitations in both RNA sequencing and RT-qPCR methods, may have hindered the ability to validate these biomarkers. This chapter, therefore, highlights the need for more reliable and accurate methods for tRFs analysis. Addressing the technical barriers could unlock the full diagnostic potential of tRFs, allowing for faster, more accurate stroke subtype identification in acute clinical settings.

Chapter 6 investigates the feasibility of a DNA-based hydrogel for sensing small-non-coding RNAs in fluids. We functionalized dextran with varying degrees of methacrylate to create hydrogels with varying crosslinking ability, and therefore, permeability. DNA was incorporated using streptavidin-biotin interactions, and the effect of varying methacrylate content and biotin content on streptavidin incorporation, and subsequently DNA incorporation was investigated. We show that higher biotin content does not correlate with higher streptavidin and DNA embedding, highlighting the importance of balancing biotin content to achieve the desired combination of surface functionality and molecular penetration in hydrogel-based applications. Importantly, the hydrogels reacted within minutes upon introduction of the target sequence, while vehicle and scrambled controls led to no reaction. Therefore, this chapter highlights the feasibility of the DNA-based hydrogel to sense specific small non-coding RNA molecules.

Chapter 7 summarizes all key findings of this thesis and outlines potential directions for future research.

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