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Impact of Medication on Quantification of Plasma MicroRNAs in Patients with Cardiovascular Disease

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1.1. <u>Non-coding RNAs in the Human Genome:</u>

Before the completion of the Human Genome Project, the genome was expected to contain at least 100,000 protein coding genes. The sequencing, however, revealed that only about 21,000 such genes can be found within the approximately 3 billion DNA bases. Although approximately 76% of the human genome is transcribed, less than 3% encodes for proteins. [1]

The remaining 97% were thought to be "junk" DNA as these sequences are not translated into protein and were not known to encode relevant information with the exception of few non-coding RNAs such as transfer or ribosomal RNAs. This assumption was based on the central dogma of molecular biology postulated by Francis Crick in 1958, which states that all relevant biological information flows unidirectionally from DNA to mRNA to protein. [2, 3] [4]

As the number of protein coding genes was unexpectedly low and similar to much simpler organisms, it was not immediately clear how the high degree of human complexity could derive from such a small protein repertoire. Additionally, the proteomes of higher organisms have been shown to be relatively stable, with humans and mice sharing 99% of their protein coding genes. Although mechanisms like alternative splicing can increase the variation of the protein repertoire, today the complexity of the regulatory network of non-coding RNAs, i.e. the control architecture of the system, is thought to be a main source of diversity. [5] [6]

This is supported by the fact that while there is only a small increase in the number of protein coding genes in humans as compared to the nearly 19,000 protein coding genes in the nematode *C. elegans*, the ratio of non-protein coding to protein coding sequences is almost 17-fold higher in humans. [6] [7] MicroRNAs (miRNAs) represent one subgroup of non-coding RNAs. More than 2000 miRNAs are encoded in the human genome, but only about 400 miRNAs can be found in *C. elegans*. [8] The miRNA system is also highly evolutionary conserved: 196 miRNA families are conserved among mammals and 34 miRNA families from *C. elegans* are conserved in humans with few examples of secondary loss and very low levels of nucleotide substitutions to the primary sequence. [9, 10]

1.2. The Role of MiRNAs:

Epigenetic control mechanisms including miRNAs, that temporarily modulate gene expression, allow the cell to respond quickly to environmental changes as miRNA molecules can be directly produced from DNA and are easily degraded. [11]

MiRNAs are one of the largest gene families and account for ~1% of the human genome, which is quite remarkable considering that about 3 % of the genome are coding for proteins. [12] Due to their mode of operation, they have the potential to target approximately 60% of all human genes and thereby influence almost all genetic pathways. [13] [14] MiRNAs are expressed in a temporal and tissue-specific manner [15], e.g. miR-208a can only be found in cardiomyocytes [16] and the miRNA content in different cells can vary from 1 to more than 30,000 copies. [17] MiRNAs play an important role in the regulation of embryonic development as well as in adult life with distinct expression profiles in every cell type at each developmental stage. [18] They show dynamic and site specific expression patterns during embryogenesis and suppression of these genes leads to death during early gestation. [19] [20]

1.3. History of MiRNAs:

The first miRNA was identified in 1993 during the study of *C. elegans* mutants that exhibited abnormal developmental timing. The gene responsible for this phenotype, *lin-4*, did not code for a protein but for two small RNAs with complementary sequences to the 3'-untranslated region of the *lin-14* mRNA. *Lin-4* had been shown previously to negatively regulate the protein level of LIN-14, creating a temporal decrease in LIN-14 during postembryonic development that regulates the execution of stage specific larval programs. [2, 21, 22]

A similar mechanism of RNA silencing had been known in plants since the beginning of the 1990s [23] but as *lin-4* was not detected in other species, this new mechanism was believed to be a process occurring only in nematodes. However, in the year 2000, a similar non-coding RNA, *let-7*, that also coordinates developmental timing in *C. elegans* was discovered. *Let-7* codes for a 21 nucleotide non-coding RNA transcript that negatively regulates the mRNA of *lin-41* through complementary Watson-Crick base pairing at the 3'-UTR and therefore influences gene expression in a way similar to *lin-4*. [20] Unlike *lin-4*, the sequence of *let-7* was found to be conserved in a wide range of species including vertebrates, making the biological significance of this finding apparent. [24] [4] Since then, over 25,000 miRNAs have been identified from more than 190 different species including algae, plants, nematodes, protozoa, viruses or vertebrates. [4]
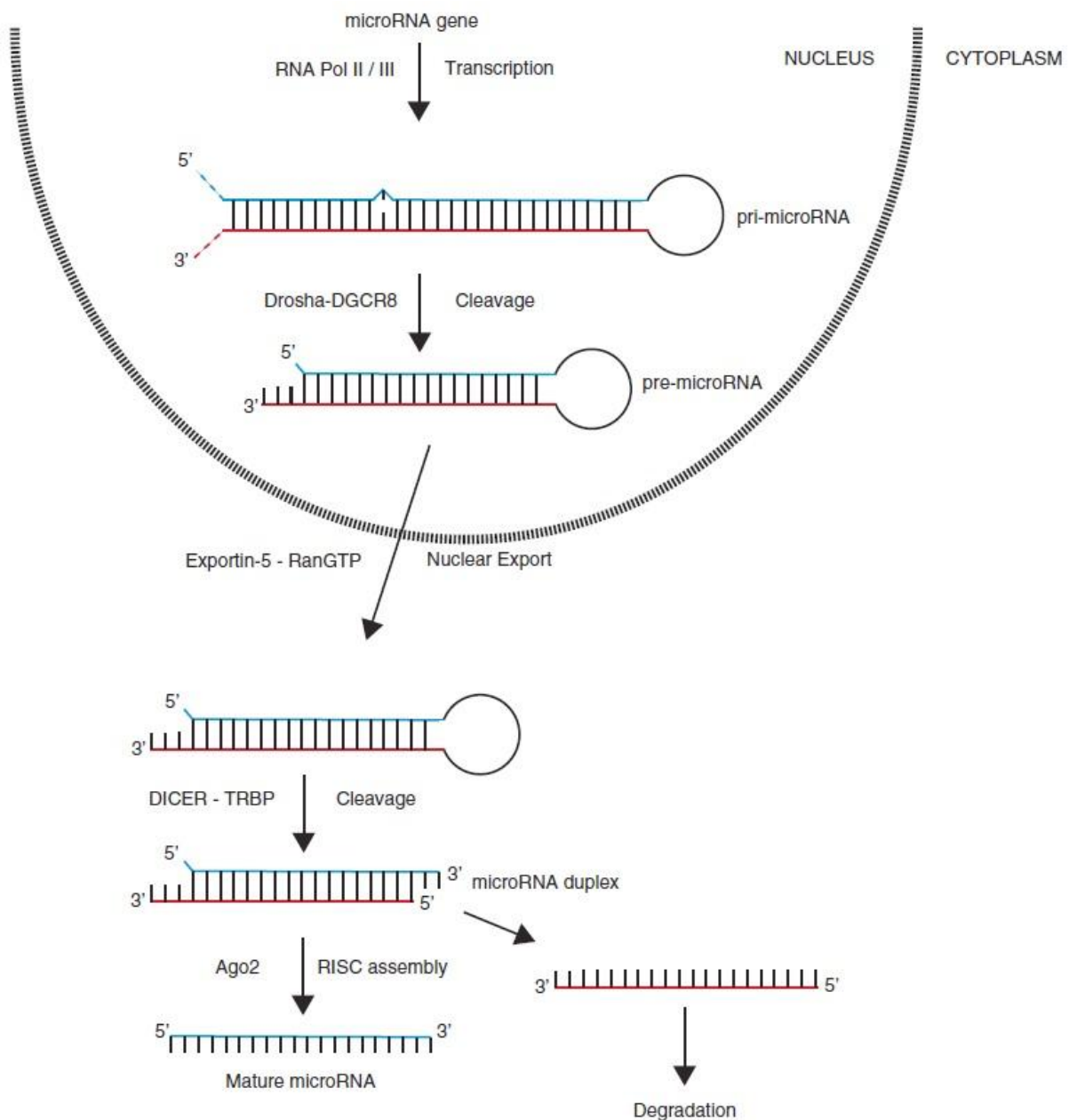
1.4. Biogenesis and Function of MiRNAs:

Figure 1 Mechanism of miRNA biogenesis. Taken from [25]

MiRNA genes are located in different parts of the human genome, and hence show a great variation in their transcriptional regulation and expression patterns. [26] The majority of human miRNAs are co-expressed together with their host gene within intronic sequences of protein-coding genes, while others are transcribed independent of coding genes. [14] More than 40% of human miRNAs appear in clusters and are transcribed together, forming a transcript that contains multiple miRNA

sequences.[27] After transcription by mainly RNA polymerase II [28], the pri-miRNA is processed by the RNaseIII Drosha, into a stem-loop precursor of ~70 nucleotides, the so called pre-miRNA [29]. As Drosha by itself cannot bind pri-miRNAs sufficiently, it acts together with the cofactor DGCR8, together forming the microprocessor complex. [30] The pre-miRNA is then exported to the cytoplasm in a Ran-GTP dependent way by Exportin-5 that specifically binds the pre-miRNA. [31] Once it has reached the cytoplasm, the pre-miRNA is cleaved by the RNaseIII Dicer together with the cofactor TRBP [32] [33] and PACT [34] into a duplex, consisting of two miRNA strands. [35] After transcription the individual miRNAs can be additionally regulated by adenosine deaminases that convert adenosine to inosine and thereby influence the hybridisation of miRNAs to their targets. [36] The RNA duplex is subsequently loaded onto an Argonaute protein together forming the RNA-induced silencing complex (RISC). After the duplex has been unwinded one of the strands is released and in most cases degraded. The other strand guides the RISC to miRNA response elements (MRE) of the target gene that are complementary to its sequence. [4] [9] MiRNAs mainly target mRNAs, but also have the potential to bind to a wide variety of other molecules including tRNAs or rRNAs. [37] Each miRNA locus therefore produces two mature miRNAs, the 'guide' strand with a prevalence of 96–99% and higher biological activity and the 'passenger' miRNA* strand. [9]

MiRNA strand selection depends on different factors including the thermodynamic stability at the ends of the miRNA:miRNA* duplex. [38, 39] Though the miRNA* strand is usually degraded, in some cases both strands can be functional [39] with either overlapping or different target sites. [40] MiRNA*-sequences are likely to have functional relevance in small RNA regulatory networks, as they are highly evolutionary conserved with e.g. more than 40% of miRNA*-sequences resisting nucleotide divergence across Drosophila evolution. [41] Given that both the 'mature' and the 'passenger' strands can be functional, the miRNA/miRNA* nomenclature has now been retired and instead the two sequences are referred to as 5-p or 3-p strand of the respective miRNA.

Once they have bound to their target mRNA, miRNAs are able to regulate gene expression in different and sometimes opposite ways depending on factors like the amount of complementarity with the target mRNA. Near-perfect complementarity of miRNAs and their targets which mainly occurs in plants, leads to direct cleavage of the target mRNA. [42] In animals, however, target recognition in most cases does not require perfect complementarity but mainly depends on pairing to the "miRNA seed", the nucleotides 2-8 of the 5' portion of the miRNA. [43] The short seed match and incomplete base pairing enable the miRNA to target different RNA molecules while a single target gene can contain multiple conserved regions of complementarity. [43] [44]

The predominant mechanism by which miRNAs reduce protein output is by triggering deadenylation of the target mRNA, which makes the mRNA more susceptible to degradation. [45] Additionally

miRNAs can inhibit eukaryotic initiation factors [46] or interfere with translational elongation [47]. In some cases the miRNA response can switch from inhibition of gene expression to enhancement thereby e.g. inducing up-regulation of target mRNAs on cell cycle arrest and repressing translation in proliferating cells. [48] [4]

These characteristics create a complex regulatory control network that changes in relation to age, developmental or pathophysiological state of the cell, and involves multiple co-operative effects on a large number of targets enabling miRNAs to control various pathways at different levels. [49]
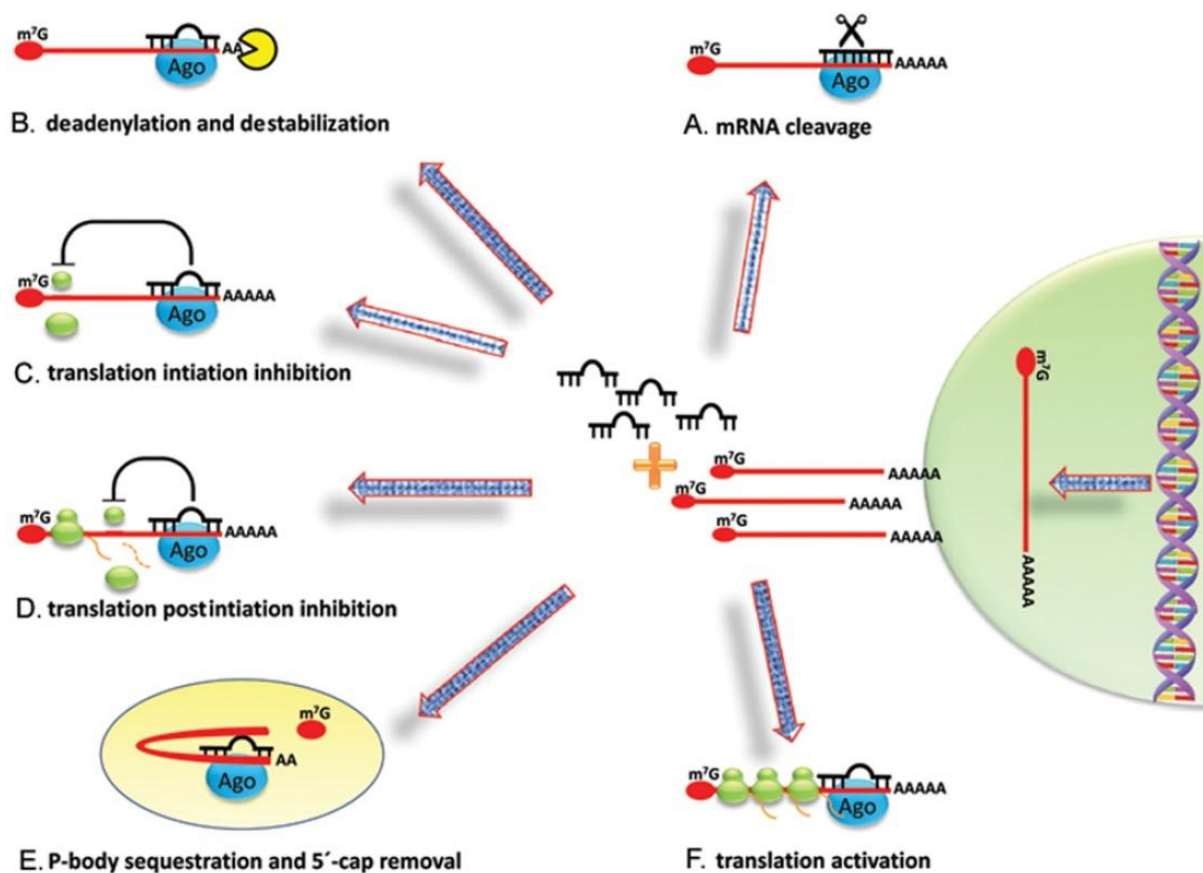

Figure 2 Schematic diagram of proposed mechanisms for miRNA function. (A) Ago-mediated cleavage of mRNA can occur when the miRNA sequence is complementary to the target gene-binding site. (B) Removal of poly(A) tail by deadenylases causes destabilization and degradation of mRNA. (C) Translation initiation inhibited by miRISC interactions with eukaryotic translation initiation factors (eIFs). (D) Inhibition of translation postinitiation. (E) Sequestration of mRNA in P-bodies. (F) miRNA-mediated translational activation. Taken from [4]

1.5. MiRNAs in Circulation:

The majority of miRNAs are located intracellularly. In 2007, however, miRNAs were found in exosomes, in which they were delivered to other cells allowing gene-based communication between cells. [50] Sequence motifs present in miRNAs can thereby enable specific interaction and loading into exosomes. [51] This transfer of RNA through exosomes may enable local and systemic intercellular exchange of biological information in a way similar to hormones. [50] In the following years, miRNAs were detected in most extracellular biological fluids including serum, plasma, saliva or urine where they showed distinct compositions. [52]

MiRNAs can be released into the blood circulation by various physiological mechanisms, including active secretion, apoptosis or necrosis. These miRNAs circulate in different types of vesicles, such as apoptotic bodies, microvesicles (100–1000 nm), and exosomes (50–100 nm). [53] Many extracellular miRNAs in circulation, however, are also independent of vesicles and are associated with RNA-binding proteins like Argonaute 2 protein, a part of the RNA-induced silencing complex. [54]
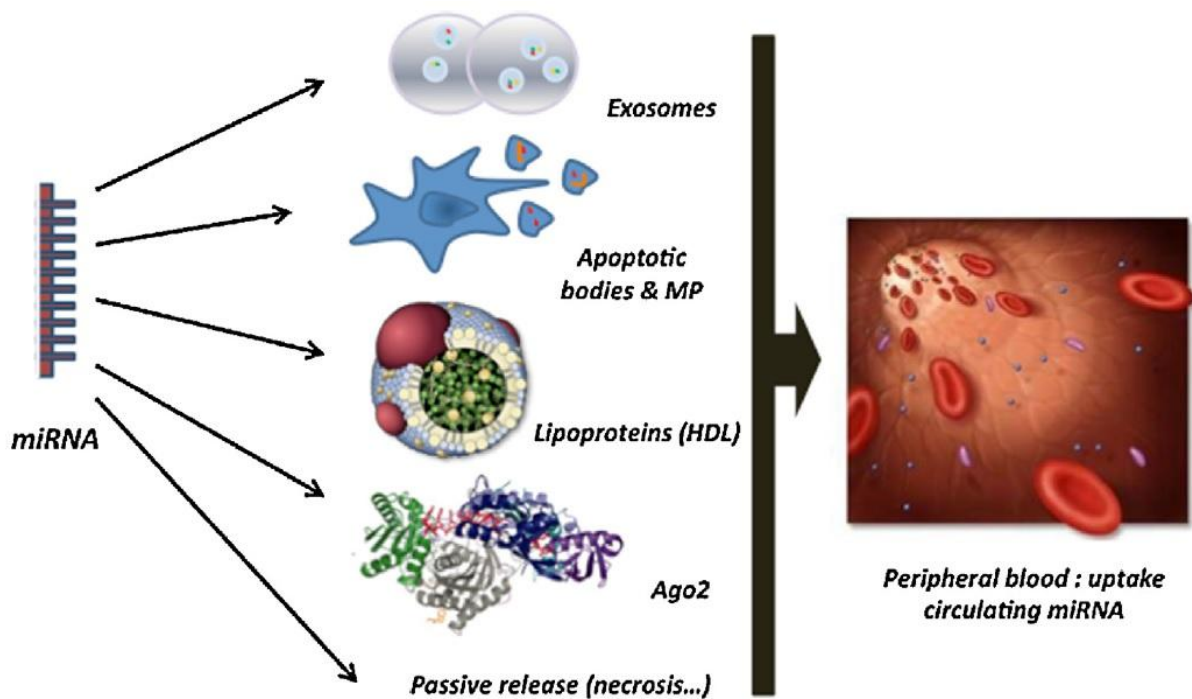

Figure 3 Mechanisms of miRNA release from cells into peripheral blood circulation and transport to target cells. MP: microparticles; HDL: high-density lipoprotein; Ago2: Argonaute 2. Taken from [14]

Human miRNAs isolated from plasma are highly stable in boiling water and resistant to very high or low pH, prolonged room temperature incubation or repeated freeze-thawing. [55] Compared to

endogenous plasma miRNAs, synthetic miRNAs are rapidly degraded when added to human plasma unless the RNase activity was inactivated beforehand. [56] Therefore, though miRNAs are susceptible to rapid degradation, circulating miRNAs are stable and resistant to RNase activity as they are secreted in a complex with other molecules such as Argonaute proteins or lipoproteins or in membrane derived vesicles. [55]

1.6. <u>MiRNAs as Biomarkers:</u>

Deregulated levels of circulating miRNAs have been linked to different disease states. [26] During cellular stress or pathophysiological conditions such as hypoxia, miRNAs can provide an efficient way of gene regulation to allow the cells to adopt and recover. [57] As they are disease-specifically modulated and easily accessible, circulating miRNAs are potential blood-based biomarkers, useful for diagnostic application e.g. in screening programs and for monitoring of treatment response or outcome prediction. [4] [53]

Based on their biology, circulating miRNAs can have a high level of sensitivity and specificity allowing early and reliable detection of pathological states. [49] Arguably, miRNAs offer some advantages over the most commonly used protein biomarkers: They interact with their target through Watson-Crick base pairing, a more predictable and stable interaction than processes involving for example proteins or lipids. [58] In addition miRNA are often expressed in a tissue-, development- or disease-specific manner and the levels of miRNAs in serum are reproducible and consistent among individuals of the same species. [53] [59]

Compared to numerous serum proteins, including various processing variants, and posttranslationally modified proteins, there are far fewer known miRNA species, making it possible to obtain a comprehensive profile. MiRNAs themselves reflect altered physiology more directly, as unlike proteins, which must be translated from mRNA to have a biological effect, they exert their effects directly. In addition, due to their small size, chemical composition and limited posttranslational modifications, miRNAs are much less complex than most other biological molecules and more stable in plasma than other RNAs such as mRNAs. [59] Also, miRNAs can be quantified cost-effectively using real-time polymerase chain reaction or microarrays and a comprehensive profile can be obtained by next generation sequencing.

An example for the potential use of miRNAs as biomarkers is the detection of myocardial infarction, where they might complement the existing biomarkers, such as cardiac troponins. There is still a need for novel biomarkers, as troponins fail to rule out myocardial infarction immediately on admission

and are not reliable in certain groups of patients. [55] [53] This is mainly due to their limited sensitivity, as their plasma levels do not change unless there is substantial damage in the heart, presenting low serum concentrations early after infarction and especially in geriatric patients often generally insufficient elevation for reliable diagnosis. Another major limitation is their lack of specificity, as unspecific elevation of troponin levels can be caused by non-ischemic conditions such as heart failure and renal disease. [60]

MiRNAs that are specifically expressed in the heart muscle, like miR-208a, which is involved in the regulation of myosin heavy chain production during cardiac development [16], have the potential to improve diagnosis of myocardial infarction as they might show greater sensitivity and specificity than existing biomarkers.

In a study with 33 consecutive AMI and 33 non-AMI patients that presented with chest pain, miR-208a remained undetectable in plasma of non-AMI patients including patients with chronic renal failure or trauma, but it was initially detected in 90.9% of AMI patients and in 100% of AMI patients within 4 h of the onset of chest pain, even in patients where cTnI levels were not yet affected. This earlier miRNA peak might be caused by a faster release of miRNAs from damaged cardiomyocytes, as miRNAs are mainly bound to protein complexes in the cytosol while most of the cTnI is bound to myofibrils. [61] [62] On the other hand, potential confounding factors of miRNA measurements remain be characterised to assess the clinical utility of miRNA biomarkers.

1.7. <u>Summary of Publications:</u>

MiRNAs are already beginning to be used therapeutically, like miravirsen, an antisense inhibitor of miR-122, which has been successfully tested in phase-II trials for the treatment of hepatitis C virus infection. [63] Nevertheless the only example where circulating nucleic acids are used as biomarkers so far is in non-invasive prenatal diagnosis. [64] [53] Apart from the lack of large cohort studies, miRNA measurements are hampered by the limited knowledge about confounding factors. Circulating levels of miRNAs have been measured in various studies related to cardiovascular disease but the effect of medication, commonly used in cardiovascular patients, specifically the effect of antiplatelet therapy and heparin administration, is unclear. This was addressed in the present thesis:

<u>Effect of anti-platelet medication:</u>

Antiplatelet therapy is widely used in prevention and treatment of cardiovascular disease. The study by Willeit and Zampetaki et al (Circ Res 2013) identified circulating platelet miRNAs that are responsive to antiplatelet therapy. In healthy volunteers, prolonged platelet inhibition over 4 weeks

affected the levels of circulating miRNAs and resulted in a reduction of several miRNAs, including miR-126 (P<0.001), miR-150 (P=0.003), miR-191 (P=0.004), and miR-223 (P=0.016). Similar results were obtained in patients with symptomatic carotid atherosclerosis (n=33) who were on 75 mg aspirin (ASA) at baseline. After initiation of dual antiplatelet therapy with either dipyridamole or clopidogrel miRNA changes were observed after 48 h. Although the miRNA content of platelets is low compared with other cells, platelets contribute substantially to the circulating miRNA pool. Antiplatelet therapy was therefore a likely confounding factor in previous case–control studies reporting a loss of miRNAs in patients with coronary artery disease. [65]

Effect of heparin:

Heparin, used in interventional cardiology, is another potential confounding factor that may influence miRNA measurements due to its known interference with polymerase chain reactions. [66] [67] In the study by Kaudewitz et al (Thromb Haemost 2013), platelet-poor plasma was obtained from patients undergoing cardiac catheterisation for diagnostic coronary angiography, or for percutaneous coronary intervention, both before and after heparin administration. Heparin had pronounced effects on the assessment of the exogenous *C. elegans* spike-in control (decrease by approx. 3 cycles), which disappeared 6 hours after the heparin bolus. Measurements of endogenous miRNAs were less sensitive to heparin medication. Normalisation of individual microRNAs with the average cycle threshold value of all microRNAs provided a suitable alternative to normalisation with exogenous *C. elegans* spike-in control in this setting. Thus, both the timing of blood sampling relative to heparin dosing and the normalisation procedure are critical for reliable miRNA measurements in patients receiving intravenous heparin.

In summary, both anti-platelet and heparin medication are confounding factors for miRNA measurements, which have to be taken into account when investigating the relation of circulating miRNAs with cardiovascular disease. As this limitation has not been previously recognised, a re-evaluation of the current miRNA literature, in particular of case-control studies in patients with cardiovascular disease or coronary interventions, is required. Future studies will need to address these shortcomings of the early literature on miRNA biomarkers.

1.8. Zusammenfassung der Publikationen:

MiRNAs werden mittlerweile bereits in ihrer therapeutischen Anwendung getestet, so z.B. miravirsen, ein miR-122-Antisense-Inhibitor, der erfolgreich in Phase-II-Studien in der Behandlung von Hepatitis C eingesetzt wurde. [63] Dennoch ist die Pränataldiagnostik bisher das einzige Beispiel für die

Anwendung zirkulierender Nukleinsäuren als Biomarker. [64] [53] Ursache dafür sind nicht nur der Mangel an hinreichend großen Kohorten-Studien, sondern auch begrenzte Kenntnisse über Störfaktoren, die miRNA-Messungen beeinflussen.

Expressionsmuster zirkulierender miRNAs, die spezifisch für kardiovaskuläre Erkrankungen sind, wurden bereits in zahlreichen Biomarker-Studien untersucht. Der Effekt von Medikamenten, die häufig bei kardiovaskulären Patienten eingesetzt werden, insbesondere von Thrombozyten-aggregationshemmern und Heparin ist jedoch bislang unbekannt.

Der Einfluss dieser Medikamente wurde in der vorliegenden Arbeit untersucht.

Effekt der Thrombozytenaggregationshemmer:

Der Einsatz von Thrombozytenaggregationshemmern ist weit verbreitet in der Prävention und Behandlung kardiovaskulärer Erkrankungen. Die Studie von Willeit und Zampetaki et al (Circ Res 2013) identifizierte zirkulierende Thrombozyten-miRNAs, die durch Thrombozyteninhibition beeinflusst werden. In gesunden Probanden beeinflusste die Einnahme von Thrombozyten-aggregationshemmern über einen Zeitraum von 4 Wochen die Spiegel zirkulierender miRNAs im Blut und führten zu einer Reduktion der Spiegel mehrerer miRNAs, einschließlich miR-126 (P<0.001), miR-150 (P=0.003), miR-191 (P=0.004) und miR-223 (P=0.016). Ähnliche Ergebnisse wurden in Patienten mit symptomatischer Karotisstenose beobachtet, die bereits zu Beginn der Studie 75 mg Aspirin (ASA) einnahmen. Nach Beginn einer dualen Antiplättchen-Therapie entweder mit Dipyridamol oder Clopidogrel wurde die Veränderung der miRNA-Spiegel nach 48 Stunden gemessen. Obwohl der miRNA-Gehalt von Thrombozyten im Vergleich zu anderen Zellen niedrig ist, tragen Thrombozyten wesentlich zum zirkulierenden miRNA-Pool bei. Gerinnungshemmende Medikamente stellen daher wahrscheinlich Störfaktoren in früheren Fall-Kontroll-Studien dar, die über eine Reduktion der miRNA-Spiegel bei Patienten mit koronarer Herzkrankheit berichten. [65]

Effekt von Heparin:

Heparin, das in der interventionellen Kardiologie eingesetzt wird, ist ein weiterer potentieller Störfaktor für miRNA-Messungen aufgrund seiner bereits bekannten Interferenz mit Polymerase-Kettenreaktionen. [66] [67] In der Studie von Kaudewitz et al (Thromb Haemost 2013) wurde thrombozytenarmes Plasma von Patienten, bei denen ein Herzkatheter gelegt wurde, um eine Koronarangiographie oder perkutane Koronarintervention durchzuführen, vor und nach Heparin-Gabe untersucht. Die Anwendung von Heparin hatte deutliche Auswirkungen auf die Messung der exogenen *C. elegans* Spike-in-Kontrolle (Rückgang um ca. 3 Zyklen), die 6 Stunden nach dem Heparin-Bolus verschwanden. Messungen endogener miRNAs zeigten sich weniger empfindlich

gegenüber der Anwendung von Heparin. Normalisierung der einzelnen miRNAs mit dem durchschnittlichen Zyklus-Schwellenwert aller miRNAs stellte eine geeignete Alternative zu einer Normalisierung mit exogener *C. elegans* Spike-in-Kontrolle in diesem Zusammenhang dar. Somit sind sowohl der Zeitpunkt der Blutentnahme relativ zur Verabreichung von Heparin und die Anwendung des geeigneten Normalisierungsverfahrens entscheidend für zuverlässige miRNA-Messungen bei Patienten, die Heparin intravenös erhalten.

Zusammenfassend sind sowohl die Gabe von Thrombozytenaggregationshemmern als auch von Heparin Störfaktoren bei miRNA-Messungen, die bei der Untersuchung von zirkulierenden miRNAs bei kardiovaskulären Erkrankungen in künftigen Studien und bei der Bewertung der bisherigen Literatur berücksichtigt werden sollten.

1.9. Abbreviations:

Ago	Argonaute
AMI	acute myocardial infarction
C.elegans	*Caenorhabditis elegans*
cTnI	cardiac Troponin I
DGCR8	DiGeorge critical region 8
miRNA	microRNA
mRNA	messenger RNA
PACT	protein activator of the interferon-induced protein kinase
Ran-GTP	Ras-related nuclear protein-Guanosine triphosphate
RISC	RNA-induced silencing complex
RNase	ribonuclease
TRBP	transactivation response RNA binding protein
tRNA	transfer RNA
UTR	untranslated region

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2. Results:

2.1. Willeit et al. *Circulation research* 2013; 112 : 595-600.

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