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Platelet miRNAs 103 and 155 are differentially expressed in patients with symptomatic coronary artery disease

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ACD	Acid citrate dextrose
ACS	Acute coronary syndrome
ASA	Acetylsalicylic acid
ACE	Angiotensin converting enzyme
ARB	Angiotensin II receptor blockers
CAD	Coronary artery disease
CCR4	Carbon catabolite repressor protein 4
CCS	Chronic coronary syndrome
CeRNA	Competing endogenous RNA
CircRNAs	Circular non coding RNAs
CRP	C-reactive protein
CK	Creatine kinase
CK-MB	Creatine kinase muscle brain type
CT	Computed tomography
ECG	Electrocardiogram
EF	Ejection fraction
EPC	Endothelial progenitor cells
FFR	Flow fraction reserve
G	Gravity-force
GFR	Glomerular filtration rate
ICAM 1	Intercellular adhesion molecule-1
ILGF 1	Insulin-like growth factor 1
Lnc RNA	Long noncoding RNA
LV	Left ventricular
MI	Myocardial infarction

MiRNA	Micro RNA
MP	Microparticles
MRI	Magnetic resonance imaging
MRNA	Messenger RNA
MRP4	Multidrug resistance protein-4
NSTEMI	Non-ST elevation myocardial infarction
PAR 4	Protease-activated receptor 4
PBMCs	Peripheral blood mononuclear cells
PC-TP	Phosphatidylcholine transfer protein
PET	Positron emission tomography
PCI	Percutaneous coronary intervention
PremiRNA	Pre micro RNA
PrimiRNA	Primary miRNA
RNA	Ribonucleic acid
SPECT	Single-photon emission computed tomography
STEMI	ST elevation myocardial infarction
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
TGF-β	Transforming Growth Factor β
UA	Unstable angina

1 Introduction

1.1 Coronary artery disease

Coronary artery disease causes over 7 million deaths annually and remains the leading cause of death worldwide (1). The disease predominantly affects male individuals and incidence rises with older age (2). CAD is generally caused by focal thickening of the coronary artery´s intima layer, a process known as atherosclerosis. Subsequently, a stenosis of the affected artery ensues which causes myocardial ischemia (3).

1.1.1 Chronic coronary artery disease

Chronic coronary syndrome (CCS) is defined as a state of reversible coronary ischemia or hypoxia. This usually results from a myocardial mismatch of metabolic demand and supply and can be caused by exercise or other stress factors. Symptoms include shortness of breath, chest discomfort, chest pain and others. However, CCS can also be asymptomatic (4).

Different clinical presentations can result from different underlying pathomechanisms. Those include atherosclerosis related stenosis of the coronary arteries, vasospasm, impaired left ventricular function (caused by prior ischemic events) or microvascular dysfunction (4).

The first step to the diagnosis of CCS is clinical evaluation and history taking. Typical pectoral angina has a substernal location, can be provoked by exercise or emotional stress and can be relieved by nitrates and rest (5). Classical risk factors like metabolic syndrome, presence of diabetes mellitus, hyperlipidemia and family history for CAD need to be evaluated. Even though, there are no direct findings in a physical examination in CAD, likely Co-morbidities like valvular heart disease, peripheral arterial disease and others should be assessed. Provocation of symptoms by palpation of the thorax makes the presence of CCS unlikely (4).

A resting electrocardiogram should be taken in every patient with suspected CCS even though normal results cannot exclude the presence of a coronary stenosis. Pathological repolarization patterns can indicate present coronary

stenosis or past ischemic events. Dynamic and reversible ST segment changes during symptoms can be useful in the diagnoses of vasospasm (4).

All patients with a new onset of symptoms should receive an echocardiography. Regional wall motion abnormalities make the presence of coronary artery stenosis more likely and can be indicative of the affected vessel. Furthermore, left ventricular function is an important prognostic parameter in patients with CCS and differential diagnoses like valvular heart disease can be ruled out (6).

Advanced non-invasive testing like stress echocardiography, stress ECG and imaging techniques like coronary CT, MRI, PET and SPECT offer alternatives to invasive methods. They should be performed depending on factors like patient comorbidity and pretest probability (4).

Invasive coronary angiography remains the gold standard for the diagnoses of CAD. Important prognostic factors like severity and location of coronary stenosis can be assessed, if necessary, with the help of flow fraction reserve (FFR) measurements. An invasive diagnostic approach should be considered depending on severity of symptoms, cardiovascular event risk and findings from non-invasive methods (4).

1.1.2 Acute coronary syndrome

The term acute coronary syndrome (ACS) includes three distinct clinical entities: unstable angina (UA), non-ST elevation myocardial infarction (NSTEMI) and ST elevation myocardial infarction (STEMI) (7).

As seen in figure 1, a therapeutic distinction needs to be made, according to ECG findings. Patients with acute symptoms and ST-elevations (STEMI) need to receive immediate reperfusion as this pathology usually reflects acute coronary occlusion and is associated with a high mortality (8).

Patients without ST elevations on ECG but with elevated troponin levels (NSTEMI) represent a broad clinical spectrum, which can range from asymptomatic individuals to cardiac arrest secondary to ischemia. Treatment and treatment urgency vary according to risk factors and clinical presentation (9).

ACS with neither ST elevation nor biomarker increase is classified as unstable angina (UA) (as seen in figure 1). Pathophysiologically, this is characterized by myocardial ischemia without myocardial cell loss (9)

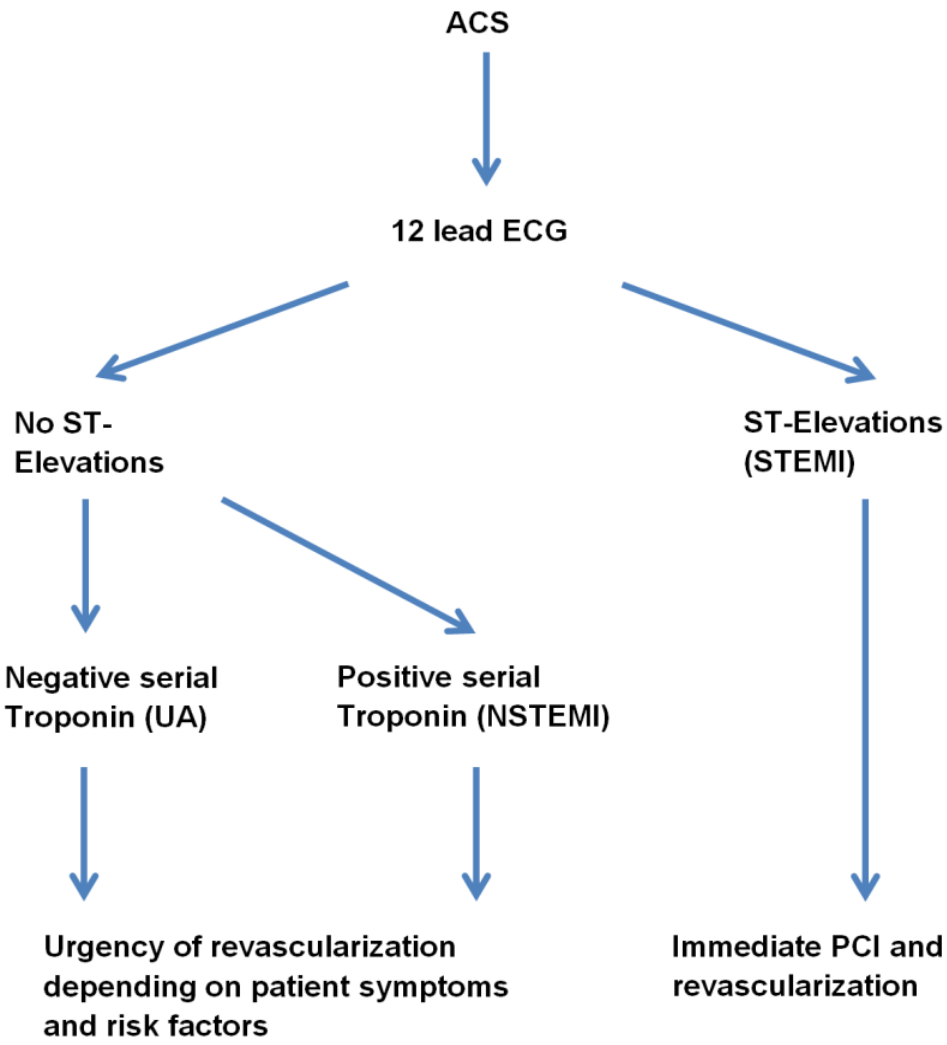

Figure 1: Diagnostic flowchart in ACS

Myocardial infarction (MI) is characterized by necrosis of cardiomyocytes in a setting of acute myocardial ischemia (10). As seen in the figure above, the presence of symptoms of ischemia, ECG changes and an increased cardiac biomarker (usually cardiac troponin) is crucial for the diagnosis of MI. From a pathophysiological point of view, MI can be subclassified into two types (9).

Type 1 MI is the result of decreased myocardial blood flow usually caused by atherosclerotic plaque rupture and subsequent intraluminal thrombus which results in myocardial necrosis (9).

Type 2 MI is caused by a mismatch in myocardial oxygen demand and supply not associated with coronary plaque instability. This can be caused by factors like anemia, arrhythmias, hypotension and others (9).

1.1.3 Established cardiac biomarkers in the diagnosis of ACS

As mentioned before, troponin is the most important biomarker in current ACS guidelines. Tropomyosin forms a continuous string along the myocardial actin helix with the troponin complex directly binding at every 7th actin molecule. Troponin itself is made up of three protein subunits: Troponin C (TnC) acts as a calcium binding site, troponin I (TnI) prevents premature actin myosin connection and troponin T (TnT) anchors the complex to tropomyosin. With rising calcium concentrations TnC releases TnI from its actin binding site and allows for actin myosin connection and muscle contraction (11).

Modern troponin essays usually measure cardiac TnT or TnI. They can be classified as sensitive (detection of blood troponin levels in 20-50% of a healthy population) or high sensitive (detection of blood troponin levels in 50-90% of a healthy population). In patients with acute MI, troponin is released due to necrosis of myocardial cells and usually can be detected within one hour if high sensitive assays are used (12, 13). However, delayed increase of troponin levels in peripheral blood has been described for 1% of patients. Because of this possible delay in blood troponin rise, a second measurement after a certain time interval is recommended (9). This time interval can be shortened by using modern high sensitive assays which can speed up the time to diagnosis. Generally, a 3-hour algorithm is recommended when using sensitive assays. This can be reduced to a 1 hour algorithm, if high sensitive troponin assays are used (9).

This algorithm results in a “rule in” versus “rule out” concept. A negative predictive value greater than 98% has been described in the “rule out” cohort and a positive predictive value of roughly 75% in the “rule in” cohort. This is due to the fact that cardiac troponin is specific for myocardial damage but not MI. Several other conditions like hypertensive emergency, heart failure, aortic stenosis, myocarditis, tako-tsubo cardiomyopathy, critical illness, cardiac

procedures, acute neurological events, aortic dissection or pulmonary embolism frequently cause elevated blood troponin levels as well (9).

Another possible biomarker is copeptin. Copeptin is part of the pro-hormone provasopressin. After release, it is proteolytically cleaved and secreted with arginine and vasopressin in equal parts. Since it is stable at room temperature and can easily be measured within a short period of time, it is suitable to be used as a biomarker. Copeptin blood levels peak early after MI, but it does not possess a satisfactory specificity and sensitivity. Recent data has shown that copeptin can be used in combination with troponin assays to improve sensitivity for MI at time of presentation (initial blood sample). Unfortunately, this lowers specificity when compared to troponin alone. However, the gain in sensitivity might be sufficient to dismiss the need for serial testing in some patients (13, 14).

To sum up, cardiac troponin and copeptin are essential as biomarkers for MI, but can lack specificity to identify patients in need of revascularization.

1.1.4 Impairment of left ventricular function in CAD

According to the European Society of Cardiology and the American Heart Association, impairment of LV function caused by CAD is not considered to be a cardiomyopathy (15, 16). Nevertheless, the disease is often referred to as ischemic cardiomyopathy in clinical practice.

Left ventricular dysfunction is associated with a poor prognosis in patients suffering from CAD (17). MI and the resulting irreversible cell death can lead to several morphological changes. The acute phase of MI results in necrosis of myofibrils as well as disintegration of collagen. The sudden tissue loss reduces the infarct area´s resistance to volume and pressure stress and leads to dilation. This thinning process of the myocardium is associated with myocardial rupture and aneurysm. Over time, cardiac remodeling ultimately leads to eccentric hypertrophy, an increase in ventricle size, wall motion abnormalities and ultimately heart failure. Established treatment options include angiotensin converting enzyme (ACE) inhibitors, aldosterone antagonists, beta blockers and more recently neprilysin inhibitors (18, 19).

The underlying pathophysiology is a complex process, which involves cellular, molecular and interstitial changes and is not yet fully understood. However, there are several biomarkers which might be candidates to reflect and possibly quantify the extent of the remodeling process. These include GLUT-1, GLUT-4, caveolin, angiotensin-converting enzyme and natriuretic peptide (19).

Early prediction of LV function recovery after revascularization and risk for adverse events is desirable. Damman et al. demonstrated, that the additional inclusion of several biomarkers to established risk factors can improve the prediction of mortality in patients with STEMI. Established risk factors were derived from the TIMI risk score and included age, body mass index, hypertension, diabetes, blood pressure, heart rate and time to balloon inflation. It was demonstrated, that the addition of blood glucose level, NT-proBNP and glomerular filtration rate (GFR) on admission help to improve prediction of mortality (20).

Hartman et al. examined the predictive strength of selected biomarkers on LVEF and infarct area measured by cardiac MRI as well as mortality in patients after STEMI. They demonstrated that Creatine kinase (CK) as well as TnI and TnT are able to predict infarct size, LVEF and mortality with the CK Muscle-Brain type (CK-MB) being the most potent marker (21). As left ventricular dysfunction is an important factor for patient outcome, reliable biomarkers which can predict recovery of systolic left ventricular function are desirable.

1.2 Micro RNA

MicroRNAs (miRNAs) are short endogenous RNAs that play important roles in plant and animal gene regulation (22).

Lee et al. first discovered, that lin-4, a gene involved in larval development in Caenorhabditis elegans, did not code for a protein, but for two small RNA strands. These two RNA strands had antisense complementarity to several sites at the 3´ untranslated region of another gene, lin-14. They went on to demonstrate, that lin-4 was able to downregulate lin-14 and suppress protein output without change in lin-4 mRNA concentration. The shorter lin-4 RNA strand is recognized as the first discovered miRNA (23).

Today we know that miRNA are non-coding and approximately 22 nucleotides in length. They downregulate gene activity by guiding so called argonaute proteins to the 3´ untranslated region of mRNAs. Argonaute proteins consist of single stranded nucleotide acids which are able to bind to their complementary sequence on the target mRNA and cause blockage of translation and mRNA degradation (24).

1.2.1 MiRNA gene location

MiRNA coding genes are found in different regions of the genome. Some can be found in regions distant from known genes implying that they possess their own independent transcription units. Others can be found in introns of precursor mRNAs (pre-mRNAs). These miRNAs do not have their own promotors, but are rather processed directly from these introns. This mechanism enables coordinated expression of mRNA and miRNA. MiRNA coding genes can also occur in gene clusters sharing functional relationships and are often co-expressed. An example for this is the miRNA 15a and miRNA 16 cluster, which is believed to be involved in tumor suppressor gene functions.
MiRNA is expressed tissue and cell specific. This is mainly regulated through transcription (22, 24).

1.2.2 MiRNA production

The first step in miRNA biogenesis is the transcription of primary miRNA (primiRNA) from miRNA coding genes by RNA polymerase II. This precursor form is altered by a protein complex called adenosine deaminase, which affects further processing or can lead to direct degradation. PrimiRNA can harbor one single miRNA but it can also be the precursor of clusters of several, often related miRNAs. Next, the primiRNA is cleaved by the enzymes Drosha and DGCR8. This process forms pre miRNA (premiRNA).
The premiRNA is identified by the protein exportin 5 by its characteristic hairpin structure and transported out of the nucleus into the cytoplasm. However, exportin 5 knockout experiments were not able to fully stop premiRNA transport suggesting an unknown secondary transport mechanism is likely to exist. In the cytoplasm, the premiRNA is further process by Ribonuclease III enzyme Dicer.

This further cleavage of the molecule results in a mature miRNA complex. Finally, one of the two strands (the guide strand) is transferred into Argonaute, while the second strand (passenger strand) is cleaved. Usually the strand with the less stable 5´ end is used as the guided strand. Mature miRNAs can be divided into families according to their specific seed sequences. The seed sequence is mainly responsible for mRNA targeting and defined as the first 2-8 nucleotides from the 5´ end (24).

1.2.3 MiRNA function

As mentioned above, miRNA mediated mRNA degradation functions via argonaute proteins. These proteins are comprised of a polypeptide chain with four characteristic domains: the amino (N)-terminal domain, the PAZ domain, the MID domain and the PIWI domain. The two latter domains contain the 5´ end of the miRNA and the PAZ domain holds the 3´ end. Four different argonaute proteins are expressed in humans: Ago 1-4 with Ago 2 being the most abundant. Ago 2 is also the only one with the ability to degrade targets which express a nucleotide strand fully complementary to the miRNA´s guided strand. This has significance for regulatory processes of a subset of miRNAs. Usually miRNAs are compatible with all argonaute protein types, however some do express certain argonaute preferences (24).

MiRNA´s target sites on mRNAs are usually found at their 3´ untranslated region. As mentioned above, the main factor for target site interaction is mRNA nucleotide complementarity to the miRNA´s guided strand seed region. An adenine molecule opposite the miRNA´s first nucleotide sharply raises target site interaction. Although miRNA itself binds with nucleotides 2-8, the first nucleotide in the sequence displays strong interaction with the argonaute protein. MRNA target site and guided strand complementarity of nucleotides 2-7 or 3-8 (instead of full 2-8 complementarity) still enables binding although the connection is considerably weaker. Binding itself is believed to be a two-step process: A weak connection is established with nucleotides 2-6, which becomes stable if complementarity of nucleotides 7-8 is also present (24).

The process described above leads to argonaute mediated recruitment of a protein called GW182 which in turn interacts with carbon catabolite repressor

protein 4 (CCR4) and poly(A)-nuclease deadenylation complex subunit 2 in order to promote mRNA degradation by exoribonuclease 1.

Recruitment of CCR4 not only leads to mRNA depletion but also to inhibition of translation, which can be mediated by RNA helicase DDX6. It is likely that mRNA degradation, which accounts for 60-90% of miRNA related gene silencing, is irreversible whereas inhibition of translation is a potentially reversible process (24).

MiRNA is also able to bind on a site neighboring the actual mRNA target site and hereby alter the original, possibly non matching target site. This process is called cooperative repression. With these mechanisms, miRNA is not only able to completely halt protein translation, but also influence it on a more subtle level. This allows for a buffering effect against fluctuating levels of mRNA transcription (24).

MiRNAs are not specific for a singular gene. In fact, a single miRNA is able to alter the expression of hundreds of genes. Inversely, multiple different miRNAs have the ability to silence the same gene (24).

Alternative cleavage by enzymes Drosha and Dicer causes different variations of miRNAs called isomirs (figure 2). These isomirs can differ in length and nucleotide sequence. This can alter their activity on the mRNA´s target sites as well as turnover and stability. Depending on the site of variation, isomirs are classified into 5´ or 3´ forms. The seed sequence is directly determined by the Dicer cleavage site on miRNAs produced from the 3´ strand (3p miRNAs). Alternative cleavage might shift the seed sequence which can result in 5´ isomir production (5p miRNAs). 3´ isomirs tend to vary in stability whereas 5´ isomir amount usually varies between tissue types (24).

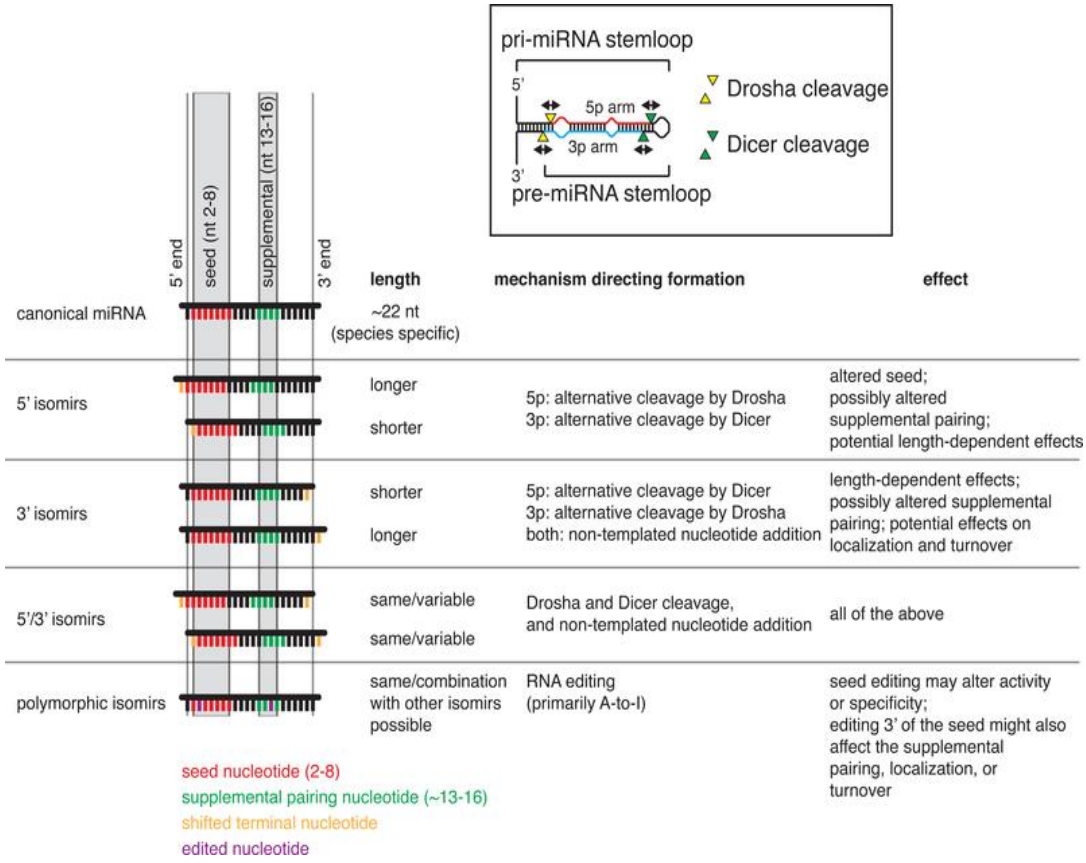

Figure 2: Isomir structures and possible physiological effects (modified from 24)

MiRNA characteristics can also be changed by sequence editing of precursor forms. This can come in the form of deamination (change from adenosine to guanosine) or via cytidine deaminase (change from cytidine to uracil). This process can alter further production steps like Dicer or Drosha cleavage or argonaute protein loading, which in turn relocates seed sequence (24).

Post translational modification of argonaute protein has also been reported to alter miRNA function. Processes like phosphorylation at different sites of the argonaute protein have been associated with both promotion and suppression of miRNA function (24).

Changes in mRNA target site can obviously also influence miRNA activity. MRNA target site editing possibly changes complementarity to miRNA´s seed sequence. Also, mRNA target sites can be added by formation of the 3´ untranslated region. One protein, able of editing mRNA target sites is the RNA binding protein Hu-antigen R. These proteins are able to modulate mRNA target sites to facilitate interactions with argonaute proteins. This seems to be an

important regulator as 75% of mRNA, that possess a miRNA binding site also exhibit a binding site for Hu-antigen R. This Hu-antigen R region often lies within close proximity to the miRNA target site, sometimes even overlapping it (24).

Another interesting miRNA regulation mechanism is the competing endogenous RNA (ceRNA) hypothesis. It suggests that high levels of miRNA-binding-RNAs are able to reduce the remaining cytoplasmic miRNA, causing derepression of specific target mRNAs. Those miRNA “sponges” could alter gene expression by causing competition for miRNA binding. However, increase in single ceRNA expression would have to be high (at least two-fold) in order to generate the postulated effect. This of course is only true, when observed within the average cytoplasmic environment; changes in local ceRNA and miRNA concentration could lower this threshold. Also, factors like RNA-directed miRNA degradation (which will be addressed later) could lower the threshold at which ceRNA becomes physiologically relevant. Possible ceRNAs include mRNAs, pseudogenes (DNA sequences related to genes, which lost protein coding function) or circular non coding RNAs (circRNAs) (24, 25).

External factors like viral infections have also been demonstrated to modify miRNA function. Viruses from the herpes family as well as hepatitis B and C have all been demonstrated to interact with miRNA. Hepatitis C, a single stranded RNA virus is able to recruit miRNA 122 to it´s 5´ end. This protects the virus from parts of the cell’s antiviral response mechanisms. Furthermore, this results in a functional downregulation of miRNA 122 (ceRNA mechanism), which is known for tumor suppressing properties. This might be one mechanism to explain hepatocellular carcinoma in hepatitis C infection. Other viruses have been reported to use miRNA binding in order to directly enhance virus replication rates (24).

1.2.4 MiRNA stability

MiRNA is stable *in vivo* and can be detected in blood and tissue. However, turnover rates vary considerably and range from minutes to days for specific miRNAs. Also, different isomirs of the same miRNA have been reported to show varying turnover rates (24).

One possible factor is non-template nucleotide addition, a multi-enzyme process, which results in uridylation or adenylation at the miRNA´s 3´ end. Uridylation has been reported to downregulate miRNA activity by decreasing its stability, whereas adenylation is associated with upregulation via decreasing miRNA turnover and improving stability. This process is reproducible across cell types, development stages and even species (24).

MiRNA type specific instability can also be explained by purely physiological reasoning. MiRNAs involved in the cell cycle need to display high turnover rates in order to allow for precise regulatory functions (24).

Stability has also been observed to be strongly tissue dependent with neuronal miRNAs displaying a higher turnover rate compared to other tissues types. Also, cell differentiation and development strongly effects miRNA stability and therefor the miRNA expression profile (24).

Interestingly, target mRNA can also mediate miRNA degradation and therefore increase turnover, this is called “target RNA-directed miRNA degradation”. During this process, target mRNAs with complementary sequences to the 3´ end of the miRNA promotes it´s destabilization and degradation. This has been associated with non-template nucleotide addition at the 3´ end and can be mediated by virus activity or as an endogenous regulatory mechanism. Contrary, there is evidence that high concentrations of seed sequence matching mRNA targets are also able to stabilize miRNA and decrease turnover rates (24).

1.2.5 MiRNA circulation

As previously mentioned, miRNA assembly starts in the cell´s nucleus before precursor forms are transported into the cytoplasm. However, certain mature miRNAs have been shown to remain in the nucleus. Owning to the hexanucleotide nuclear localization signal, 20% of total miRNA 21 is never transported into the cytoplasm. Since important processing molecules like argonaute and Dicer can be found in the nucleus, the mature miRNA is functional although understanding of its purpose is limited (24).

MiRNA can be detected in human plasma and serum. However, it is unclear, which amount of the circulating miRNA travels within exosomes. Exosomes are

cellular vesicles, which can be found in eukaryotic fluids and play crucial roles in intercellular signaling. It appears, that in plasma, roughly 10% of miRNA travels within exosomes whereas in serum up to 99% travels exosome dependent. At the moment, this discrepancy is not yet fully understood (24, 26).

Circulating miRNAs are postulated to have regulatory functions. However, targeted exosomal miRNA transport and exosomal miRNA sorting is necessary in order to provide controlled biological functionality. Exosomal long non coding RNA (lnc RNA) and exosomal RNA binding proteins have been proposed as sorting mechanisms. So-called “exomotifs”, nucleotide sequences at the miRNA´s 3´ end, could possibly interact with proteins like “heterogeneous nuclear ribonucleoproteins A2/B” or “nuclease-sensitive element-binding protein 1” in order to mediate miRNA sorting into exosomes. The earlier mentioned process of non-template nucleotide addition is able to edit the 3´ end of the miRNA. This could change binding ability to the proteins mentioned above and therefore play a role in exosome sorting. Adenylation is associated with lowered miRNA exosome levels whereas uridylation is associated with elevated miRNA exosome levels in human B-cells (24).

1.2.6 Clinical use of miRNA

MiRNAs ability to alter translation and protein production as well as potential cell signaling functions make them interesting candidates for therapeutic use. There are two general concepts: stimulating miRNA activity with the use of “miRNA mimetics” and inhibiting miRNA activity with the use of “anti-miRs”. The former consist of short, synthetic double stranded RNAs with corresponding sequences to the target mRNA, the later consist of single stranded antisense oligonucleotides which bind and neutralize the desired miRNA. Advances in RNA delivery and improvement of stability have made these therapeutic options feasible and there are several ongoing clinical trials (27).

As mentioned previously, miRNA profiles are cell and tissue specific and sensitive to biological changes. Additionally, miRNA is stable and can be measured not only in tissue but also serum and plasma. This makes it ideal for usage as potential a biomarker (28).

1.2.7 Circulating miRNA in CAD

MiRNAs are known to have key roles in cardiovascular disease and especially atherosclerosis (27). Several authors compared the miRNA expression profile from CAD patients to controls or within the cohort of CAD (ACS vs. non-ACS) in mostly observational studies (29).

Most studies tested for miRNA concentrations in plasma or serum. Other miRNA sourced included whole blood, peripheral blood mononuclear cells (PBMCs), endothelial progenitor cells (EPC) or microparticles (MP) from plasma (29). Results varied widely with different miRNA measurements, differences in sample preparation and handling as well as differences in storage. This makes a comparison of different studies difficult and could in part explain contradicting results. Furthermore, data obtained by quantitative real-time polymerase chain reaction needs to be normalized post measurement and there is no consensus on the optimal method at the moment (30).

However, some results were reproducible. Upregulation of miRNA 21 was described in several studies by different authors (31-37). It appears that miRNA 21 is upregulated in patients suffering from CAD compared to healthy controls as well as in ACS and myocardial infarction when compared to stable CAD (31-35, 37, 38). Pathophysiologically, miRNA 21 is known to play an important role in heart failure by modulating signaling pathways in cardiac fibroblasts which eventually leads to fibrosis and hypertrophy (39).

MiRNA 146 increase was reported by several authors in MP, PBMCs and serum. Upregulation took place when comparing patients with CAD to healthy controls as well as ACS and MI to CCS (31, 35, 37, 40).

MiRNA 208 upregulation has been associated with myocardial injury in studies by several authors (35, 36, 41-44). This miRNA is known to be expressed by the same gene responsible for transcription of the myosin heavy chain and is tissue specifically expressed in the heart. Animal models were able to demonstrate that miRNA 208 is elevated exclusively by myocardial but not skeletal muscle damage (38). However, there were also studies not able to reliably measure miRNA 208 concentrations in MI patients. This might be due to

the fact that absolute concentrations are rather low and measuring methods like TaqMan PCR might not possess a sufficient sensitivity (45).

MiRNAs 1, 133 and 499 have also been associated with myocardial injury by multiple authors (34-36, 41-44, 46-48). Unlike miRNA 208, these miRNAs are not myocardium specific but are also expressed in skeletal muscle and elevations have been observed in patients suffering from Duchenne muscular dystrophy. MiRNA 1 and 133 are known to belong to a specific miRNA cluster and therefore are often co-transcribed whereas MiRNA 499 is related to the myosin chain. Measuring transcoronary gradients, the partial myocardial origin of miRNA 133 has been proven (45).

Potential advantages for miRNAs compared to established biomarkers like troponin could be their early release. In both animal and clinical studies, the aforementioned muscle related miRNAs demonstrated a favorable kinetic with an early release. MiRNA 499 upregulation has been demonstrated as early as 15 minutes after coronary ligation in mice and miRNA 208 might be superior to troponin in the early stages of acute MI (38, 46).

MiRNA could also aid in identifying patients suffering from UA, a condition where established biomarkers like troponin are not helpful. A panel of miRNAs 132, 150 and 186 has been associated with a high discriminatory power to identify patients suffering from UA compared to non-coronary chest pain (49).

MiRNA 126 is in part expressed in endothelial cells. Changes in expression could represent endothelial activation which is known to take place early during the formation of atherosclerotic plaques. This could provide a tool for early non-invasive screening for patients at risk of developing CAD (45).

Downregulation appears to be much rarer than upregulation. MiRNA 155 downregulation was reported by three authors: one group compared miRNA concentrations in PBMCs of CAD and non-CAD patients, the second group found a correlation between miRNA concentration and patient´s Gensini score (a score quantifying CAD), while the third group found miRNA downregulation in whole blood when comparing CAD patients to heathy controls. MiRNA 155 is hypothesized to be down regulated due to a possible uptake of atherosclerotic lesions, thus decreasing it´s levels in the circulation (37, 42, 50, 51).

To date, the complex changes and regulatory patterns in miRNA expression in CAD are only partially understood. Irregularities in miRNA expression between studies might be attributed to small cohorts, different patient population (no age or sex match) and small sample sizes. However, data suggests that miRNAs can offer additional value as biomarkers, but there is a lack of large prospective studies. Also, miRNA isolation and measurement can be challenging and might hinder miRNA usage in a broad clinical setting (29, 45).

1.2.8 MiRNA in platelets

Platelets are well known to play a central role in thrombotic disorders and cardiovascular disease. Since thrombocytes do not possess a cell nucleus, they are not able to conduct transcription (30). Nevertheless, genes are abundant in platelets at the mRNA level. Possessing the necessary components for mRNA translation, thrombocytes are also able to synthesis proteins.

Platelet miRNA regulation starts in platelet synthesis. Murine models suggest that miRNAs 146b and 105 are important stimulating factors in megakaryopoiesis, whereas miRNA 142 and miRNA 486-3p show megakaryopoiesis inhibiting effects (52).

The presence of miRNA in adult platelets was first described by Merkerova et al. in 2008 when they compared miRNA expression profiles of different cell types (53). Shortly after that, Landry and colleagues demonstrated that thrombocytes are able to process miRNAs independently of their megakaryocyte progenitors (54). Knockout of the protein Dicer in megakaryocyte leads to a reduction in platelet miRNA and subsequently to a severe alteration in thrombocyte protein expression. This suggests that miRNAs possess regulatory functions at the translational level (52, 55, 56). Nagalla et al. were able to demonstrate an inverse correlation between selected platelet miRNAs and their target mRNAs and subsequent protein downregulation. This also suggests normal miRNA function in platelets (57). Moreover, the lack of transcription in platelets could be a reason for their reliance on miRNA as a post transcriptional mechanism for regulating gene expression.

Studies comparing miRNA profiles in healthy volunteers over a certain time frame were able to demonstrate both inter-individual stability as well as stability

in miRNA expression over time. However, this inter-individual stability apparently only applies to individuals of the same age and sex group as differences in platelet miRNA expression between male and female individuals as well as persons of different age groups have been described (58, 59).

Sequencing data reveals the occurrence of isomir forms as well as post transcriptional modifications in platelet miRNA too (55).

Figure 3 shows the 25 most abundant miRNAs in a collective of patients of 8 different studies (54, 55, 57, 58, 60-63). They are sorted by expression levels from top to bottom (with miRNA 126-3p demonstrating the highest abundance). Numbers indicate the relative rankings within the single studies, colors help to visualize these rankings (modified from (64)). A clear variation in miRNA expression between the study cohorts can be seen.

Single miRNA functions within the platelet can be important for our understanding of disease pathogenesis. For example, a difference in the survival of CAD between Caucasian and African-Caribbean people has been reported. Phosphatidylcholine transfer protein (PC-TP) is able to activate platelets via Protease-activated receptor 4 (PAR 4), which is more pronounced in people of African descent. MiRNA 376c has been shown to regulate PC-TP and might be responsible for increased platelet aggregation rates as it is differently expressed in African and Caucasian individuals (64).

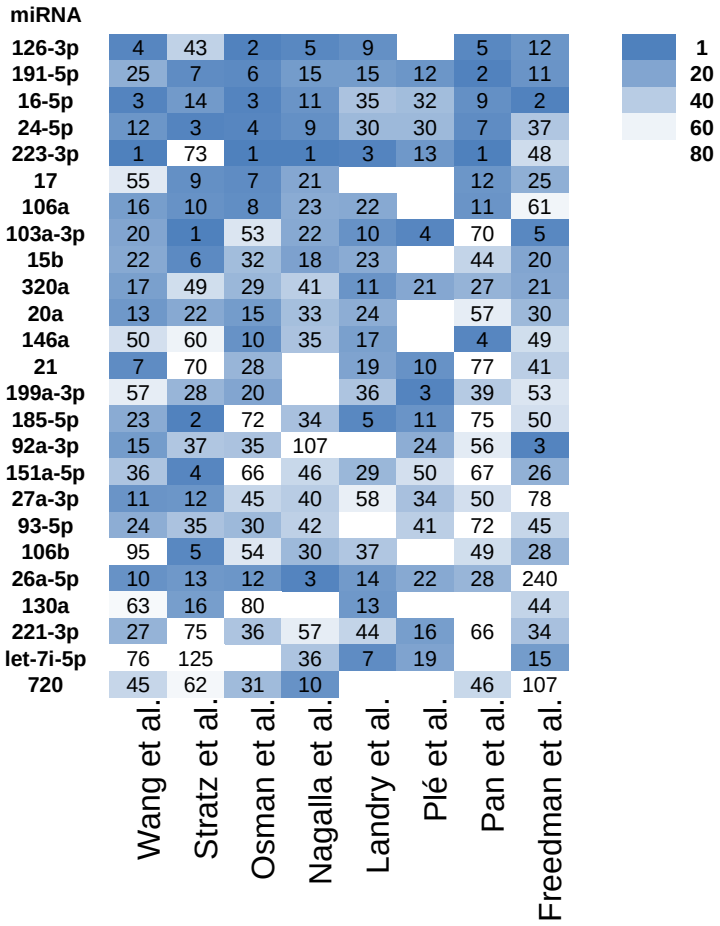

Figure 3: The 25 most abundant miRNAs in 8 different studies sorted from top to bottom. Numbers indicate the miRNA rankings within the single studies (modified from 64)

Risk factors for CAD like diabetes might also affect platelet reactivity through miRNA expression. Calpain is a calcium-dependent protease which can inactivate the protein Dicer and is predominantly activated in platelets in patients suffering from diabetes. This results in a decrease of miRNA 223, miRNA 26b, miRNA 126, and miRNA 140 (among several others) in both diabetic humans and mice. In the murine model, this effect was reversible by administering a Calpain inhibitor. MiRNA 223 deficient mice showed larger clots in response to injury, increased numbers of microcirculatory emboli and modest increase of in vitro thrombus formation (65, 66).

Reduced levels of miRNA 30c in diabetic humans and mice can cause upregulation of plasminogen activator inhibitor-1 (PAI-1). Artificial overexpression of miRNA 30c was able to reduce arterial thrombosis in mice suggesting a relationship between miRNA 30c levels and platelet function (67).

Platelet miRNA 27b downregulation was observed after activation of platelets via thrombin. Inversely, artificial increase of miRNA 27b using mimics led to Thrombospondin-1 downregulation which enhances angiogenesis in endothelial progenitor cells (68). Similarly, platelet miRNA 21 is able to enhance fibrosis in other tissues via modulation of TGF-β1 secretion (69).

The miRNA profiles of plasma and serum closely match miRNA profiles observed in platelets. This might indicate that platelets are an important source for circulating miRNA. The fact that antiplatelet medication was found to influence the levels of several circulating miRNAs also points towards this. Inversely, circulating miRNA profiles have been demonstrated to be associated with platelet function (30, 64).

The vast amount of platelet miRNA is likely to act outside of thrombocytes in other cells and tissues. Upon stimulation, platelets are able to produce microvesicles which can contain miRNA. These miRNAs are then actively transported into macrophages, smooth muscle cells, endothelial cells and others. This is demonstrated in figure 4 (70).

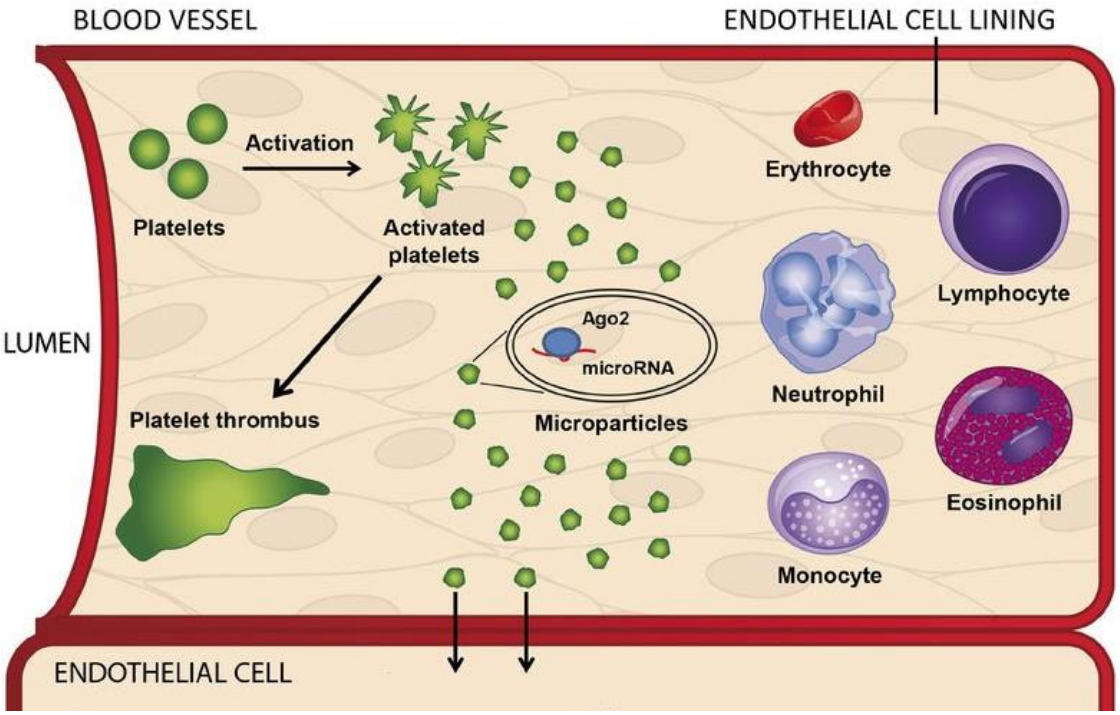

Figure 4: Microvesicles production and transport after platelet activation (modified from 70)

In those target cells, several miRNAs like miRNA 320b and miRNA 223 are able to bind to mRNA and regulate functions like cell adhesion and inflammatory response (via intercellular adhesion molecule-1, ICAM 1), smooth muscle cell proliferation (via platelet-derived growth factor receptor, PDGF receptor) and apoptosis (via insulin-like growth factor 1 receptor, ILGF 1 receptor) (62, 70-73).
MiRNA 126 was thought to be endothelial specific. However, evidence suggests that circulating miRNAs 126 levels could in part originate from platelets. De Boer et al. were able to demonstrate that treatment of patient plasma with arachidonic acid (a platelet activator) resulted in an increase of plasma miRNA 126 levels. This effect could be slowed down with Acetylsalicylic acid (ASA), a platelet inhibitor. The levels of miRNA 126 also correlated with p-selectin levels (a marker of platelet activation). This strongly suggests platelets as a source for plasma miRNA 126 levels (74).
Another cell type capable of taking up platelet microvesicles are macrophages. Murine models suggest that miRNA 4306 is able to alter macrophage functionality and reduce cardiac tissue infiltration after infarct. The authors also found miRNA 4306 to be associated with poor prognosis in patients suffering from CAD (75).
As mentioned above, there is evidence of possible paracrine effects of micro particle associated miRNA and platelets could be an important source. However, most data stems from in vitro studies and it is not known if the observed transfer is functionally relevant. Firstly, amplification methods have to be used in order to measure the transferred miRNA. It is possible that low in vivo miRNA concentrations are not sufficient to have a physiological effect. Secondly, it is not known whether the protein content of MP is functionally more important than the miRNA. Thirdly, in situ hybridization staining patterns show tissue specificity for certain miRNAs (for example an endothelial pattern for miRNA 126). If sufficient transfer would occur (as *in vitro* data suggests), one would expect a more diffuse staining pattern (64).

An obvious field of interest is the change of platelet miRNAs in the presence of antiplatelet drugs like clopidogrel and ASA. MiRNA 223 is one of the most

abundant miRNAs in platelets. It is able to interact with the mRNA responsible for translation of the P2Y12 receptor. This receptor is vital for platelet functions like aggregation and granulation and the therapeutic target for drugs like clopidogrel. Decrease in miRNA 223 levels has been associated with high platelet reactivity. Downregulation of miRNAs 223, 191, 126 and 150 in response to pharmacological platelet inhibition have been reported in both healthy volunteers as well as patients suffering from symptomatic atherosclerosis of the carotid artery (76). For miRNAs 223, 221, 126 and 21, a correlation between platelet responsiveness to clopidogrel and miRNA expression levels was observed (64, 77-80). In mice, a reduction in platelet aggregation was observed after knock out of miRNA 126 (77). The platelet inhibiting agent also seems to alter miRNA expression profiles. After switching from clopidogrel to ticagrelor, a downregulation of miRNAs 150, 223 and 126 was observed whereas miRNA 96 was increased. Further downregulation of miRNA 126 could be observed after administrating a loading dose of ticagrelor. This suggests a correlation between miRNA 126 expression levels with amount of platelet inhibition (81).

Antiplatelet drug resistance is an important factor in clinical treatment. A potential mechanism for platelet resistance to ASA could be the expression profile of miRNA 26b. Downregulation of miRNA 26b was observed in patients who had been subjected to chronic ASA treatment. This increases levels of multidrug resistance protein-4 (MRP4), a protein which has been linked to platelet ASA resistance (82, 83).

Most clinical studies mentioned miRNA of partly platelet origin measured in other compartments like plasma or serum. To our knowledge, there is little data on miRNA measured directly in platelets of patients suffering from CAD. Ward et al. compared expression profiles of 343 miRNAs in 13 STEMI and NSTEMI patients in several compartments, including platelets. They found an upregulation of miRNA 25-3p and 221-3p and a downregulation of miRNA 186-5p and 342-3p in STEMI patients compared to individuals suffering from NSTEMI (84).

Platelet miRNA profiles of 5 different miRNAs in 20 STEMI patients compared to 40 healthy volunteers were compared by Li et al. in 2017. They found an upregulation of miRNA 150 and 223 and a downregulation of miRNA 21 and miRNA 126 in the STEMI cohort. However, there was no correlation between miRNA expression and platelet function testing (85).

Sondermeijer et al. looked for platelet miRNA as a diagnostic tool to identifying patients with premature CAD. They were measuring 214 miRNAs in platelets of 12 patients with premature CAD and 12 healthy controls. They found 7 differently expressed candidate miRNAs which were then measured in two validation cohorts. Validation cohort one consisted of 40 patients suffering from premature CAD with 40 matched controls, validation cohort two consisted of 27 patients suffering from premature CAD with 40 healthy family members. The findings were a significant upregulation of miRNA340 and miRNA624 in patients with premature CAD (86).

2 Study aims

The aim of this study was to examine the distribution of miRNAs with known cardiovascular function in platelets of patients with different manifestations of CAD. The different pathophysiology of ACS compared to CCS might lead to different miRNA distribution which could be used as a possible diagnostic biomarker.

Furthermore, follow up data on LVEF% was acquired. Hence, we aimed to investigate associations between miRNA expression at study inclusion and course of LVEF% in CAD patients. This approach yielded identification of distinct miRNAs which correlated with worsening or improvement of LVEF% over time. These miRNAs might be useful for identifying CAD patients at high risk for progressive myocardial failure and thus allow for tailoring therapies in those patients.

3 Materials and Methods

3.1 Chemicals

Chemical	Source
Tri sodium citrate dihydrate	Merck KGaA, Darmstadt, Germany
Citric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
D-(+)-Glucose	Sigma-Aldrich Chemie GmbH, Munich Germany
Sodium hydroxide	Merck KGaA, Darmstadt, Germany
Sodium chloride	AppliChem GmbH, Darmstadt, Germany
Sodium hydrogen carbonate	Merck KGaA, Darmstadt, Germany
Potassium chloride	Carl Roth, Karlsruhe, Germany
Bovine Serum Albumin (BSA)	Thermo Fisher Scientific, Waltham, Massachusetts
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Carl Roth, Karlsruhe, Germany
Hydrochloride acid	Carl Roth, Karlsruhe, Germany

3.2 Buffers

Buffer	**Components**
ACD buffer	12,5g tri sodium citrate dihydrate 6,82g Citric acid 10g D-(+)-Glucose 500ml distilled water Sodium hydroxide until (ph 4.69)
Tyrodes pH 6,5 + 0,1% BSA/Glucose	80g Sodium chloride 10,15g Sodium hydrogen carbonate 1,95g Potassium chloride 1000ml distilled water 0.1% Bovine Serum Albumin 0.1% D-(+)-Glucose (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) until pH 7.4 1N Hydrochloride acid until pH 6,5

3.3 Laboratory materials

Laboratory Material	Source
20 ml syringe	B. Braun, Melsungen, Germany
15 ml Falcon tube	Greiner Bio One, Frickenhausen, Germany
50 ml Falcon tube	Greiner Bio One, Frickenhausen, Germany
Pasteur pipette	RatioLab GmbH, Dreieich, Germany
Eppendorf Safe-Lock Tubes 1.5 ml	Eppendorf AG, Hamburg, Germany

3.4 Laboratory devices

Device Name	Source
Haraeus Multifuge 3S Plus Centrifuge	Thermo Fisher Scientific, Waltham, Massachusetts
PIPETBOY acu 2	INTEGRA Biosciences AG
Sysmex KX-21N™ Automated Hematology Analyzer	Sysmex Deutschland GmbH, Nordertstedt, Germany
pH/Ionen-Messgerät SevenCompactTM S220, pH-Meter SevenCompactTM S220-basic	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sartorius extend scientific scale	Sartorius AG, Göttingen, Germany
Eppendorf centrifuge	5417 Eppendorf AG, Hamburg, Germany
Eppendorf pipette 100/200/1000µl	5417 Eppendorf AG, Hamburg, Germany

3.5 Study design

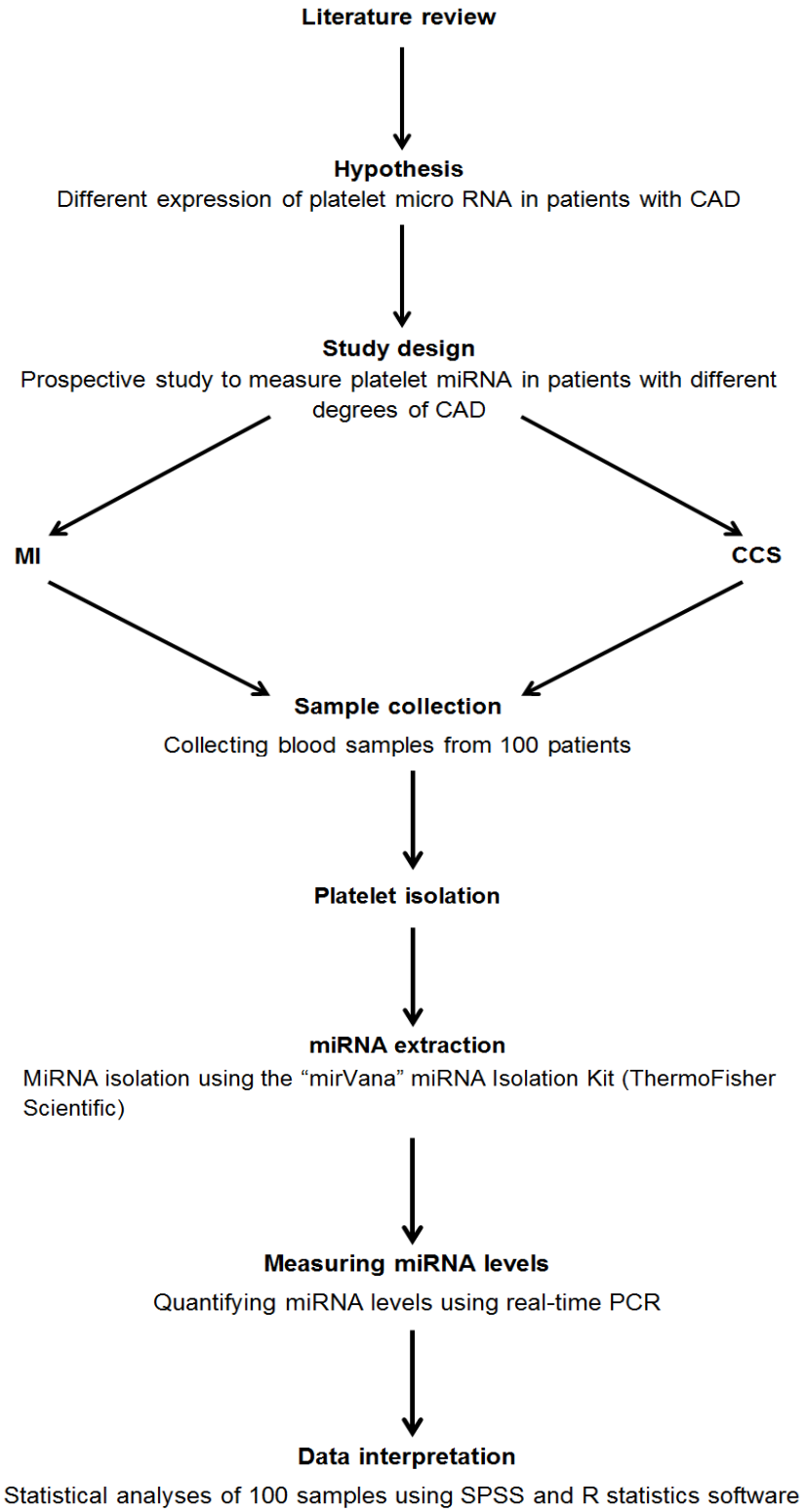

Figure 5: Flow chart of the study design

3.6 Study population

In this prospective cohort study, we included 100 patients who presented to the department of Cardiology at the University Clinic of Tuebingen, Germany from May to July of 2017. 33 patients presented with acute MI, 67 patients were classified as CCS. MI was defined using the universal definition of myocardial infarction of the 2015 and 2017 ESC guidelines (8, 9). These individuals either presented for a scheduled coronary angiography or were transferred for invasive diagnostics to the catheter laboratory via the clinic´s emergency room. 60 ml of arterial blood were drawn periprocedurally via the catheter sheet and directly transferred to our laboratory for platelet isolation. Inclusion criteria were patient age >18 years and invasively diagnosed coronary artery disease. All patients gave written informed consent. The study complies with the declaration of Helsinki and good clinical practice guidelines and was approved by the responsible ethics committee (270/2011B01) and (238/2018BO2).

3.7 Thrombocyte isolation

As mentioned above, 60 ml of arterial whole blood was taken in 3 syringes during coronary angiography and immediately transferred to the in-house laboratory. There, the blood was pooled in a ratio of 1:4 with Acid-Citrate-Dextrose (ACD) buffer in 15 ml falcons and centrifuged at 330g for 10 minutes at room temperature without breaks. Using a Pasteur pipette, the platelet rich plasma was pooled at a ratio 1:2 with Tyrodes ph 6.5 buffer in a 50 ml falcon and redistributed to 15ml falcons. Next, the solution was centrifuged at room temperature without breaks at 240g for 10 minutes. The supernatant was transferred to new 15 ml falcons and a 50 µl aliquot was scanned for leukocyte contamination using a clinical hematology analyzer (Sysmex). If leukocyte contamination could be excluded, the probes were centrifuged at room temperature without breaks at 430g for 10 minutes. The supernatant was discarded and the thrombocyte pellet re-suspended in Tyrodes ph 6.5 buffer solution using an Eppendorf pipette. A 50 µl aliquot was used to calculate thrombocyte count using the clinical hematology analyzer. Finally, the probe was split into two aliquots and transferred to 1.5 ml eppendorf cups and

centrifuged at 4° Celsius at 420 g for 5 minutes. Afterwards, the probes were labeled and stored at -80° Celsius.

3.8 Micro RNA extraction and measurement

Micro RNA measurements as described below were done in cooperation with Professor Matthias Schwab, Dr. Stefan Winter and Dr. Elke Schaeffeler at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology.

Total RNA including miRNA was extracted from the thrombocyte pellets using mirVana miRNA isolation kit (Applied Biosystems, Thermo Fisher Scientific, USA) following the manufacturer's protocol and stored at −80° Celsius. MiRNA selection for quantification by real-time PCR as previously described was based on literature data related to cardiovascular disease (87, 88). MiRNAs were reverse transcribed according to the manufacturer's protocol using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, USA) and pooled individual stem-loop reverse transcription primers were included in the pre-developed TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific, USA).

Next, a pre-amplification reaction was performed to improve the sensitivity of miRNA quantification, using TaqMan PreAmp Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA) and pooled pre-developed TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific, USA). Pre-amplification PCR was run according to the manufacturer's protocol. The resulting PCR product was diluted 1:5 with suspension buffer (Teknova AS, Norway) and stored at −20 °C until quantification by real-time PCR. The miRNA expression levels were quantified by real-time PCR using TaqMan® Universal Master Mix II (no UNG) and pre-developed TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific, USA) on a real-time PCR BioMark system (Fluidigm Corporation, USA) following the manufacturer's protocol. Relative levels of miRNA expression were calculated by normalization to expression levels of miRNA 24-3p, miRNA 320a and miRNA-451a (delta-delta CT value). Based on a literature study, 62 miRNAs with known effects on cardiovascular functions were measured.

3.9 Statistical analysis

Data processing and statistical evaluation were conducted in cooperation with Dr. Bernhard Drotleff (Institute of Pharmaceutical Sciences, Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany). We used Excel 2019 (Microsoft, Redmond, WA, USA), SPSS Statistics 23 (IBM, Armonk, NY, USA), Origin 2019 (OriginLab, Northampton, MA, USA), and RStudio 1.2.1335 (R version R-3.5.1, R Foundation for Statistical Computing, Vienna, Austria) (89). Missing miRNA data (1.48 % of values for all patients and 1.15 % of values for the matched controls dataset) were imputed using a random forest-based method (90). The resulting data was used to compare miRNA expression between groups (ACS vs CCS, Δ LVEF%>1 vs Δ LVEF%<1). Calculation of relative miRNA expression profiles for the whole population was based on non-imputed data. For hypothesis testing of the matched dataset, Wilcoxon signed rank tests were used, whereas Mann-Whitney U tests were applied for the other datasets. Accordingly, fold changes between experimental groups were calculated via the Hodges-Lehmann estimator (i.e. pseudomedian). Concerning the baseline characteristics, chi-square tests and McNemar tests were applied to compare dichotomous variables whereas Mann-Whitney U tests and Wilcoxon signed rank tests were used for metric variables. All statistical tests were two-sided and statistical significance level was defined as 5%. No adjustment for multiple testing was performed.

3.10 Follow up for measuring LVEF%

If available, periprocedural echocardiography or ventriculography was analyzed and LVEF% measured. One year after sample preparation the electronic database was searched for possible readmissions and follow up echocardiography or ventriculography. Eligibly image studies were revisited and LVEF% measured.

4 Results

4.1 Baseline characteristics

Baseline characteristics of the full study cohort are shown in table 1. Normally distributed data are presented as mean ± standard deviation. Chi-square tests were performed to compare dichotomous variables, whereas T-tests were calculated to compare means. C-reactive protein (CRP) and CK were significantly higher in the MI group. Difference in CRP can be attributed to a systemic inflammatory reaction caused by myocardial necrosis in MI whereas CK is a known marker for myocardial damage.

Patients also had a significantly higher frequency of treatment with clopidogrel and statins in the CCS cohort. This is likely due to the fact that some of these individuals were administered for a scheduled coronary angiography and had known CAD which they already received medical treatment for. Furthermore, LVEF% on admission was lower in the MI cohort. This can also be attributed to myocardial necrosis cause by infarction which impairs systolic function.

As miRNA profiles are influenced by numerous factors, 28 patients suffering from CCS were matched to MI patients with respect to age, sex, diabetes and history of chronic kidney failure. Five MI patients could not be matched since we could not provide suitable matches within the CCS cohort. Baseline characteristics of this cohort are shown in table 2. As with the full cohort, normally distributed data are presented as mean ± standard deviation. Chi-square tests were performed to compare dichotomous variables whereas T-tests were calculated to compare means. Similarly to the full cohort, patients in the MC group showed a significantly higher frequency of treatment with statins. Although more patients in the MC group were treated with clopidogrel, in this cohort the difference in medication was not significant. Surprisingly, CRP was higher in the MC group then in the MI group although the difference was not significant. As expected, CK was higher in patients suffering from MI. However, the difference was also not significant.

Table 1: Baseline characteristics of the full study cohort stratified according to patients with MI vs. CCS

	Total (n=100)	MI (n=33)	CCS (n=67)	p-value
Gender (male%)	76 (76%)	23 (70%)	53 (79%)	p=0.300
Age (years)	72.5 (45)	76 (54)	71 (41)	p=0.800
CV Risk Factors				
Hypertension	76 (78%)	22 (67%)	55 (83%)	p=0.060
Diabetes	29 (30%)	10 (30%)	19 (29%)	p=0.876
Previous MI	22 (22%)	4(12%)	18 (27%)	p=0.094
GFR (ml/min)	73.3 (240)	73.9 (22)	72.8 (171)	p=0.822
Smoking	19 (20%)	6 (18%)	14 (21%)	p=0.723
Hyperlipidemia	42 (42%)	9 (27%)	33 (49%)	p=0.310
Laboratory values				
Troponin positive	33 (33%)	33 (100%)	0 (0%)	**p<0.001**
CK (U/l)	113.0 (3642)	145 (3604)	81 (655)	**p<0.001**
CRP (mg/dl)	0.33 (23.28)	0.5 (23.28)	0.19 (8.45)	**p=0.019**
Medication on Admission				
ASA	54 (57%)	13 (45%)	41 (63%)	p=0.098
Clopidogrel	8 (9%)	0 (0%)	8 (12%)	**p=0.048**
Prasugrel	5 (5%)	0 (0%)	5 (8%)	p=0.125
Ticagrelor	12 (13%)	2 (7%)	10 (15%)	p=0.255
Statins	67 (71%)	14 (48%)	53 (82%)	**p=0.001**
ACE Inhibitors	41 (43%)	10 (35%)	31 (48%)	p=0.233
ARBs	25 (27%)	6 (21%)	19 (29%)	p=0.387
Beta Blockers	56 (60%)	14 (48%)	42 (65%)	p=0.136
Diuretics	39 (42%)	12 (41%)	27 (42%)	p=0.988
Coronary Artery Disease				
MI	33 (33%)	33 (100%)	0 (0%)	**p<0.001**
CCS	67 (67%)	0 (0%)	67 (0%)	**p<0.001**
LVEF%				
LVEF% admission	55 (52.5)	50 (45)	55 (52.5)	**p=0.041**
LVEF% follow up	55 (40)	55 (30)	57.5 (40)	p=0.414

Table 2 Baseline characteristics of the study cohort stratified according to patients with MI vs. MC

	MI (n=28)	MC (n=28)	p-value
Gender (male%)	22 (79%)	22 (79%)	p=1
Age (years)	74 (39)	74 (33)	p=0.828
CV Risk Factors			
Hypertension	19 (68%)	24 (86%)	p=0.180
Diabetes	8 (29%)	8 (29%)	p=1
Previous MI	3(11%)	6(21%)	p=0.508
GFR (ml/min)	72.3 (93)	76.7 (140)	p=0.662
Smoking	6 (21%)	6 (14%)	p=0.688
Hyperlipidemia	8 (29%)	14 (50%)	p=0.263
Laboratory values			
Troponin positive	28 (100%)	0 (0%)	**p<0.001**
CK (U/l)	134.5 (3601)	116 (629)	p=0.073
CRP (mg/dl)	0.46 (23.23)	1.10 (8.45)	p=0.166
Medication on Admission			
ASA	10 (42%)	18 (64%)	p=0.424
Clopidogrel	0 (0%)	4 (14%)	p=0.248
Prasugrel	0 (0%)	3 (11%)	p=0.248
Ticagrelor	2 (8%)	7 (25%)	p=0.688
Statins	12 (50%)	25 (89%)	**p=0.006**
ACE Inhibitors	9 (38%)	14 (50%)	p=0.607
ARBs	4 (17%)	9 (32%)	p=0.508
Beta Blockers	11 (46%)	20 (71%)	p=0.302
Diuretics	10 (42%)	12 (43%)	p=1
Coronary Artery Disease			
MI	28 (100%)	0 (0%)	**p<0,001**
CCS	0 (0%)	28 (100%)	**p<0,001**
LVEF%			
LVEF% admission	52.5 (40)	55 (40)	p=0.084
LVEF% follow up	55 (30)	57.5 (25)	p=0.628

4.2 MiRNA abundance within the population

Table 3: Relative miRNA expression profiles ranked by average delta-delta-CT value

miRNA	Min	Max	Average	SD
miRNA 1207-5p	-1.80	5.00	2.29	±1.79
miRNA 638	-1.40	5.20	1.84	±1.87
miRNA 206	-1.10	4.30	1.31	±1.46
miRNA 208a-3p	-2.00	4.10	0.83	±1.33
miRNA 539-5p	-2.20	3.30	0.65	±1.09
miRNA 20b-5p	-0.92	1.40	0.33	±0.46
miRNA 93-5p	-1.00	1.20	0.32	±0.46
miRNA 146b-5p	-1.10	1.30	0.30	±0.47
let7c5p	-2.20	2.10	0.29	±0.82
miRNA 30c-5p	-0.87	1.20	0.27	±0.42
miRNA 146b-3p	-1.60	1.30	0.25	±0.60
let7e5p	-1.60	1.60	0.23	±0.66
miRNA 133a-3p	-2.10	3.50	0.19	±1.05
miRNA 103a-3p	-0.96	1.20	0.19	±0.40
miRNA 125a-5p	-2.00	1.80	0.16	±0.84
miRNA 24-3p	-1.10	1.30	0.16	±0.48
miRNA 30b-5p	-1.00	0.93	0.16	±0.38
miRNA 221-3p	-1.00	1.40	0.14	±0.43
miRNA 22-5p	-1.50	1.40	0.14	±0.48
let7b5p	-2.10	2.40	0.12	±0.84
miRNA 16-5p	-1.30	1.40	0.12	±0.48
miRNA 451a	-2.00	2.40	0.11	±0.79
miRNA 452	-3.50	3.50	0.09	±1.54
miRNA 126-3p	-1.90	1.20	0.09	±0.50
miRNA 378a-3p	-1.80	1.40	0.07	±0.59
miRNA 191-5p	-1.30	1.10	0.06	±0.46
miRNA 15b-5p	-1.10	1.10	0.06	±0.39
miRNA 20a	-2.20	2.40	0.06	±0.98
miRNA 1-3p	-5.20	4.10	0.04	±1.83
miRNA 423-5p	-1.10	1.00	0.02	±0.34
miRNA 29a-3p	-2.50	2.30	0.02	±0.90

miRNA	Min	Max	Average	SD
miRNA 17-5p	-1.20	0.90	0.00	±0.39
miRNA 30a-5p	-1.00	0.86	-0.00	±0.44
miRNA 25-3p	-1.10	0.80	-0.07	±0.36
miRNA 92a-3p	-1.10	0.78	-0.01	±0.38
miRNA 320c	-0.86	0.82	-0.08	±0.38
let7a5pp	-2.10	1.30	-0.11	±0.80
miRNA 155-5p	-1.90	1.10	-0.12	±0.60
miRNA 223-3p	-1.60	0.80	-0.13	±0.43
miRNA 21-5p	-1.10	1.10	-0.17	±0.41
miRNA 15a-5p	-3.20	1.50	-0.18	±0.86
miRNA 185-5p	-2.00	1.10	-0.18	±0.49
miRNA 342-3p	-1.70	1.40	-0.24	±0.72
miRNA 125b-5p	-2.40	1.30	-0.24	±0.73
miRNA 320a	-1.30	0.86	-0.27	±0.44
miRNA 425-3p	-2.70	1.60	-0.28	±0.71
miRNA 29c-3p	-2.00	0.99	-0.30	±0.59
miRNA 27a-3p	-1.50	0.53	-0.31	±0.46
miRNA 26b-5p	-1.80	0.86	-0.44	±0.56
miRNA 22-3p	-1.70	0.59	-0.49	±0.49
miRNA 132-3p	-2.50	1.00	-0.64	±0.82
miRNA 140-3p	-5.00	1.30	-0.65	±1.04
miRNA 212-3p	-2.90	0.95	-0.66	±0.75
miRNA 31-5p	-7.10	3.10	-0.74	±1.79
miRNA 195-5p	-3.30	2.40	-0.78	±1.02
miRNA 29b-3p	-3.50	0.91	-1.00	±0.92
miRNA 23a-3p	-3.70	0.87	-1.06	±0.86
miRNA 199a-5p	-4.30	0.80	-1.08	±0.91
miRNA 23b-3p	-3.10	0.42	-1.24	±0.82
miRNA 133b-3p	-9.70	4.60	-1.29	±3.23
miRNA 886-3p	-5.30	3.40	-2.13	±1.88
miRNA 34a	-4.00	3.80	-3.79	±3.41

Relative miRNA expression profiles within the full cohort of 100 patients are demonstrated in table 3. They are ranked by relative abundance from top to bottom.

As shown in table 3, miRNAs 1207-5p, 638 and 206 were most abundant in the tested population whereas miRNAs 133b-3p, 886-3p and 34a were the least expressed. All 6 miRNAs demonstrate a very heterogenic expression (with a high standard deviation) so the high/low expression profiles are driven partly by statistical outliers.

Nine of the 10 previously described most common miRNAs in platelets (table 3) were also measured in our study (miRNAs 103a-3p, 24-3p, 16-5p, 126-3p, 191-5p, 17-5p, 223-3p, 15a-5p, 320a). However, none of them was among the higher expressed miRNAs. This points towards a difference in platelet miRNA expression in patients with CAD compared to healthy individuals, which were investigated in figure 3.

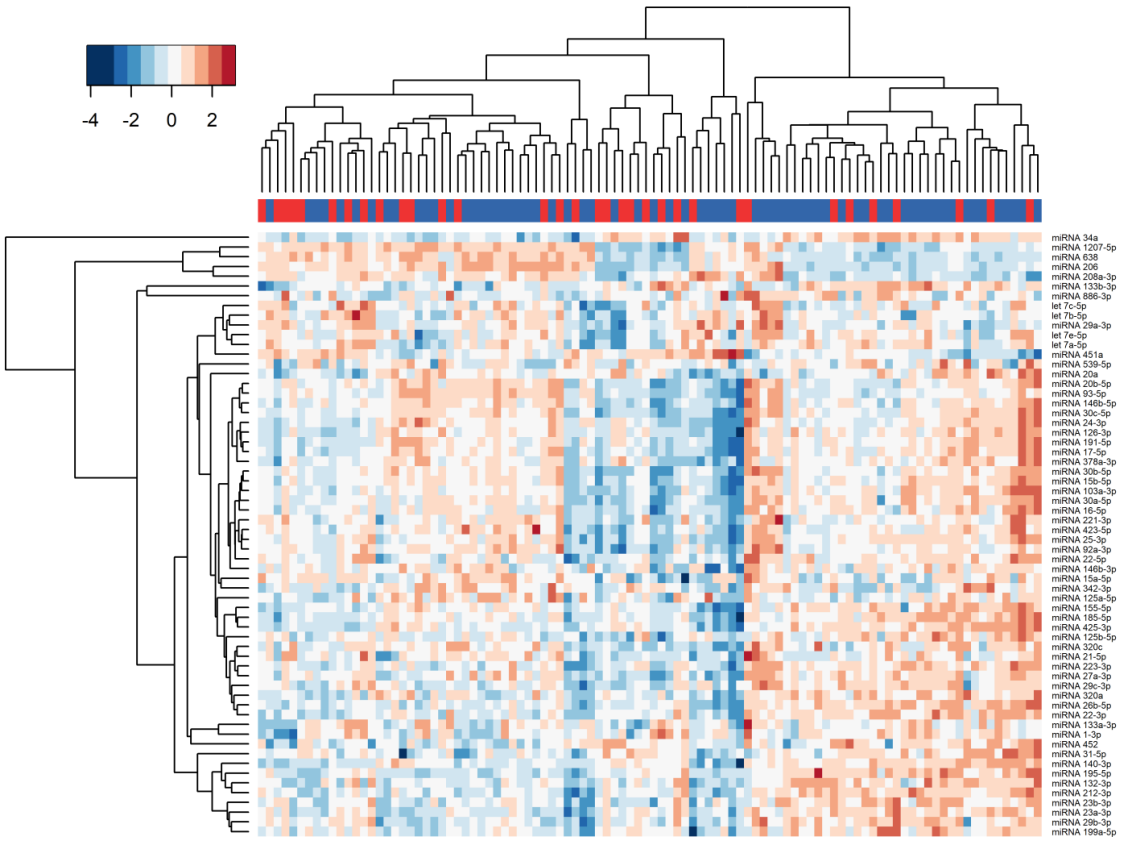

Figure 6: Heatmap of unsupervised hierarchical clustering of miRNAs

Next, unsupervised hierarchical clustering was performed and is shown in a heat map (figure 6). Colors represent miRNA expression according to delta-delta-CT values (see legend in the top left corner). Columns coded in red (row above heat map) indicate patients suffering from CCS, columns in blue, patients suffering from MI. Data is based on *z*-scores for the $\log_2$-transformed dataset. Hierarchical clustering was calculated using the Manhattan method as distance method and Ward's method as agglomeration method. The distribution of the 62 measured miRNAs within the patient collective appears to be rather heterogenic and no global trend in expression between CCS and MI patients could be observed.

4.3 Individual miRNA expression

To illustrate expression profiles of individual miRNAs between the two groups, volcano plots and box plots were generated. Analyses show a significantly lower expression of miRNA 103a-3p and 155-5p in MI compared to CCS patients (figures 7 and 8).

Since miRNAs are influenced by several factors like age, sex and others, we repeated the analyses with a subgroup of patients with matched CCS controls (MC) and MI (see baseline characteristics). The subgroup analyses were able to confirm the lower expression of miRNAs 103a-3p and 155-5p. Additionally, miRNAs 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p demonstrated lower expression in MI patients compared to the matched control population (see figures 9 and 10).

A Volcano plot comparing individual miRNA expression levels between CCS and MI patients is shown in figure 7. The Mann-Whitney U test was used to compare individual miRNA expression profiles. The x-axis presents fold change on a $\log_2$ scale (positive values represent a higher expression of miRNAs in CCS compared to MI), the y-axis presents statistical significance (blue dots above the green line ($\alpha = 0.05$) represent significantly differently expressed miRNAs). As mentioned above, miRNAs 103a-3p and 155-5p are lower expressed in MI patients compared to CCS.

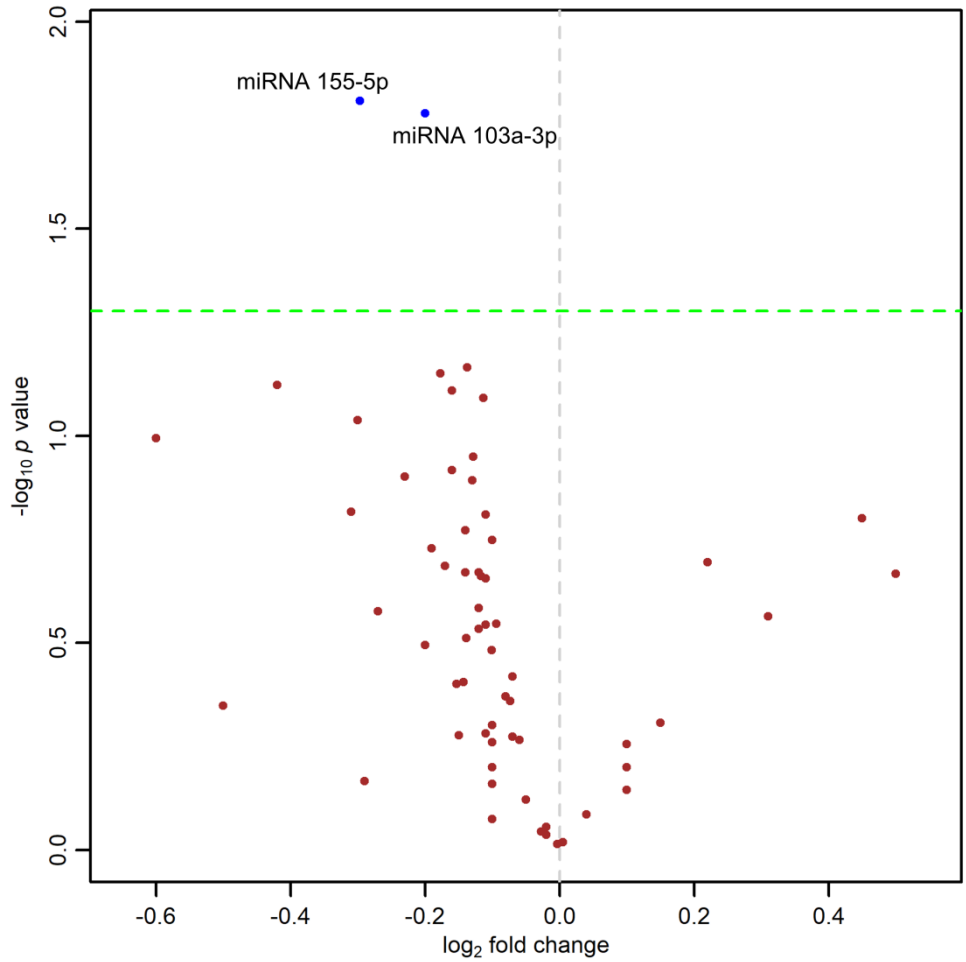

Figure 7: Comparing expression levels of individual miRNAs in patients with MI and CCS

Next, box plots were generated to compare expression of miRNA 103a-3p and miRNA 155 in MI and CCS. Corresponding *p* values and fold changes (fc) are indicated on top of the boxes. The y-axis indicates relative miRNA expression (delta-delta CT value), the white dots are miRNA measurements in individual patients. Even though the difference in expression between the two groups is significant, the margin (fold-change) is moderate (see figure 8).

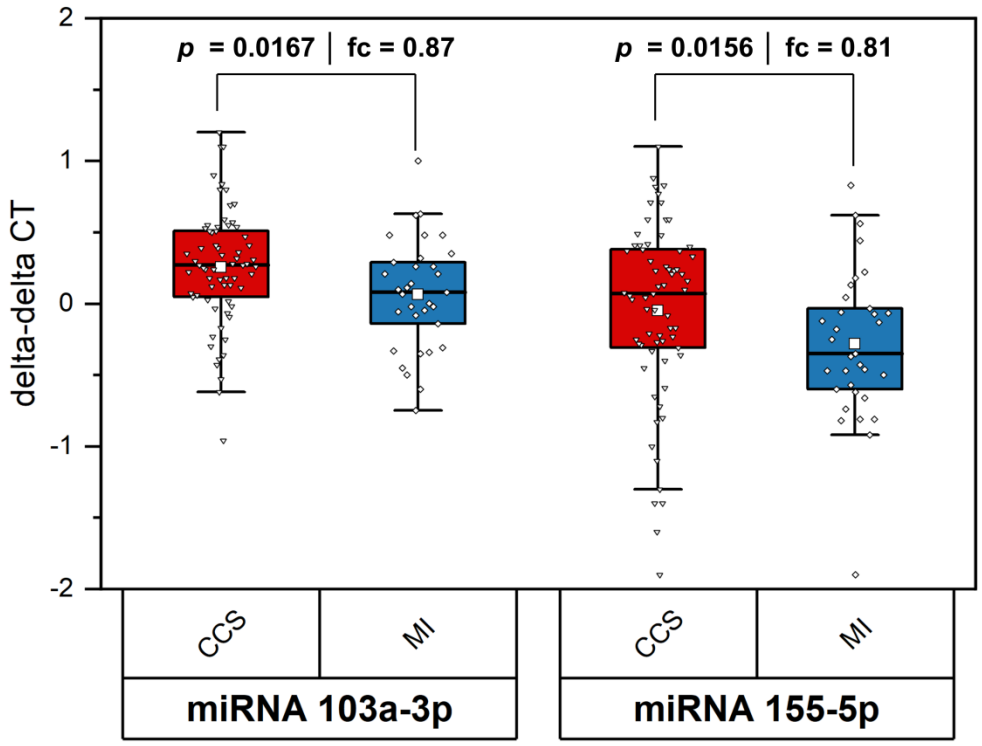

Figure 8: Box plots of miRNA 103-3p and 155-5p expression in patients with MI and CCS

As mentioned earlier, the analysis was repeated with a matched cohort in order to validate our findings. A Volcano plot comparing individual miRNA expression levels between MC (matched control) and MI patients is shown in figure 9. Because the data was matched, the Wilcoxon signed rank test was used to compare individual miRNA expression profiles. Similar to figure 7, the x-axis presents fold change on a $\log_2$ scale (positive values represent a higher expression of miRNAs in MC compared to MI), the y-axis presents statistical significance (blue dots above the green line ($\alpha = 0.05$) represent significantly differently expressed miRNAs). We were able to reproduce the results for miRNAs 103a-3p and 155-5p in this cohort. Additionally, miRNAs 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p showed lower expression in MI compared to the MC cohort.

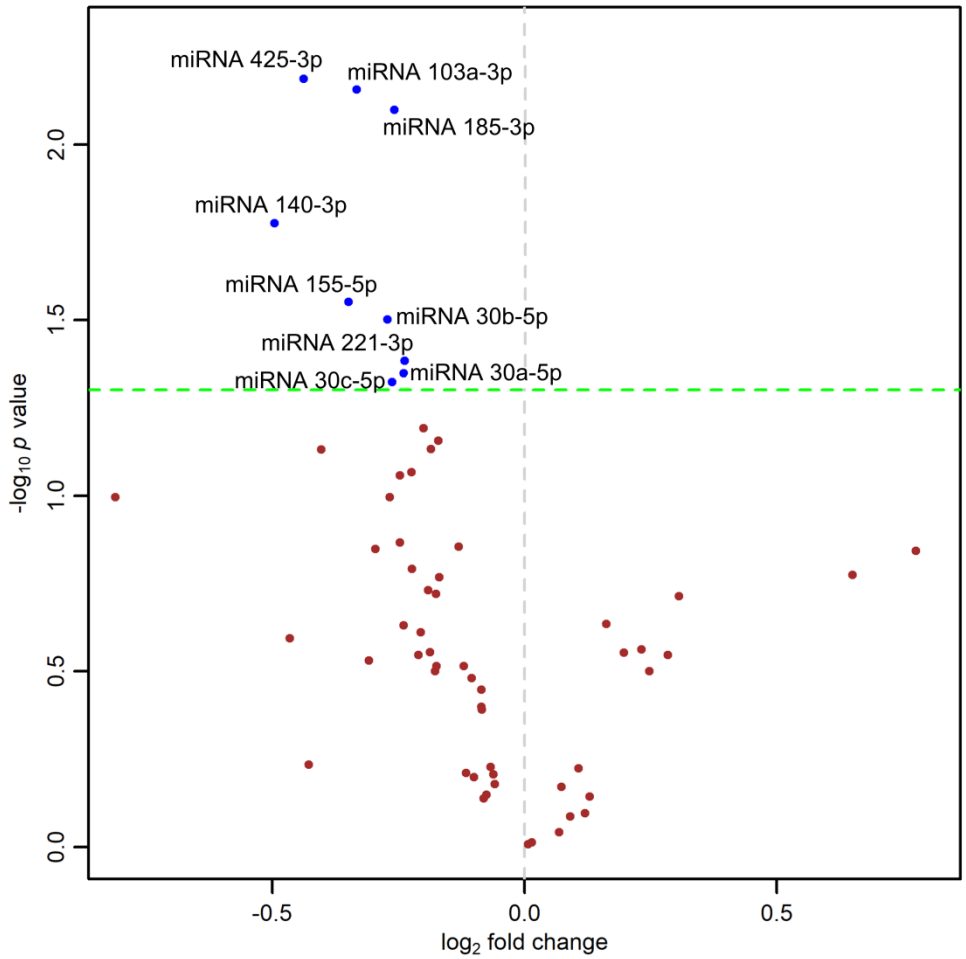

Figure 9: Comparing expression levels of individual miRNAs in patients with MI and MC

Similar to the analysis of the full cohort, box plots were generated to compare expression of the significantly differently regulated miRNAs in the MI and MC cohort (figure 10). Corresponding *p* values and fold changes (fc) are indicated on top of the boxes. The y-axis indicates relative miRNA expression (delta-delta CT value); the white dots are miRNA measurements in individual patients. The margin of change (fold-change) was similar to the observed fold changes in the full cohort. The level of significance varied with miRNA 425-3p showing the lowest *p* value. However, miRNA 425-3p demonstrated a rather broad distribution. Other highly significantly differently expressed miRNAs were miRNA 103a-3p and 185-3p.

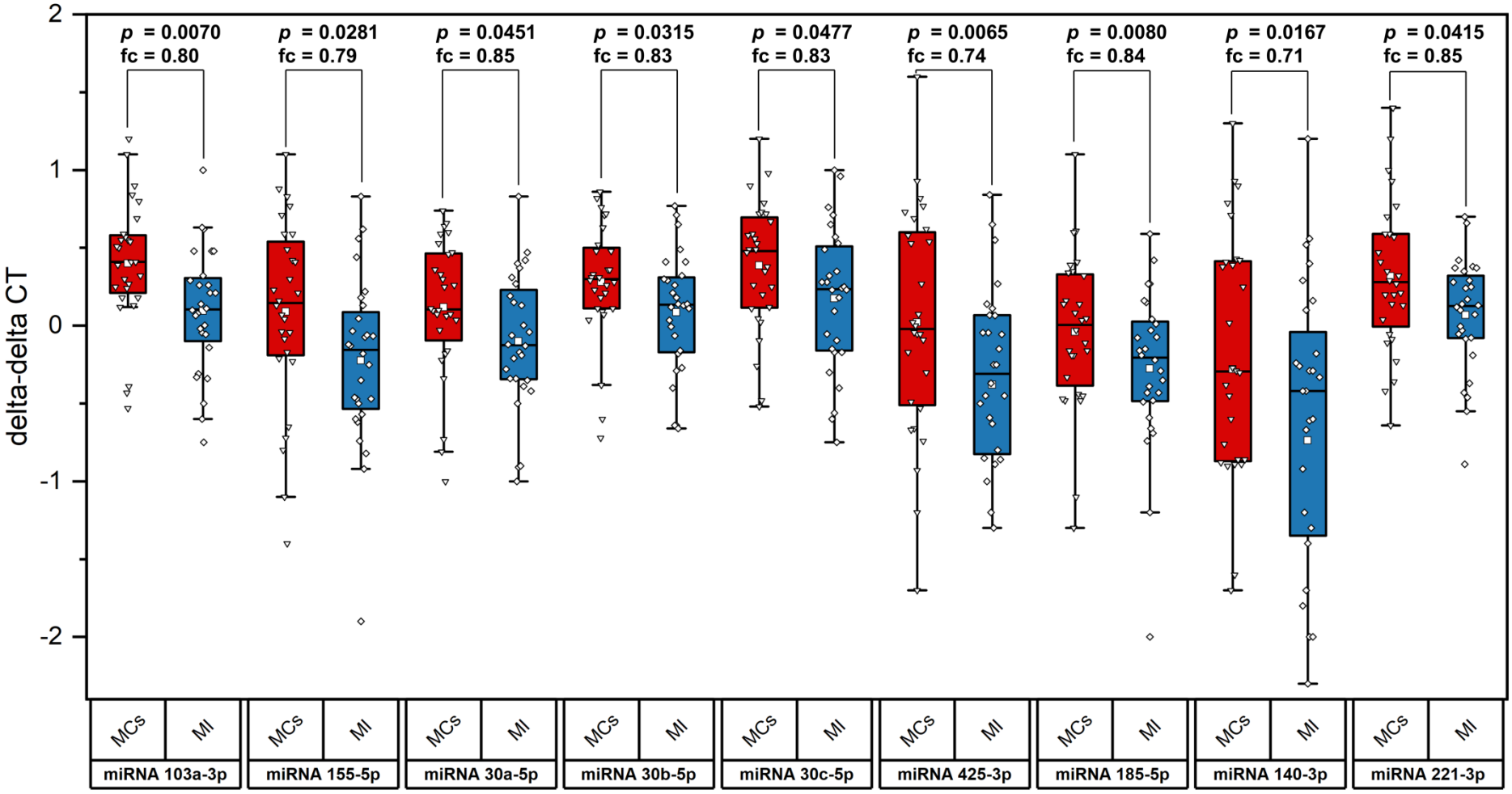

Figure 10: Box plots of miRNA 103-3p, 155-5p, 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p expression in patients with MI and MC

4.4 Changes in miRNA expression depending on course of LVEF%

We were able to follow-up LVEF% in 48 patients. Patients with improved or worsened LVEF% (Δ<1 or >1) over time were compared and individual miRNA expression was examined. Unfortunately, the follow up resulted in different group sizes (figure 11).

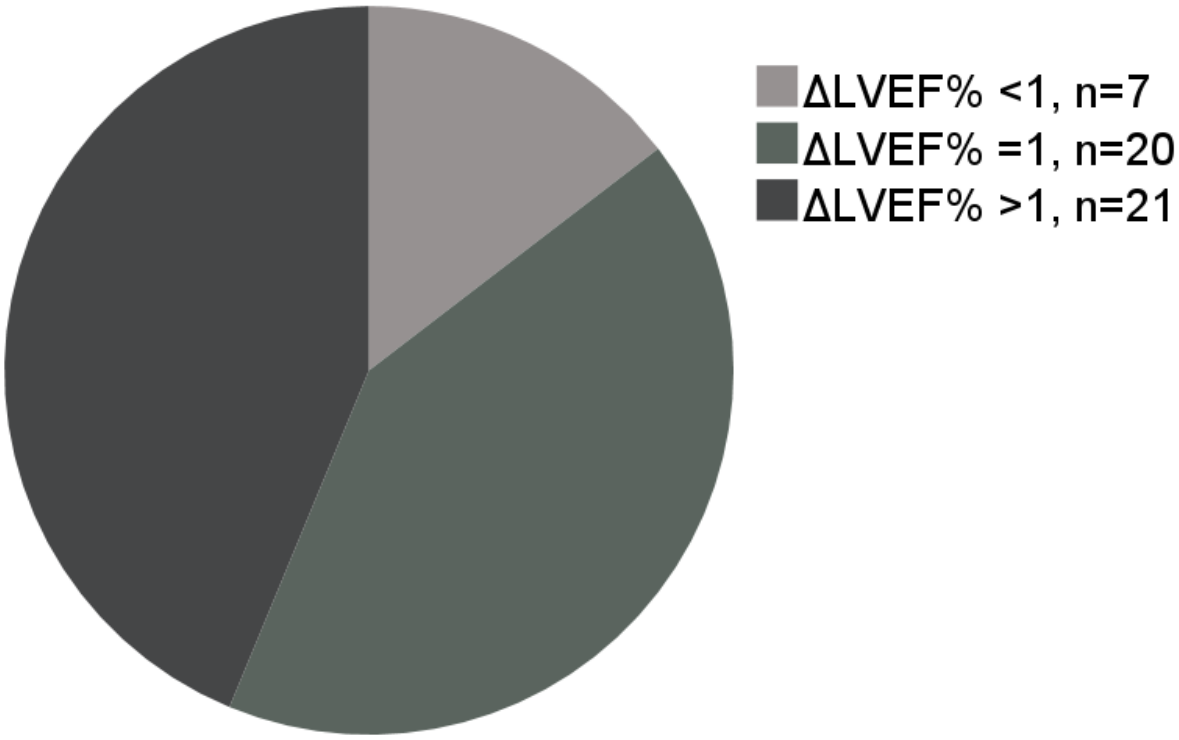

Figure 11: Pie chart of ΔLVEF% in 48 patients

Next, analyses of individual miRNA expression of patients with a ∆ LVEF% <1 and a ∆ LVEF% >1 using the Mann-Whitney U test were carried out. Similarly to the MI vs CCS and MI vs MC cohort, the results are demonstrated using a volcano plot (figure 11). The x-axis presents fold change on a log2 scale (positive values represent a higher expression of miRNAs in ∆ LVEF% <1 compared to ∆ LVEF% >1), the y-axis presents statistical significance (blue dots above the green line (α = 0.05) represent significantly differently expressed miRNAs).

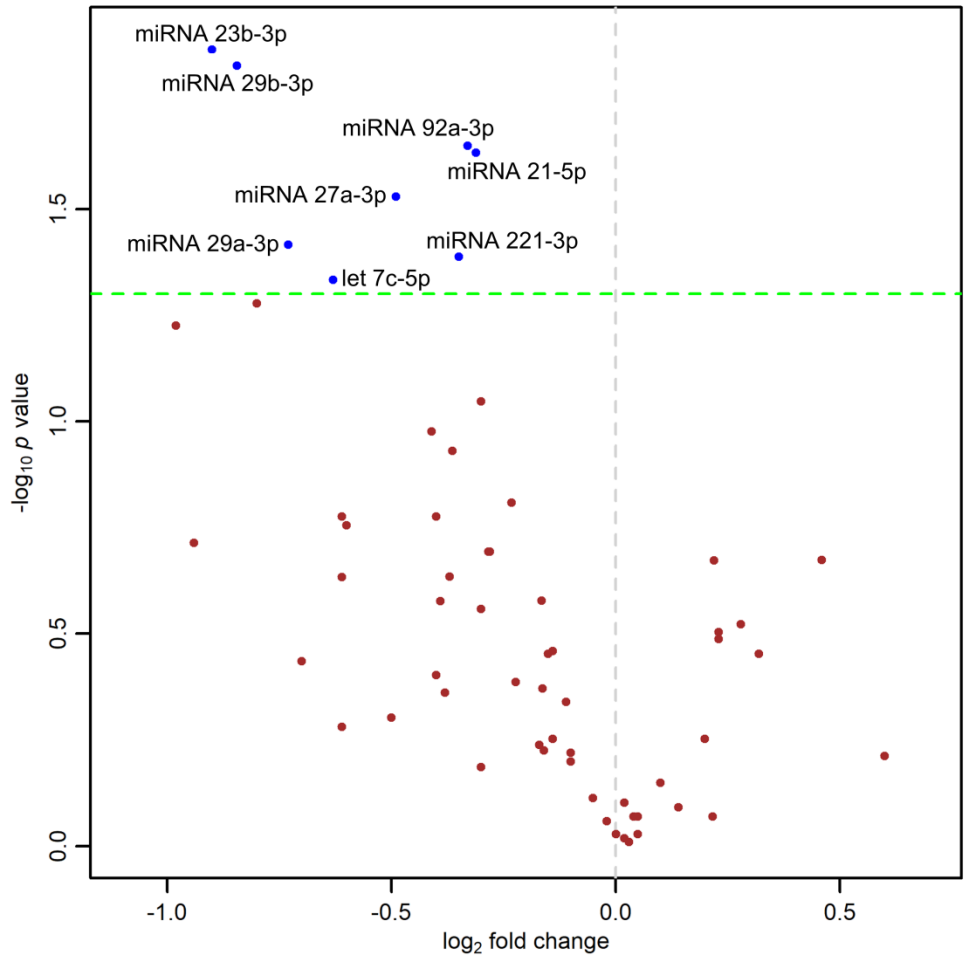

Figure 12: Comparing expression levels of individual miRNAs in patients with Δ LVEF% <1 and Δ LVEF% >1

As shown in figure 12, miRNAs 21-5p, 23b-3p, 27a-3p, 29a-3p, 29b-3p, 92a-3p, 221-3p and the miRNA precursor let7c-5p showed significantly lower expression profiles in the cohorts of worsened LVEF% when compared to improved LVEF%.

MiRNAs 23b-3p and 29b-3p demonstrated both the greatest margin of difference and the highest degree of significance. Boxplots of their expression in all three groups (Δ LVEF% <1, Δ LVEF% =1 and Δ LVEF% >1) are shown in figure 13.

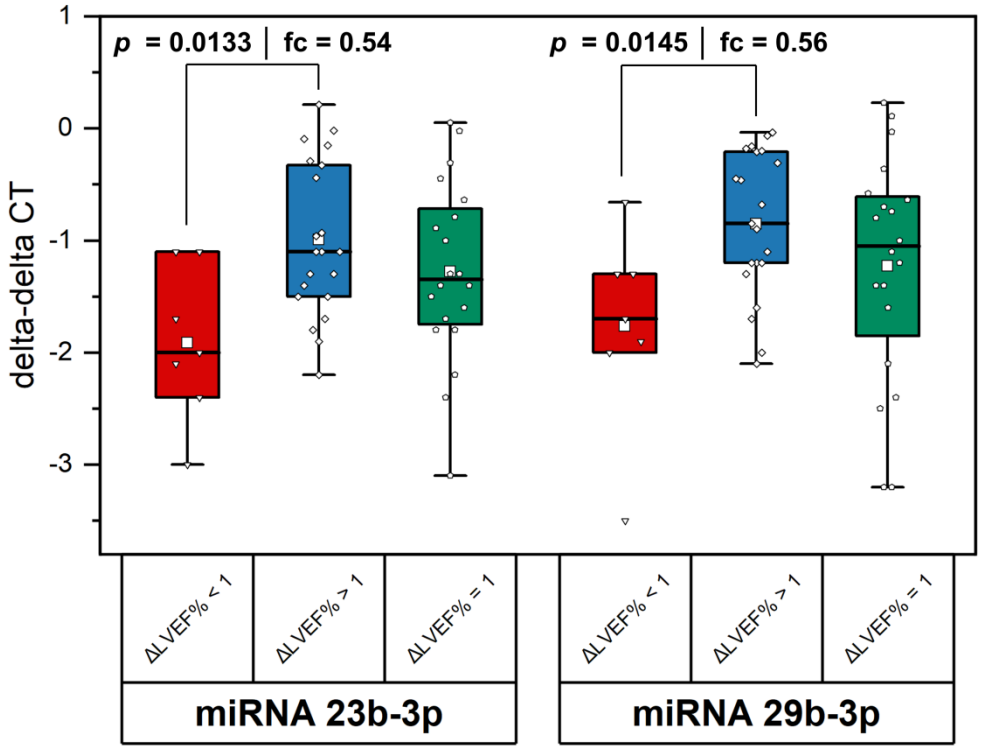

Figure 13: Comparing expression levels of miRNAs 23b-3p and 29b-3p in patients with Δ LVEF% <1, Δ LVEF% =1 and Δ LVEF% >1

Corresponding *p* values and fold changes (fc) are indicated on top of the boxes. The y-axis indicates relative miRNA expression (delta-delta CT value), the white dots are miRNA measurements in individual patients.

5 Discussion

In this observational cohort study, we compared platelet miRNA expression profiles of 62 selected miRNAs in 33 patients with MI and 67 patients with CCS. The major findings of the present study are:

(1) MiRNAs 103a-3p and 155-5p showed a lower expression in platelets in MI patients.

(2) MiRNAs 103a-3p and 155-5p showed a significantly lower expression in patients with MI compared to a matched CCS cohort.

(3) Abundance of miRNA 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p was significantly lower in MI patients compared to the matched CCS cohort.

(4) MiRNAs 21-5p, 23b-3p, 27a-3p, 29a-3p, 29b-3p, 92a-3p, 221-3p and the miRNA precursor let7c-5p showed significantly lower expression profiles in the cohort with worsened LVEF% when compared to the cohort with improved LVEF% over time.

Suggested cardiovascular effects for miRNA 103 and 155 are shown in Table 4. MiRNA 103 is believed to play a role in cardiomyocyte necrosis and atherosclerosis (91, 92). Wang et al. reported cardiomyocyte necrosis induced by miRNA 103 via activation of FADD (Fas-Associating Death Domain-Containing Protein). Hartmann and colleagues found miRNA 103 mediated endothelial inflammation and atherosclerosis by suppression of KLF4 (Kruppel-like factor 4). However, miRNA 103 might also exhibit cardioprotective properties. Chen et al. reported angiogenic effects by inhibition of suppression of VEGF mRNA and Wang et al. described miRNA 103 mediated autophagy in CAD after H2O2-induced oxidative stress (93, 94).

MiRNA 155 has been demonstrated to promote atherosclerosis via activation of endothelial cells and might inhibit megakaryopoiesis (95, 96). However, clinical data on miRNA 155 is contradictory. Downregulation in peripheral blood mononuclear cells (PBMCs) of ACS compared to CCS patients and downregulation in plasma of individuals suffering from CAD compared to healthy controls have been reported (37, 42, 97). Other data suggests, that plasma upregulation of miRNA 155 is associated with coronary slow flow (98).

Table 4: Differently expressed miRNAs between MI and CCS and known cardiovascular effects

miRNA	Cardiovascular effects	Pathway (if known)	Authors
miRNA 103	Promotes cardiomyocyte necrosis through FADD activation	FADD activation	Wang et al. Circ Res. 2015 (91)
	Endothelial Cell activation induces endothelial inflam-mation and atherosclerosis	Suppression of KLF4	Hartmann et al. Nat. Commun. 2015 (92)
	Mediates hypoxia induced angiogenesis	Desuppression of VEGF mRNA	Chen et al. J. Clin. Invest. 2013 (94)
	Promotes end-stage auto-phagy in coronary artery endothelial cells after H2O2-Induced oxidative stress	Inhibition of BNIP3	Wang et al. Oxid. Med. Cell. Longev. 2020 (93)
miRNA 155	Associated with inhibition of murine megakaryopoiesis		Georgantas et al. Proc. Natl. Acad. Sci. U S A. 2007 (95)
	Promotes atherosclerosis via endothelial cell activation		Virtue et al. J. Biol. Chem. 2017 (96)
	Downregulation in plasma of CAD patients compared to healthy controls		Fichtlscherer et al. Circ Res. 2010 (42)
	Downregulation in PBMCs of ACS patients compared to stable CAD		Yao et al. Cell. Mol. Immunol. 2011(37)
	Upregulation in plasma associated with coronary slow flow		Su et al. Dis Markers. 2018 (98)
	Low plasma levels associated with CAD		Jia et al. Cell Physiol Biochem. 2017 (97)

Suggested cardiovascular effects for miRNA 30, 140, 185, 221 and 425 are shown in Table 5. Ceolotto and colleagues report promotion of early atherosclerosis by miRNA 30 via inhibition of oxLDL mediated macrophage IL-1b release, caspase-3 expression, and apoptosis (99). Furthermore, mediation of hypoxia induced cardiomyocyte apoptosis by upregulation of cell death regulator Protein Aven has been reported for miRNA 30 (100). This might explain clinical findings of miRNA 30 upregulation in whole blood of STEMI patients compared to healthy controls (101). However, those findings are not reproducible in other cellular compartments. Woo et al. report lower miRNA 30 expression in vascular smooth muscle cells in MI patients compared to non-MI patients and hypothesize regulatory functions in smooth muscle cell differentiation via downregulation of RNA splicing protein muscleblind like

splicing regulator 1 (102). In contrast, downregulation of miRNA 30 in CAD patients compared to controls and reduction of murine arterial occlusion via downregulation of plasminogen activator inhibitor-1 have been reported (51, 67).

In the mouse model, upregulation of miRNA 140 increased oxidative stress and reactive oxygen species levels by suppression the transcription factor nuclear factor erythroid 2-related factor 2 and enzyme sirtuin 2 (103). Clinical data suggests an upregulation of circulating miRNA 140 in ACS patients. High miRNA 140 levels are furthermore associated with an increased mortality risk. Li and colleagues suggested circulating endothelial cells and monocytes as a likely source for circulating miRNA 140 (104, 105). Fejes et al. looked specifically into platelet miRNA 140. They found reduced levels in platelets of patients suffering from diabetes which resulted in upregulation of P2Y12 receptor and SELP mRNAs (which induces translation of p-selectin). This could contribute to the altered platelet function in this patient collective (66).

Gidlöf et al. investigated miRNA 185 in platelets of ACS patients. They found lower expression levels in patients with ACS compared to healthy controls. Additionally, they described an extracellular shift upon platelet activation and uptake by endothelial cells (71). Interestingly, treatment with extracellular vesicles containing miRNA 185 promotes atherosclerosis in mice by inhibition of the protein mothers against decapentaplegic homolog 7 (SMAD 7).

Upregulation of miRNA 221 in several cellular compartments has been described by several authors. Coskunpinar et al. reported upregulation in plasma of ACS patients compared to controls with an inverse correlation of miRNA 221 levels to LVEF (106). Other authors described higher levels in endothelial progenitor cells in patients suffering from CAD compared to controls, upregulated miRNA 221 in platelets of STEMI compared to NSTEMI patients and increased plasma levels in patients suffering from pulmonary embolism (84, 107, 108). Conversely, Jia and colleagues reported decreased plasma levels of miRNA-221 in patients suffering from CAD compared to controls (97). Interestingly, Peng et al. were able to use platelet miRNA 221 levels to distinguish between clopidogrel responding and non-responding ACS patients

(78). Furthermore, plasma levels of miRNA 221 may distinguish between heart failure patients with reduced and preserved ejection fraction (109).

Li et al. recently reported on inhibition of myocardial inflammation and cardiomyocyte apoptosis in mice by miRNA 425 by suppression of cytokine transforming growth factor β (TGF-β) and proteins p-smad2 and p-smad3 (110). Furthermore, lower expression of exosomal miRNA 425 in heart failure patients was described by Wang et al. This resulted in suppression of collagen 1, actin protein α-SMA and cytokine TGF-β1 (111). Paradoxically, higher levels of circulating miRNA 425 were reported in patients suffering from diabetes. This led to downregulation of monocarboxylate transporter 4 and apoptosis of endothelial cells (112).

Table 5: Differently expressed miRNAs between MI and MC and known cardiovascular effects

miRNA	Cardiovascular effects	Pathway (if known)	Authors
miRNA 30	Reduction of murine arterial occlusion (after induced arterial thrombosis)	Downregulation of PAI-1	Luo et al. Sci Rep. 2016 (67)
	Upregulation in whole blood of STEMI patients compared to healthy controls		Meder et al. Basic Res Cardiol. 2011 (101)
	Downregulation in whole blood of patients with CAD compared to healthy controls		Weber et al. Cardiology Research and Practice. 2011 (51)
	Prevention of aortic valve calcification	Suppression of Runx2, Smad1, and caspase 3	Zhang et al. J. Thorac. Cardiovasc. Surg. 2014 (113)
	Mediation of hypoxia induced cardiomyocyte apoptosis	Upregulation of Aven	Zhang et al. Cell. Mol. Biol. Lett. 2019 (100)
	Reduction in microparticles promotes early atherosclerosis	Inhibits macrophage IL-1b release, caspase-3 expression and apoptosis.	Ceolotto et al. Cardiovasc. Res. 2017 (99)
	Lower expression in vascular smooth muscle cells in MI compared to non-MI patients	Downregulation of MBNL1 in VSMCs	Woo et al. Int. J. Mol. Sci. 2020 (102)
	Inhibits capillary morphogenesis	Stimulation of TGF-β2.	Howe et al. Plos One 2017 (114)
miRNA 140	High serum expression associated with high mortality in ACS patients		Karakas et al. Eur Heart J. 2017 (105)

	Upregulation increases reactive oxygen species levels in mice	Suppression of nuclear factor erythroid 2-related factor 2 (Nrf2) and sirtuin 2 (Sirt2)	Liu et al. Int. J. Mol. Med. 2017 (103)
	Upregulated in ACS patients. Originating mostly from circulating endothelial cells and monocytes		Li et al. Plos One. 2017 (104)
	Reduced in platelet of patients with diabetes	Low levels cause Upregulation of P2-RY12 and SELP mRNAs	Fejes et al. Thromb. Haemost. 2017 (66)
miRNA 185	Lower expression in platelets of patients with ACS compared to controls followed by extracellular shift and uptake by endothelial cells		Gidlöf et al. Blood. 2013 (71)
	Treatment with extracellular vesicles containing miRNA 185 promote atherosclerosis in mice	Inhibition of Smad7	Li et al. Int. Immunopharmacol. 2021 (115)
	Inhibition of Angiotensin II-mediated proliferation of Human Aortic Vascular Smooth Muscle Cell	Downregulation of P2Y6 protein	Wang et al. DNA Cell Biol. 2017 (116)
miRNA 221	Upregulated in Clopidogrel high responsive ACS patients		Peng et al. Thromb. Res. 2017 (78)
	Upregulation in plasma of ACS patients compared to controls, inversely correlated with LVEF		Coskunpinar et al. Gene. 2016 (106)
	Decreased plasma levels in CAD compared to controls		Jia et al. Cell. Physiol. Biochem.2017 (97)
	Higher levels in endothelial progenitor cells in CAD compared to controls. Lowering effect with atorvastatin		Minami et al. Eur. J. Clin. Invest. 2009 (107)
	Increased plasma levels in patients suffering from pulmonary embolism		Liu et al. Med. Sci. Monit. 2018 (108)
	Upregulated in platelets of STEMI patients compared to NSTEMI patients		Ward et al. Fam Med Med Sci Res. 2013 (84)
	Decreased in plasma of patients with systolic heart failure compared to healthy controls. Able to differentiate between heart failure with reduced and preserved LVEF%		Watson et al. Eur J Heart Fail. 2015 (109)
miRNA 425	Lower expression in plasma of ACS patients with colchicine compared to		Barraclough et al. J. Cardiovas. Pharmacol. Ther. 2020 (117)

	colchicine naïve ACS patients		
	Inhibition of myocardial inflammation and cardiomyocyte apoptosis in mice	Suppression of TGF-β1, p-smad2 and p-smad3	Li et al. Immun. Inflamm. Dis. 2020 (110)
	Lower expression in exosomes of heart failure patients	Suppression of collagen 1, a-SMA and TGFb1	Wang et al. Kaohsiung J Med Sci. 2018 (111)
	Upregulation in patients suffering from diabetes	MCT4 downregulation and apoptosis of endothelial cells	Luo et al. Mol. Cell. Endocrinol. 2020 (112)

Suggested cardiovascular effects of miRNA 21, 23, 27, 29, 92 and the miRNA precursor let-7 are shown in Table 6. MiRNA 21 has been linked to heart failure. Thum et al. have reported elevated miRNA 21 levels in fibroblasts of the failing heart where it promotes fibrosis and heart failure via activation of ERK–MAP kinase activity (39). However, circulating miRNA 21 also seems to promote fibrosis via TGF-β1 platelet release and Inhibition of Wiskott-Aldrich syndrome protein (69). Clinically, Zhang et al. were able to demonstrate increased plasma levels of miRNA 21 in patients with heart failure (118). It seems like platelets are a source for circulating miRNA 21. Tan et al. reported murine miRNA 21 platelet excretion upon activation and subsequent inhibition of SMC proliferation via inhibition of platelet-derived growth factor receptor beta (73). Plasma expression levels of miRNA 21 are increased in ACS compared to CCS patients and return to baseline at 90 days after the index event. On the other hand, a decrease of miRNA 21 in platelets of patients with STEMI compared to healthy controls was observed (85, 119, 120). Similarly to miRNA 221, upregulation in clopidogrel responding compared to clopidogrel non-responding ACS patients has also been reported for miRNA 21 (78).

Compared to miRNA 21, data on cardiovascular effects of miRNA 23 is relatively sparse. MiRNA 23 has been reported to enhance the oxLDL-induced inflammatory response of macrophages via activation of the NF-κB signaling pathway, which could promote atherosclerosis (121). Furthermore, van Rooij et al. described an upregulated of miRNA 23 in cardiomyocytes after inducing hypertrophy in mice (122).

MiRNA 27 seems to exhibit regulatory functions within platelets. Miao and colleagues reported enhancement of de novo synthesis of thrombospondin-1 after platelet activation via thrombin (68). In a clinical setting, miRNA-27 demonstrated higher expression in PBMCs of patients with CAD compared to controls. MiRNA 27 levels correlate with forkhead box protein O1 levels, a transcription factor linked to sugar metabolism and adipogenesis (123). Furthermore, a lower abundance of miRNA 27 in plasma of patients with acute heart failure was reported (124).

MiRNA 29 is linked to ventricular hypertrophy. Roncarati et al. found a correlation between miRNA 29 plasma levels and fibrosis and hypertrophy in patients with HCM when compared to healthy controls (125). Also compared to healthy controls, Huang et al. described an association of plasma levels with hypertension and left ventricular hypertrophy in a collective of patients suffering from essential hypertension (126). Furthermore, a downregulation of miRNA 29 in whole blood of CAD patients compared to healthy individuals was reported by Weber et al. (51).

Marfella et al. investigated changes in miRNA levels in patients with chronic heart failure before and after successful cardiac resynchronization therapy. They demonstrated an initial decrease of miRNA 29 and 92 plasma levels which rose after successful CRT implantation. Those changes in plasma miRNA levels could predict improvement of LV function after resynchronization therapy (127).

Other data on miRNA 92 is mostly clinical. Binderup et al. demonstrated that low miRNA 92 plasma levels are associated with low platelet response to ASA (128). Associations of miRNA 92 with CAD and diabetes were reported by several authors. Wang et al. showed higher prevalence in plasma derived exosomes of CAD patients compared to healthy controls as well as an upregulation in plasma of patients with diabetes and ACS compared to ACS patients without diabetes (129, 130). Cheng and colleagues reported an association with CAD and linked miRNA 92 to regulation of the genes GATA2, MAP1B and ARG1 (131).

Finally, there is little data on the direct role of miRNA precursor let-7 in the cardiovascular system. High serum concentrations were linked to low risk of

fatal MI by Bye and colleagues (132). Furthermore, Brennan et al. reported anti-atherosclerotic properties as well as inhibition of proliferation and monocyte adhesion in smooth muscle cells via inhibition of nuclear factor-kB (133).

Table 6: Differently expressed miRNAs between ΔLVEF and known cardiovascular effects

miRNA	Cardiovascular effects	Pathway (if known)	Authors
miRNA 21	Excreted in murine platelet exosomes upon activation and inhibits SMC prolife-ration	Inhibition of PD-GFRβ	Tan et al. Cell. Physiol. Biochem.2016 (73)
	Increased in fibroblasts of the failing heart promoting fibrosis and heart failure	Promotion of ERK–MAP kinase activity	Thum et al. Nature. 2008 (39)
	Circulating miRNA promotes fibrosis via TGF-β1platelet release	Inhibition of Wiskott-Aldrich syndrome protein	Barwari et al. JCI Insight. 2018 (69)
	Upregulated in clopidogrel high responsive ACS patients		Peng et al. Thromb. Res. 2017 (78)
	Higher plasma levels 5 days post myocardial infarction, return to baseline at day 90		Zile et al. Circ Cardiovasc Genet. 2011 (119)
	Increased plasma ex-pression in ACS compared to CCS	Correlation with matrix metallopro-teinase-9	Darabi et al. Mol Cell Biochem. 2016 (120)
	Decreased in platelets of patients with STEMI compared to healthy controls		Li et al. J Thromb Thrombolysis. 2017 (85)
	High serum levels are associated with systolic heart failure		Zhang et al. Mol Med Rep. 2017 (118)
miRNA 23	Enhances oxLDL-induced inflammatory response of macrophages	Promotion of the A20/NF-κB signaling pathway	He et al. Biosci Rep. 2018 (121)
	Upregulated in cardiomyo-cyte after inducing hyper-trophy in mice		Van Rooij et al. Proc Natl Acad Sci U S A. 2006 (122)
miRNA 27	Reduced levels in platelets after stimulation with throm-bin	Enhancement of de novo synthesis of thrombospondin-1	Miao et al. J Thromb Haemost. 2018 (68) .
	Increased in PBMC of patients with CAD	Correlation with FOXO1	Babaee et al. Life Sci. 2020 (123)
	Decreased in plasma of patients with acute heart failure		Ovchinnikova et al. Eur J Heart Fail. 2016 (124)
miRNA 29	Plasma levels are decreased in chronic heart failure but increase after successful		Marfella et al. Eur J Heart Fail. 2013 (127)

	treatment with a cardiac resynchronization device		
	Downregulation in whole blood of CAD patients compared to healthy controls		Weber et al. Cardiol Res Pract. 2011 (51)
	Correlation with fibrosis and hypertrophy in plasma of patients with HCM compared to healthy controls		Roncarati et al. J Am Coll Cardiol. 2013 (125)
	Association of plasma levels with hypertension and left ventricular hypertrophy		Huang et al. Clin Exp Hypertens. 2017 (126)
miRNA 92	Lower plasma levels associated with low platelet ASA response		Binderup et al. Clin Biochem. 2016 (128)
	Circulating levels show association with CAD	Regulation of genes GATA2, MAP1B and ARG1	Cheng et al. Gene. 2017 (131)
	Upregulation in plasma of patients with diabetes and ACS compared to ACS patients without diabetes and controls		Wang et al. Lipids Health Dis. 2019 (129)
	Higher prevalence in plasma derived exosomes of CAD patients compared to healthy controls	Targeting ATP binding cassette (ABC)A1	Wang et al. Mol Med Rep. 2019 (130)
	Plasma levels are decreased in chronic heart failure but increase after successful treatment with a cardiac resynchronization device		Marfella et al. Eur J Heart Fail. 2013 (127)
Let-7	High serum concentration associated with low risk of fatal MI		Bye et al. J Mol Cell Cardiol. 2016 (132)
	Inhibits atherosclerotic in-flammatory response	Inhibition of nuclear factor-kB	Brennan et al. Diabetes. 2017 (133)

Even though numerous studies have investigated cardiovascular effects of the differently expressed miRNAs found in our study, the direct regulatory functions and properties, especially within platelets, mostly remain unknown. Clinical studies show contradictory results. Therefore, it is difficult to interpret the results of our observatory study. As mentioned previously, the lack of a cell nucleus in platelets and their inability to execute transcription render them prone to rely on miRNA to regulate protein synthesis (134). Data shows that platelet activation promotes exosome and microparticle depended release of miRNA. Paracrine effects in smooth muscle cells, macrophages and endothelial cells have been observed (24, 26, 70-73). Similar to our study, Gidlöf et al. found a lower

platelet expression of miRNA-185 in patients with ACS compared to healthy controls. Interestingly, they described an extracellular shift upon platelet activation and uptake by endothelial cells. Those paracrine effects could explain our finding of lower abundance of pro-atherosclerotic miRNAs in platelets of patients suffering from MI. Platelets could potentially serve as a source for circulatory miRNA 103a-3p and 155-5p which is released upon activation and mediates atherosclerosis and cardiomyocyte necrosis through the mechanisms described in the literature. The differently expressed miRNAs between the MI and MC cohort (miRNA 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p) might also become excreted from platelets upon activation in patients suffering from MI.

However, this hypothesis is highly speculative since we did not measure miRNA levels in plasma or serum and warrants further research.

Additionally, we investigated possible associations of platelet miRNAs with regeneration of LVEF% after MI and in patients suffering from CCS. We found 8 miRNAs with higher abundance in individuals with an improvement of LVEF% after the initial admission. Multiple studies investigate the pathophysiologic and prognostic role of circulating miRNAs both in acute and chronic systolic heart failure (135). To our knowledge, this is the first study to investigate platelet miRNAs as a possible prognostic factor for course of systolic heart failure. Several of the differently expressed miRNAs in the current study have already been associated with heart failure or fibrosis. As mentioned previously, miRNAs 221, 21, 23, 92 and 29 have been linked to either acute heart failure, chronic heart failure, cardiac fibrosis or cardiac hypertrophy. Surprisingly, prior research links the aforementioned miRNAs to worsening of heart failure and fibrosis (with the exception of the study of Marfella et al. (127)). However, in our collective, high miRNA levels were associated with improvement of LVEF. This might possibly be a consequence due to the different compartments analyzed since our study was the only one measuring miRNAs in platelets. This is again speculative since we did not measure corresponding miRNA plasma or serum levels.

Because of our study design, we cannot make any assumptions about the pathophysiological rationale for the shifts in expression of certain miRNAs depending on course of LVEF%. However, a reliable prognostic marker for regeneration of systolic LV function would be highly desirable and further research is warranted to confirm the current findings.

6 Conclusion

MiRNA 103a-3p and miRNA 155-5p demonstrate lower expression levels in patients with MI compared to CCS. In addition to miRNA 103a-3p and miRNA 155-5p, miRNAs 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p and 425-3p were reduced when comparing MI patients to MC. Finally, 8 differently expressed miRNAs in patients with improved LVEF% compared to worsened LVEF% over time were identified, serving as a potential prognostic marker in high risk collectives suffering from systolic heart failure.

7 Limitations

Our study has several limitations. Due to the observational nature of the study, we cannot adjust for all confounders since miRNA expression is influenced by multiple factors like cardiovascular risk, medication and several others.

Furthermore, our study collective is heterogeneous. Even though all patients suffered from invasively diagnosed CAD, not all individuals received PCI (percutaneous coronary intervention). Also, the MI cohort consisted of both patients with type I and II MI.

Due to the limited follow up data, the sample size for investigating effects on LVEF% is small. In order to maximize the sample size, individuals with varying follow-up duration were included into the study. Finally, LVEF% on admission varied considerably.

8 Deutsche Zusammenfassung

Die koronare Herzerkrankung (KHK) ist statistisch gesehen nach wie vor die häufigste Todesursache weltweit. Sie verursacht jährlich über 7 Millionen Todesfälle und wird durch atherosklerotische Veränderung und Stenosierung beziehungsweise Verschluss von Koronargefäßen verursacht (1, 3). Eine Einteilung kann je nach Ausprägung und Pathophysiologie der Erkrankung in das akute Koronarsyndrom (ACS) und das chronische/stabile Koronarsyndrom (CCS) erfolgen. Eine weitere Subgruppe des ACS stellt der akute Myokardinfarkt dar. Dieser ist durch eine akute Myokardschädigung charakterisiert (4, 8, 9).

MircoRNAs (miRNAs) sind kurze RNA Fragmente, welche wichtige Rollen in der Genregulation von Pflanzen und Tieren übernehmen (22). Sie besitzen eine Länge von circa 22 Nukleotiden und kodieren selbst keine Proteintranslation. Ihre genregulatorischen Effekte kommen über die Bindung, Inhibierung und Degeneration von messenger RNA (mRNA) über so genannte Argonautproteine zu Stande (24).

Die Rolle von miRNAs in der Pathogenese der Atherosklerose und somit der Entstehung der KHK ist bekannt (27). Mehrere Autoren konnten bereits unterschiedliche Expressionsmuster von miRNAs im Plasma sowie im Serum von Patienten mit unterschiedlichen Ausprägungen der KHK nachweisen (29).

Thrombozyten spielen eine zentrale Rolle in der Pathophysiologie der KHK. Da sie keinen Zellkern besitzen, ist die Transkription nicht möglich. Trotzdem enthalten Thrombozyten große Mengen an mRNAs. Dies erlaubt ihnen eine Translation und Proteinsynthese (30, 52). Da die regulatorische Möglichkeit der Transkription entfällt, liegt eine Regulation durch miRNAs nahe. Dennoch gibt es aktuell nur wenige Studien, die das Expressionsprofil von miRNAs in Thrombozyten von Patienten mit KHK vergleichen.

Nach ausführlicher Literaturrecherche erfolgte der Studieneinschluss von 100 Patienten, welche sich notfallmäßig oder elektiv in der Abteilung für Kardiologie der Universitätsklinik Tübingen zur Koronarangiographie vorstellten.

Einschlusskriterien waren ein Alter über 18 Jahren sowie eine invasiv (mittels Koronarangiographie) diagnostizierte KHK. Bei 33 Patienten wurde ein akuter Myokardinfarkt diagnostiziert (MI), 67 wurden als chronisches Koronarsyndrom klassifiziert (CCS) (anhand der entsprechenden ESC Leitlinien) (8, 9). 60ml Blut wurden periinterventionell abgenommen. Eine sofortige Isolation und Lagerung der Thrombozyten bei -80° Celsius erfolgte. Die Studie entspricht der Helsinki Deklaration sowie den Leitlinien für Gute klinische Praxis. Eine Zustimmung der verantwortlichen Ethik Kommission (270/2011B01) sowie (238/2018BO2) wurde eingeholt.

MiRNA Isolation sowie Messungen wurden in Kooperation mit Professor Matthias Schwab, Dr. Stefan Winter und Dr. Elke Schaeffeler am Dr. Margarete Fischer-Bosch Institut für klinische Pharmakologie (Auerbachstraße 112, 70376 Stuttgart, Deutschland) mittels quantitativer Echtzeit-PCR durchgeführt. Die Expression von 62 miRNAs mit aus der Literatur bekannter physiologischer oder pathophysiologischer Funktion im Herz-Kreislaufsystem wurden gemessen.

Die statistische Auswertung erfolgte in Kooperation mit Dr. Bernhard Drotleff (Pharmazeutisches Institut, Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Deutschland). Die Datenverarbeitung wurde mit den Programmen Excel 2019 (Microsoft, Redmond, WA, USA), SPSS Statistics 23 (IBM, Armonk, NY, USA), Origin 2019 (OriginLab, Northampton, MA, USA), sowie RStudio 1.2.1335 (R version R-3.5.1, R Foundation for Statistical Computing, Wien, Österreich) durchgeführt (112). Fehlende Daten (1.48% des gesamten Datensets) wurden mittels einer „random forrest“ Methode ergänzt (90).

Ergänzend erfolgte ein Jahr nach Probenisolation eine Durchsicht der elektronischen Klinikdatenbank zur Erfassung von erneuten Echokardiographien beziehungsweise erneuten Lävokardiographien. Durch Messung der systolischen LV Funktion konnten für 48 Patienten Verlaufsdaten generiert werden.

Die Patientencharakteristika zeigten Unterschiede zwischen den beiden Gruppen (MI und CCS): CRP war in der MI Gruppe höher und Patienten in der CCS Kohorte wurden öfter mit Clopidogrel und Statinen behandelt (Dauertherapie vor Klinikeinweisung). Zudem zeigten Patienten der MI Kohorte eine niedrigere systolische LV Funktion bei Aufnahme. Da das miRNA Expressionsprofil von multiplen klinischen Faktoren beeinflusst werden kann, wurden 28 Patienten aus der CCS Gruppe (Matched Control) mit 28 Patienten aus der MI Gruppe nach Alter, Geschlecht, Diabetes sowie Vorliegen eines chronischen Nierenversagens gematcht. Fünf Patienten aus der MI Gruppe konnten nicht gematcht werden und wurden für diese Gruppenvergleiche ausgeschlossen.

Um Trends in der globalen Expression der gemessenen miRNAs im Patientenkollektiv zu untersuchen, wurde eine hierarchische Clusteranalyse durchgeführt und eine Heatmap erstellt. Hier zeigte sich ein heterogenes Verteilungsmuster. Es war kein Trend im Verteilungsmuster zwischen den beiden Gruppen (MI und CCS) erkennbar.

Um Unterschiede der miRNA Expressionsprofile zwischen den beiden Gruppen der einzelnen miRNAs zu untersuchen, wurden Volcano plots generiert: Es stellten sich eine signifikant niedrigere Expression der miRNAs 103a-3p und 155-5p in Patienten aus der MI Gruppe dar. Dieses Ergebnis konnte in einem Vergleich der Matched Control mit der MI Gruppe verifiziert werden. Zusätzlich zeigte sich in dieser Analyse die Expression der miRNAs 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p und 425-3p in der MI Kohorte verglichen mit der Matched Control Kohorte signifikant erniedrigt.

Als nächstes erfolgte ein Vergleich des miRNA Expressionsprofils zwischen Patienten mit einer Verbesserung und einer Verschlechterung der systolischen LV Funktion (Δ LVEF% <1 verglichen mit Δ LVEF% >1). Hier zeigte ich eine erniedrigte Expression der miRNAs 21-5p, 23b-3p, 27a-3p, 29a-3p, 29b-3p, 92a-3p, 221-3p und der miRNA Vorstufe let7c-5p in der Gruppe der Patienten mit verschlechterter LV Funktion.

In der Literatur finden sich Hinweise auf proatherosklerotische Effekte für die, in dieser Studie untersuchten miRNAs 103a-3p und 155-5p (92, 96). Wie oben bereits erwähnt, zeigten sich diese miRNAs in unserem Kollektiv bei Patienten mit Myokardinfarkt jedoch signifikant erniedrigt. Eine mögliche pathophysiologische Erklärung könnte die Ausschüttung von miRNAs durch aktivierte Thrombozyten darstellen. Ein miRNA Transport aus Plättchen und resultierende parakrine Effekte an glatter Muskulatur, Makrophagen und Endothelzellen wurde von mehreren Autoren beschrieben (24, 26, 70-73). Diese Mechanismen könnten auch die erniedrigte Expression der miRNAs 30a-5p, 30b-5p, 30c-5p, 140-3p, 185-3p, 221-3p und 425-3p in der MI Gruppe verglichen mit der matched control Kohorte erklären. Um diese Annahme zu erhärten, ist allerdings die Durchführung weiterer Untersuchungen erforderlich.

Auch die, zwischen den Gruppen der verbesserten und verschlechterten systolischen LV Funktion unterschiedlich exprimierten miRNAs wurden bereits mit Herzinsuffizienz in Verbindung gebracht: MiRNA 27a-3p Erhöhung wurde bei Patienten mit akuter Herzinsuffizienz nachgewiesen und miRNA 29a-3p und 92a-3p Konzentrationen stiegen nach Behandlung von chronisch herzinsuffizienten Patienten mittels Resynchronisationstherapie (124, 127). MiRNA 221 Spiegel waren bei chronisch herzinsuffizienten Patienten verglichen mit einer gesunden Kontrollgruppe erniedrigt. Zudem erlaubten die Expressionslevels eine Unterscheidung zwischen Herzinsuffizienz mit reduzierter und erhaltener systolischer Funktion (109). Letztlich wurden erhöhte miRNA-21 Spiegel bei Herzinsuffizienz mit reduzierter systolischer LV Funktion in Verbindung gebracht (118). Keine dieser Messungen wurden jedoch an Thrombozyten durchgeführt.

Leider erlaubt unser Studiendesign keine pathophysiologischen Rückschlüsse und dient zum jetzigen Zeitpunkt lediglich der Hypothesengeneration. Da ein potenter Biomarker für die Regeneration der systolischen LV Funktion allerdings breite, klinische Anwendung finden würde, sind weitere Studien zu diesem Thema erstrebenswert.

9 References

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10 Erklärung zum Eigenteil

Die Arbeit wurde in der Medizinischen Klinik III am Universitätsklinikum Tübingen unter Betreuung von Prof. Dr. med. Meinrad Gawaz durchgeführt.

- Das Studiendesign, Identifikation und Rekrutierung von Patienten erfolgten von Herrn Andreas Goldschmied.
- Probenakquirierung für die Studienkohorte von Patienten wurden von Herrn Andreas Goldschmied durchgeführt.
- Erhebung der Patientencharakteristika und Isolation von Probenmaterial (Thrombozytenisolation) wurden von Herrn Andreas Goldschmied durchgeführt.
- MiRNA Extraktion sowie real-time PCR Messung wurden in Kooperation mit Prof. Schwab, Dr. Stefan Winter und Dr. Elke Schaeffeler (Dr. Margarete-Fischer-Bosch Institut für klinische Pharmakologie, Stuttgart, Deutschland) durchgeführt.
- Die Statistische Auswertung wurde in Kooperation mit Herrn Bernhard Drotleff (Pharmazeutisches Institut der Universität Tübingen, Tübingen, Deutschland) durchgeführt.

Ich versichere, die Arbeit selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

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