Aus dem Institut für Pharmakologie und Toxikologie
der Technischen Universität München
Fakultät für Medizin
Direktor: Prof. Dr. Dr. Stefan Engelhardt

MicroRNAs and platelet function in cardiovascular disease

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Summary

Small non-coding RNAs (ncRNAs) have generated an increasing interest as potential novel biomarkers that are predictive of cardiovascular events such as myocardial infarction and stroke. One class of these RNA molecules are microribonucleic acids (miRNA), which have a length of 18-25 nucleotides and are also present in platelets. Upon activation, platelets release miRNA-containing microvesicles into circulation, where they can be collected and easily quantified. Differences in circulating miRNA profiles were noticed in several physiological and pathological conditions. Antiplatelet therapy is a medical standard of care for treating patients with a history of acute coronary syndrome (ACS). The effects of several antiplatelet agents on circulating miRNAs are currently unclear.

In this work, next-generation sequencing was used to identify the distribution of small ncRNAs in circulation. Platelet function was assessed with three different platelet function tests. These results were correlated with expression of miRNAs and YRNAs in 125 patients with a history of ACS under administration of different schemes of antiplatelet therapy. Moreover, selected YRNA fragments and miRNAs as well as markers that reflect platelet activation and the distribution of a specific single nucleotide polymorphism (SNP) were measured in samples of the Bruneck cohort (n=669). Additionally, miRNA expression was measured in stroke patients pre and post thrombolysis as well as in healthy volunteers in a radial injury experiment.

The work at hand shows an existing correlation between platelet function and miRNA and YRNA expression in circulation. Furthermore, a strong dependency of small ncRNAs, including YRNAs, miR-126 and miR-223 on platelets has been shown, which is influenced by antiplatelet therapy. These small ncRNAs also correlated with plasma levels of platelet activation proteins. Furthermore, alterations of several miRNAs were shown upon thrombolysis therapy and as a result of endothelial denudation. Moreover, it has been shown that the rs4636297 SNP genotype has an impact on endogenous expression of miR-126 and, further, on plasma concentrations of platelet activation proteins.

Zusammenfassung

In den vergangenen Jahren hat die Bedeutung von nicht-kodierenden RNA-Molekülen (ncRNAs) für große Aufmerksamkeit in der Wissenschaftswelt gesorgt. Auch im Zusammenhang mit Herz-Kreislauferkrankungen scheinen microRNAs (miRNAs) eine Schlüsselrolle zu spielen. Es handelt sich dabei um kurze Moleküle von 18-25 nt Länge, die auch zahlreich in Thrombozyten vorhanden sind und beispielsweise bei der Thrombozytenaktivierung freigesetzt werden. Diese im Blut zirkulierenden Moleküle können mittels quantitativer Echtzeit-Polymerase-Kettenreaktion gemessen werden. Weitgehend unklar ist der Zusammenhang zwischen solchen miRNAs im Blutkreislauf und ihren Einfluss auf die Wirksamkeit von Thrombozytenaggregationshemmern.

Mittels moderner Sequencing-Methoden wurde analysiert, welche ncRNA-Moleküle in Blutplasmaproben nachweisbar sind. Des Weiteren wurde die Plättchenfunktion mit verschiedenen Testmethoden erhoben und auf Korrelationen zur miRNA-Expression in 125 Patienten nach akutem Koronarsyndrom unter unterschiedlicher Medikation analysiert. In 669 Blutproben der Bruneck Studie wurden zudem Zusammenhänge zwischen ausgewählten YRNAs bzw. miRNAs und Thrombozytenproteinen sowie der Häufigkeit eines speziellen Einzelnukleotid-Polymorphismus untersucht. Des Weiteren wurden die Modulationen in der miRNA Expression vor und nach Lysetherapie mit Alteplase in Blutproben von Schlaganfallpatienten untersucht; gleichfalls wurden Veränderungen infolge experimentell zugefügter Endothelverletzungen in gesunden, freiwilligen Probanden untersucht.

In der vorliegenden Arbeit konnte gezeigt werden, dass eine Korrelation zwischen der Thrombozytenfunktion und zirkulierenden miRNA- bzw. YRNA-Molekülen existiert. Ebenso konnte eine Wechselwirkung zwischen Blutplättchen und verschiedenen kleinen ncRNAs – unter anderem YRNAs, miR-126 und miR-223 – gezeigt werden, die außerdem durch Antiplättchentherapie beeinflusst wird. Einige der untersuchten YRNA- und miRNA-Moleküle zeigten darüber hinaus eine Korrelation mit ausgewählten Thrombozytenproteinen. Zudem wurden durch Trombolyse oder experimentell verursachte Endothelverletzung ausgelöste Veränderungen in der miRNA Expression im Blutkreislauf aufgezeigt. Schließlich konnte ein Effekt des untersuchten Einzelnukleotid-Polymorphismus rs4636297 auf die Expression von miR-126 und verschiedenen Thrombozytenproteinen nachgewiesen werden.

List of abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ASA	Acetylsalicylic acid
BD	Bis in die (lat.), twice a day
CAD	Coronary artery disease
CVD	Cardiovascular disease
cDNA	Complementary deoxyribonucleic acid
Cel/ *C. elegans*	*Caenorhabditis elegans*
CT	Cycle threshold
DLK1	Notch 1 inhibitor delta-like 1 homolog
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
g	Gram
g_n	Earth’s gravitational acceleration, 9.80665 m/ s^2
GP IIb/ IIIa	Glycoprotein IIb/ IIIa
h	Hour
HDL	High-density lipoprotein

LDL	Low-density lipoprotein
LTA	Light transmittance aggregometry
µL	Microlitre
mL	Millilitre
mRNA	Messenger ribonucleic acid
miRNA	Microribonucleic acid
MI	Myocardial infarction
min	Minute
n	Number
ncRNA	Non-coding ribonucleic acid
NGS	Next-generation sequencing
nM	Nanomolar
nm	Nanometre
nt	Nucleotide
OD	Once daily
p	P-value
PCA	Principal component analysis
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PF4	Platelet factor 4

PPBP	Pro-platelet basic protein
PPP	Platelet-poor plasma
PreAmp	Preamplification
pre-miRNA	Precursor microribonucleic acid
PRI	Platelet reactivity index
pri-miRNA	Primary microribonucleic acid
PRP	Platelet-rich plasma
RNA	Ribonucleic acid
RT	Reverse transcription
rtPA	Recombinant tissue plasminogen activator
RT-qPCR	Real-time quantitative polymerase chain reaction
SD	Standard deviation
SELP	P-selectin
SNP	Single nucleotide polymorphism
T2DM	Diabetes mellitus type 2
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
VASP	Vasodilator-stimulated phosphoprotein phosphorylation
WHO	World Health Organization

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Some of the tables and figures stem from other publications. For each case it is noted in the corresponding subtitle whether the table or figure has been used in its original form (“originated from”) or whether it has been modified for this thesis (“adapted from”).

1 Introduction

1.1 Cardiovascular diseases

Disorders and diseases of the cardiovascular system are the leading cause of death and in its various manifestations the most frequent reasons for medical consultations (Lozano et al. 2012). In 2016, 17.9 million people died from cardiovascular disease (CVD), out of these approximately 15 million people died due to heart attack or stroke.[1] Therefore, it is essential to improve prevention, diagnosis and treatment of CVD. CVDs involve pathological conditions in the heart and blood vessels and include hypertension, heart failure, carditis, stroke, coronary artery disease (CAD), cardiomyopathy, arrhythmia, (ischaemic) heart disease, aortic aneurysm, peripheral artery disease, angina pectoris, myocardial infarction, arrhythmia, rheumatic fever, renal artery stenosis, heart valve problems and sudden cardiac death. CVDs occur in diverse forms in clinical practice but, in most cases, atherosclerosis is the primary cause.

1.2 Introduction to and pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory process, which occurs in medium- and large-sized arteries and is accompanied by a loss of vessel elasticity. Lipid deposits lead to a narrowing of the vessel lumen. Atherosclerosis preferentially occurs at sites with non-laminar blood flow such as arterial trees or vessel branching points. An occlusion by a platelet thrombus can further attenuate blood flow and induce tissue ischaemia (Gistera and Hansson 2017).

Atherogenesis often starts in childhood already. Individuals suffering from metabolic syndrome – which is characterised by central adiposity, elevated fasting glucose, hypertension, increased triglycerides and low high-density lipoprotein (HDL) cholesterol – are at high risk of developing atherosclerosis. The cholesterol accumulation predominantly occurs in the intima and triggers chronic inflammation. Disturbances in blood flow, mainly at predilection sites like vessel branching points, promote changes in local haemostasis and immunothrombosis

[1] URL: "https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)", visited 02.09.2017, 10.35 CET.

processes (Gistera and Hansson 2017, Fiedler et al. 2018). Leukocytes, such as monocytes and T-lymphocytes, are attracted to endothelial cells (EC) covering the predilection site. The former differentiate into macrophages while the latter release cytokines, proteases, growth factors and chemokines. When macrophages endocytose excessive low-density lipoprotein (LDL) particles via scavenger receptors, they undergo cell death and form the necrotic core of atheromas. Thus, plaques consist of an inner lipid core covered by a fibrous cap (Steinberg et al. 1989, Hansson and Hermansson 2011). Concomitantly, injuries to the intima disrupt endothelial integrity. The exposure of collagen and other thrombogenic molecules induce platelet aggregation (Gistera and Hansson 2017). The perilous consequence of advanced atherosclerosis is plaque rupture, triggering thrombosis at its origin or embolisation, if the thrombus is dislodged and occludes blood vessels distant from its origin for example in brain arteries leading to cerebral infarction. Often, clinical symptoms of CVDs first manifest at the stage of acute life-threatening events. There are several factors increasing individual risk, which can be subdivided into non-modifiable – such as sex, age and genetic predisposition – and modifiable risk factors – such as smoking or lifestyle. Besides, one can group together classical risk factors, such as male gender, age, high LDL, low HDL, smoking, (systolic) hypertension, positive family history, diabetes and high triglycerides (WHO 1979, Murray and Lopez 1997). However, these parameters do not capture all patients at risk and there might be unknown risk factors. The evaluation of electrocardiogram (ECG), echocardiogram, percutaneous coronary intervention (PCI), cardiovascular magnetic resonance imaging and cardiac-specific lab parameters – like troponin point-of-care-test – are currently state of the art for diagnosing CVD events. Over the last decades, several types of serum and genetic markers have been suggested for the improvement of prediction and detection of CVDs, but none of them had sufficient diagnostic power for clinical utility thus far (Zampetaki et al. 2012, Fiedler et al. 2018).

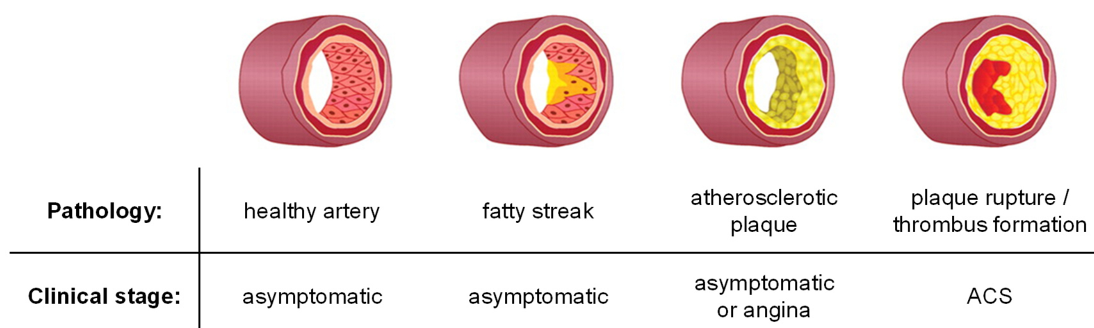


Figure 1 Atherosclerosis progress and clinical symptoms
Figure adapted from Klingenberg et al. (Klingenberg and Hansson 2009).

1.3 Acute coronary syndrome

Acute coronary syndrome (ACS) refers to a spectrum of diseases whose leading clinical symptom is acute chest pain caused by reduced blood flow in one or more coronary arteries. It occurs in the form of unstable angina pectoris, ST-elevation myocardial infarction (STEMI) or non-ST-elevation myocardial infarction (NSTEMI). Inadequate perfusion of heart muscle tissue initiates an ischaemic cascade that ultimately results in cardiomyocyte death. ACS is mainly caused by spontaneous plaque rupture and thrombus formation. Besides stroke, ACS is the most frequent medical instance of a cardiovascular emergency and is classified on the basis of characteristic changes in 12-lead ECG (Ibanez et al. 2018).

Typically, the main symptom of ACS is chest pain, often radiating to the left shoulder and limb, accompanied by unequivocal changes in ECG, corresponding patients' history and alterations in cardiac enzymes in peripheral blood (WHO 1979, Ibanez et al. 2018). Interestingly, a substantial part of patients suffers from a silent myocardial infarction, which occurs with few or even without any symptoms (Arenja et al. 2013, Thygesen et al. 2018). An important breakthrough proved to be the invention of a point-of-care test method invented by the German cardiologist Prof. Hugo Katus, which is based on detecting troponin T (Katus et al. 1989). Still, identification and differentiation of ACS patients remain a diagnostic challenge. Cardiac markers such as troponins are only released when cardiac cell damage has already occurred. Nevertheless, researchers have been aiming to identify novel biomarkers for early, specific and sensitive diagnosis or even identification of high-risk patients before acute events occur. However, such laboratory parameters are not available to date in clinical settings (Meder et al. 2011, Zampetaki et al. 2012, Fiedler et al. 2018).

"Time is muscle" – for this reason, ACS patients require immediate intensive medical treatment. Thereby, adequate cardiac perfusion is restored and, furthermore, the process of ischaemic heart muscle cell damage is inhibited. Usually, the standard of care procedure comprises application of oxygen, nitric oxide, beta blockers or ACE-inhibitors and dual antiplatelet therapy with acetylsalicylic acid (ASA) and a $P2Y_{12}$ inhibitor. If necessary, morphine is administered in order to reduce chest pain, diminish (cardiac) stress and, thereby, improve heart muscle oxygenation. If a patient presents with characteristic ECG changes and clinical symptoms within 90-120 min after onset of symptoms, immediate emergency cardiac catheterisation is required for both diagnosis and treatment purposes. Patients who undergo PCI

require intravenous anticoagulation in order to decrease the risk of further thrombotic events. After percutaneous access into either the radial, femoral or brachial artery, a balloon catheter is carefully advanced towards the site of the stenosis. Usually, a bare metal or drug-eluting stent is inserted to keep the stretched artery in shape and prevent re-occlusion. Dual antiplatelet therapy is recommended for at least one month after bare-metal stent implantation and 12 months after drug-eluting stent implantation (Singh et al. 2015, Valgimigli et al. 2017, Ibanez et al. 2018).

1.4 Stroke

Stroke is the second highest cause of mortality worldwide. 87% are ischaemic, thus, resulting from a vessel occlusion of a brain-supplying artery, 13% are resulting from acute intracerebral or subarachnoid bleeding. Stroke puts an enormous strain on national healthcare systems as it is the leading cause of long-term disability and has extensive personal and economic consequences (Writing Group et al. 2016). Ischaemic stroke is caused by cerebral thrombosis or embolism, which initiates the so-called ischaemic cascade.

Thrombolysis with alteplase is the medical standard of care for treating an ischaemic stroke. Alteplase, a recombinant human tissue plasminogen activator (rtPA), is a serine protease. It acts by converting plasminogen to plasmin, thus, enhancing fibrinolysis in order to dissolve the thrombus. In a routine clinical setting, the alteplase powder is dissolved with water and then diluted with sodium chloride (NaCl). If patients are selected for thrombolysis therapy, rtPA is applied intravenously within an appropriate time window. RtPA is metabolised hepatically and eliminated renally. Application within 3 h is the US Food and Drug Administration (FDA) approved standard of care for treating an ischaemic stroke. Other countries' guidelines allow its use until 6 h after onset of symptoms. However, "time is brain" and, thus, earlier application, ideally within 3 h, significantly reduces mortality and long-term morbidity without an increased risk resulting from (intracerebral) haemorrhage, which is the main harmful side effect (Yepes et al. 2009, Wardlaw et al. 2014, Powers et al. 2018).

1.5 Platelets and platelet function

Platelets, also called thrombocytes, are 2-3 µm-sized, biconvex, discoid anucleate blood cells, which are released by megakaryocytes in the bone marrow. Their average life span is 7 to 10 days before elimination in the spleen and liver. Their ability to aggregate and clot makes them – alongside von Willebrand factor and the coagulation system – essential for haemostasis (Choi et al. 1995). Thrombogenic mediators and molecules are capable of initiating platelet activation, which on the one hand is a vital process for stopping bleeding but on the other hand can lead to major thrombotic events like pulmonary embolism or ischaemic stroke (Engelmann and Massberg 2013).

There are different mechanisms leading to platelet activation, such as via receptors of the P2Y family, protease activated receptors, thromboxane or 5-hydroxytryptamine receptors. Activation is mainly triggered by certain molecules, namely collagen, tissue factor, thrombin, fibrinogen and the von Willebrand factor. Signal cascades are initiated via G_q, G_i and G_{12}/ G_{13} proteins and, consequently, stimulatory signal molecules and cytokines like CD40 ligand or P-selectin (SELP) are released. Thus, more platelets are recruited, their aggregation skills are facilitated and vessel constriction is mediated (Kaplan et al. 2015, Thomas et al. 2015). Over the last decades, research has deepened the knowledge of the various roles of platelets as they were proven to be of importance for many physiological and pathological processes. For instance, they impact on inflammation and modulate the recruitment of immune cells by releasing cytokines. Furthermore, they are believed to influence tumour cell growth (Warshaw et al. 1967, Merhi-Soussi et al. 2005, Boilard et al. 2010, Coupland and Parish 2014). Moreover, activated platelets were shown to contribute to CVDs by formation of platelet aggregates such as platelet-leukocyte aggregates and the release of platelet microparticles (Heijnen et al. 1999, Weber and Noels 2011, Risitano et al. 2012, Chandraratne et al. 2015).

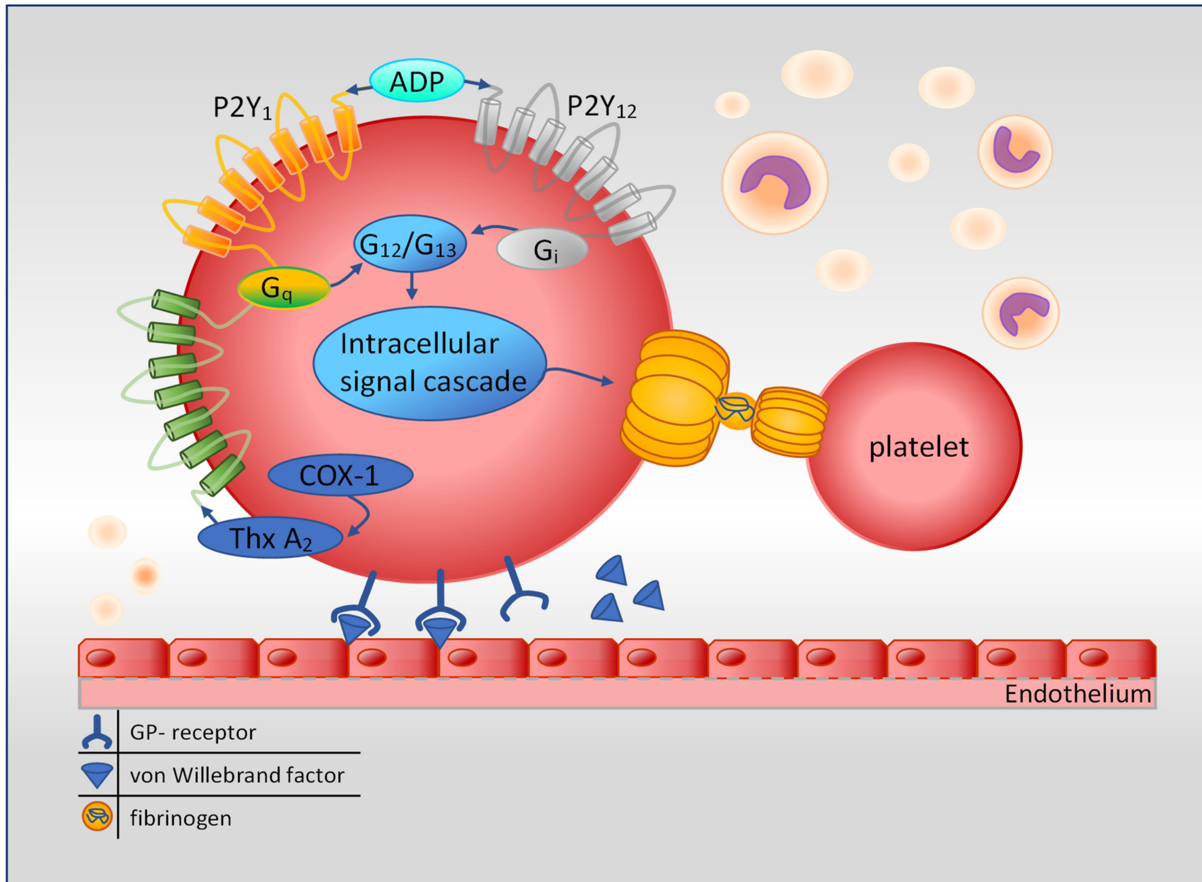


Figure 2 Platelet receptors
Illustration of platelets and simplified scheme of different signal cascades leading to platelet activation.

1.6 Platelet inhibition

The formation of thrombi and platelet aggregates promotes various pathological disorders. In patients showing advanced stages of atherosclerosis and high risk of major cardiovascular events, prolonged antiplatelet therapy decreases the incidence of ischaemic events. In post myocardial infarction (MI) patients, antiplatelet drugs are part of the clinical standard of care to attenuate thrombus formation and, thereby, lower mortality rates (Hochtl and Huber 2014, Joshi et al. 2014, Ibanez et al. 2018). Furthermore, as secondary prevention therapy, dual antiplatelet therapy – consisting of ASA and a $P2Y_{12}$ inhibitor, such as clopidogrel, prasugrel or ticagrelor – was shown to significantly decrease the event rates in patients suffering from major cardiac events (Tantry et al. 2013, Thomas et al. 2015, Valgimigli et al. 2018). Dual antiplatelet therapy can work by way of inhibiting leukocytes and SELP, which trigger inflammation and activate coagulation. This reduces the formation of platelet leukocyte

aggregates and decreases the risk of major thromboembolic events (Thomas et al. 2015). Thus, CVD patients benefit from dual antiplatelet inhibition. ASA and clopidogrel are both on the World Health Organization's (WHO) list of essential medicines.[2] Nevertheless, the use of antiplatelet therapy needs to be carefully considered as on the one hand it prevents thrombosis and ischaemic events but on the other hand increases the risk for major bleeding. To date, the guidelines of antiplatelet therapy are mainly based upon clinical observational studies and, thus, recommendations vary in different countries (Cannon et al. 2010, Levine et al. 2016, Ibanez et al. 2018, Valgimigli et al. 2018).

Table 1 Antiplatelet agents
Therapeutic targets and commonly administered antiplatelet drugs.

inhibition	drug
COX-1	ASA
$P2Y_{12}$	clopidogrel, prasugrel, ticagrelor, cangrelor
GP IIb/ IIIa	abciximab, tirofiban

1.6.1 Acetylsalicylic acid

Acetylsalicylic acid (trade name: aspirin) primarily suppresses the synthesis of prostaglandins and thromboxane A2 as discovered by noble price laureate Sir John Robert Vane (Vane 1971). It is listed in the WHO essential medical drugs catalogue[3] for treating headaches, pain, fever, heart attacks and inflammation. It inhibits activation of platelets via the thromboxane pathway. Thromboxane receptor activation initiates an intracellular signal cascade via G_q and G_{12}/ G_{13} proteins enhancing platelet aggregation. The inhibitory effect of ASA on COX enzymes lasts the entire platelet lifetime. Hence, its clinical use has been widely established, for instance, for primary and secondary prevention in mid- and high-risk heart disease patients. In post-MI patients, low-dose aspirin administration decreases the rate of future thromboembolic events (Vane 1971, Tantry et al. 2013, Thomas et al. 2015). ACS patients benefit from dual antiplatelet

[2] URL: "http://www.who.int/medicines/publications/essentialmedicines/en/", 20th edition, March 2017, visited 20.10.2018, 11.50 CET.
[3] Ibid.

therapy and, thus, ASA administration combined with a $P2Y_{12}$ inhibitor is a standard procedure in acute MI therapy and strongly recommended after arterial stent implantation for the prevention of (stent) thrombosis and re-occlusion (Hochtl and Huber 2014, Joshi et al. 2014, Levine et al. 2016, Ibanez et al. 2018, Valgimigli et al. 2018).

1.6.2 Clopidogrel

Clopidogrel (300-600 mg loading dose, 75 mg once daily (OD) maintenance dose, trade name: Plavix), a second-generation thienopyridine drug, acts by irreversibly inhibiting the adenosine diphosphate (ADP) $P2Y_{12}$ receptor, which is predominantly expressed on the surface of platelets. Clopidogrel is a prodrug and undergoes metabolic activation in the gastrointestinal epithelium and hepatic cells. It requires two metabolic activation steps after oral application. Once activated, it inhibits an intracellular signal cascade via G_i and G_{12}/ G_{13} proteins inhibiting Glycoprotein IIb/ IIIa (GP IIb/ IIIa) activity and, thus, the aggregation of thrombocytes. An estimate of 16 to 50% of patients treated with clopidogrel are suspected of not adequately responding (Gurbel et al. 2003, Gurbel et al. 2007, Roberts and Nawarskas 2013). Early identification of these non-responders is important to ensure sufficient platelet inhibition (Tantry et al. 2010, Grove et al. 2012, Hochtl and Huber 2014). This phenomenon is suspected to be caused by lower expression of CYP2C19. CYP2C19 belongs to the family of cytochrome P450 enzymes, which are haemoproteins that participate in numerous oxidation processes. They are involved in the enzymatic metabolism of several hormones, vitamins, lipids, toxins and drugs. The CYP2C19 isoform in particular is mainly expressed in hepatic cells and acts on approximately 10 to 15% of clinically used drugs such as clopidogrel. Polymorphisms influence the enzymatic activity and the effectiveness of clopidogrel therapy in ACS patients. Thus, the use of clopidogrel is replaced in first-line therapy by newer drugs such as prasugrel and ticagrelor with improved efficacy (Gurbel et al. 2003, Brandt et al. 2007, Stone et al. 2013).

1.6.3 Prasugrel

Prasugrel (60 mg loading dose, 10 mg OD maintenance dose, trade name: Efient), a third-generation thienopyridine drug, acts by irreversibly binding to the $P2Y_{12}$ receptors and, thus, inhibiting platelet activation and aggregation after a single metabolic activation step by enzymes of the cytochrome P450 family. Its effect is more pronounced with a more rapid onset and, therefore, prasugrel partially replaced clopidogrel as the first choice in ACS therapy. Moreover, compared to clopidogrel, prasugrel shows a more sufficient platelet inhibition in healthy individuals and ACS patients and a low number of non-responders. However, prasugrel increases the risk of major bleeding complications and is, therefore, contraindicated in patients older than 75 years or with a history of a transient ischaemic attack or stroke (Brandt et al. 2007, Wiviott et al. 2007).

1.6.4 Ticagrelor

Ticagrelor (180 mg loading dose, 90 mg maintenance dose bis in die (BD), trade name: Brilique) is a cyclopentyl-triazolo-pyrimidine and acts by inhibiting $P2Y_{12}$ receptors. In contrast to clopidogrel and prasugrel, it is a reversible allosteric antagonist and is administered BD because of its shorter half-life of 6-12 h. It is already orally active without requiring enzymatic activation (Teng et al. 2010). Especially in patients older than 75 years, ticagrelor showed benefits compared to prasugrel due to fewer bleeding complications. Compared to prasugrel and clopidogrel, ticagrelor treatment has also been associated with a survival benefit that was not explained by its platelet inhibitory effect (Storey et al. 2011, Steg et al. 2013, Larmore et al. 2015). However, no survival benefit compared to clopidogrel was observed in recent studies in patients with symptomatic peripheral artery disease (Hiatt et al. 2017).

1.6.5 Other CVD therapeutics

Statins act by competitive inhibition of the enzyme 3-hydroxy-3methylglutaryl-CoA (HMG-CoA) reductase, which enables the reduction of HMG-CoA to mevalonate. They effectively reduce cholesterol synthesis, which mostly (up to 70%) occurs in hepatic cells (Goldstein and Brown 1990). Additionally, statins inhibit the prenylation of proteins involved in the modulation of immune responses in chronic inflammation. Overall, statins were shown to be beneficial, when they were administered for primary prevention purposes in high-risk CVD patients (Hansson and Hermansson 2011, Taylor et al. 2013). Newer lipid lowering reagents

include Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors that can further reduce cholesterol when combined with statins (Robinson et al. 2014). Other medical treatment applicable for CVD therapy includes beta blockers – invented by James Black in 1964 – which reduce the progression of atheroma growth in CAD. Widely prescribed medication comprises ACE- and renin inhibitors, angiotensin receptor blockers, antiarrhythmic drugs, calcium channel blockers, nicotinic acids, fibrates, diuretics and vasodilators (Taylor et al. 2013, Wald et al. 2013, Hochtl and Huber 2014, Bednar et al. 2015, Levine et al. 2016, Ibanez et al. 2018). In advanced cases of CVD, interventional and surgical therapies, such as heart valve replacement or heart transplantation, become necessary in order to treat the perilous consequences of CVDs, such as papillary muscle rupture or heart failure (Levine et al. 2016, Ibanez et al. 2018).

1.7 New challenges

Biomarkers are an essential diagnostic tool in clinical routine providing information concerning disease formation and progression. Better biomarkers for risk stratification and therapeutic regimes would be desirable to combat CVDs and its consequences. C-reactive protein, cardiac troponines, growth differentiation factor-15 (GDF-15) and n-terminal prohormone of brain natriuretic peptide (NT-proBNP) have already been evaluated for their use in cardiovascular risk prediction. Many other molecules are in discussion for potentially being predictive of CVDs, among these sphingolipids, chemokines, myeloperoxidases or apolipoproteins. In addition to these biomarkers, ribonucleic acids (RNA) could present a novel entity of molecular biomarkers used for the early recognition of CVD (Olson 2014, Barwari et al. 2016, Fiedler et al. 2018).

1.8 Small non-coding RNAs

The decryption of the human genome revealed that only 2% of the human deoxyribonucleic acids (DNA) encode proteins. The remaining 98% were considered to be regions without any coding function (Mattick 2001). However, a vast amount of transcribed non-coding ribonucleic acids (ncRNA) were found to be distinctively expressed in various cells and tissue types and were shown to play key roles in the process of posttranscriptional gene regulation, for instance, by targeting and controlling messenger ribonucleic acids (mRNA). Microribonucleic acids (miRNAs) constitute a class of small, non-coding RNA molecules, which regulate expression

of target genes by mRNA degradation or translational repression and, thereby, contribute to the regulation of protein biosynthesis. MiRNAs with a length of 18 to 25 nt were found in both animals and plants with a high degree of evolutionary conservation. It has been proposed that they are involved in regulation of over 60% of human genes, mainly by imperfectly binding to the 3' untranslated region (UTR) of mRNAs (Carrington and Ambros 2003, Ambros 2004, Bartel 2004).

1.8.1 History

In 1992, Victor Ambros and co-workers Rosalind Lee and Rhonda Feinbaum discovered a small RNA molecule in non-parasitic nematode *Caenorhabditis elegans* as a product of the gene lin-4, a gene known to be involved in controlling its larval development. Surprisingly, this small RNA molecule did not have any protein-coding function but gave rise to a pair of small ncRNAs, whose functions were unclear (Lee et al. 1993). In the following years, under the leading influence of Victor Ambros and Gary Ruvkun, more of such small ncRNA molecules, derived from genes lin-4 and let-7, were detected in various species. They were formerly termed small temporal RNAs (Wightman et al. 1993, Pasquinelli et al. 2000, Lee and Ambros 2001). Since 2001, the term miRNA is used to describe these single-stranded RNA molecules with a length of 18 to 25 nt as an important category of small ncRNAs. Small ncRNAs comprise transfer, ribosomal, small-interfering and piwi-interacting RNAs, which can be distinguished by their unique pathways of biogenesis (Lagos-Quintana et al. 2001, Lee and Ambros 2001, Aalto and Pasquinelli 2012).

1.8.2 MicroRNA biogenesis

The majority of eukaryotic miRNA genes is transcribed by enzymes RNA polymerase II and III in the cell nucleus either directly from intron regions of the DNA or in antisense-orientated annotated genes (Lagos-Quintana et al. 2001, Lee and Ambros 2001, Cai et al. 2004). The processed primary microribonucleic acid (pri-miRNA) with about 500 to 3000 nt length obtains a poly-A-cap at the 3' end and a 7-methyl-guanosine-tail at the 5' end and forms a characteristic hairpin loop. This terminal loop might offer a target region for the subsequent cleavage by the microprocessor complex (Michlewski et al. 2008, Morlando et al. 2008). The microprocessor-complex, consisting of the Class 2 ribonuclease III enzyme Drosha and cofactors like protein Di-George syndrome chromosomal region 8 (DGCR8), directly binds to the RNA substrate and

cleaves the pri-miRNA into an approximately 70 nt long precursor micro ribonucleic acid (pre-miRNA) molecule (Gregory et al. 2004, Landthaler et al. 2004, Yeom et al. 2006). DGCR8 recognises the pri-miRNAs due to its “labelling” by methyltransferase-like 3 and stabilises the pri-miRNA for processing by Drosha (Alarcon et al. 2015). Next, Exportin-5, a Ran-dependent nuclear transporter, exports the pre-miRNA from the nucleus to the cytoplasm (Yi et al. 2003, Bohnsack et al. 2004). Dicer, a cytoplasmic enzyme with endonuclease activity, mainly encoded by the DICER-1 gene in humans, converts cellular miRNA precursors into short double-stranded RNA molecules (Bernstein et al. 2001, Hutvagner et al. 2001). The conversion is facilitated by several cofactors such as protein argonaute-2 before the miRNA duplex is loaded onto the RNA-induced silencing complex (RISC) (Gregory et al. 2005, Chakravarthy et al. 2010, Lee et al. 2013). Thermodynamic stability determines which of the two strands becomes an active mature strand. It seems that for most miRNAs the strand with the higher stability at the 5'-anti-sense terminal base pair (bp) is more likely to be incorporated into the RISC (Khvorova et al. 2003, Schwarz et al. 2003, Okamura et al. 2008). In contrast, the remaining strand degrades more rapidly. Recently, however, it has been shown that for some miRNAs this remaining strand also mediates miRNA functions (Ro et al. 2007, Shin 2008, Schober et al. 2014).

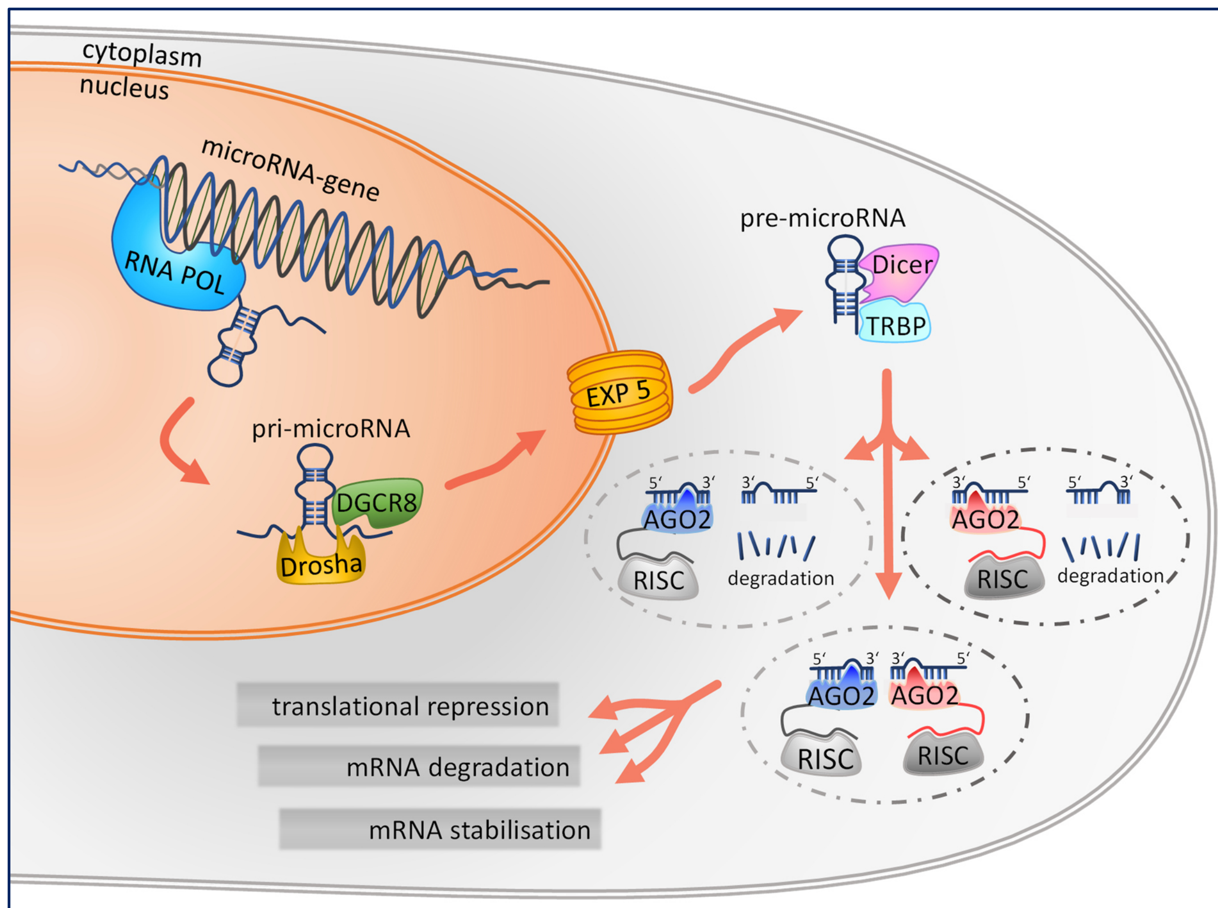


Figure 3 MiRNA biogenesis
Pri-miRNA is transcribed in the nucleus and is processed by Drosha to a pre-miRNA. After its export into the cytoplasm and cleavage by Dicer, two mature miRNA strands exist. There are different possibilities as to which of the mature strand will be incorporated into RISC.

1.8.2.1 Nomenclature of microRNAs

A coherent nomenclature is essential for setting up a basis for experimental comparability and interdisciplinary exchange worldwide. Therefore, common guidelines for miRNA typing were introduced in 2003 (Ambros et al. 2003): the species of origin is encoded in a three-letter prefix, such as hsa (*Homo sapiens*) or cel (*Caenorhabditis elegans*). The following prefix “mir” or capitalised “miR” differs between precursor and mature variant of the miRNA, e.g. miR-29. Differences in sequences with up to two varying nucleotides are annotated with a lower-case letter, e.g. “hsa-miR-29a”. Paralogous miRNAs, which are identical mature miRNAs with different gene loci, have an additional number appended to the three-digit code with a dash, e.g. miR-29b-1. The suffixes 3p or 5p are used to label the single-stranded miRNAs, which originate from opposite arms of their precursor stage. Formerly, they were distinguished in literature by adding an asterisk or naming them “sense” and “antisense”, “star” and “non-star”, “guide” and

"passenger" strand. Now, labelling as 3p or 5p strand is strongly recommended. MiRNAs that originate from the same precursor but differ in their mature sequences are called isomiRs. Several mechanisms lead to these sequence variations: 5' or 3' trimming, 3' nucleotide addition and nucleotide substitution (Morin et al. 2008). All detected miRNAs are recorded in a public database, miRbase[4], founded as part of the RNA family database in 2003 and hosted by the University of Manchester (Griffiths-Jones 2004).

1.8.3 MicroRNA function

Since the late 1990s, scientists have unveiled various fundamental functions of miRNAs in both physiological and pathophysiological pathways. Dysregulation of miRNA pathways is involved in the pathogenesis of chronic inflammation, fibrosis and sclerosis processes as well as cancers (Carrington and Ambros 2003, Ambros 2004, Bartel 2004, Cai et al. 2004, Chen et al. 2004, Gregory et al. 2005, Ro et al. 2007, Aalto and Pasquinelli 2012). Research on the function of Dicer improved the understanding of miRNAs: exemplary, experimental loss-of-function mutations and deletions in the Dicer-encoding region in zebrafish and mice resulted in fatal cardiac problems ending in embryonic or early death after birth (Bernstein et al. 2003, Wienholds et al. 2003). Since then, miRNAs have been identified in most tissues and fluids like blood, saliva, breast milk, urine and semen, where they are part of vesicles (exosomes or microparticles) or protein complexes, such as argonaute-2 and HDL. MiRNAs play diverse roles in metabolism, stem cell division and differentiation, cell cycle control, apoptosis and even tumour biogenesis (Bartel 2004, Weber et al. 2010). They promote transcript destruction, sequestration of P-bodies and repression of translational processes and trigger both deadenylation and decapping of mRNAs and, thus, lead to their decay. Interaction and pairing between miRNA and mRNA happen mostly between the seed sequence in the 5' region between nucleotides 2-8 of the miRNA and the 3'-UTR of the target mRNA (Bartel 2004, Gregory et al. 2004, Okamura et al. 2008). Many groups found levels of various miRNAs to be associated with CVD: MiR-1 was among the first found to be associated with cardiac function in CAD. Myocardial cell damage in CAD is associated with overexpression of miR-1, whereby

[4] URL: "http://www.mirbase.org/", visited 20.10.2018, 12.15 CET.

elimination of miR-1 by an antisense inhibitor in infarcted rat hearts was found to facilitate arrhythmogenesis (Yang et al. 2007).

1.8.4 Circulating microRNAs

In 2008, Mitchell and collaborators first published on the stability of miRNAs in blood plasma samples from prostate cancer patients. These circulating miRNAs were protected from degradation and reliably detected by polymerase chain reaction (PCR). In contrast, exogenously supplied synthetic miRNA underwent rapid degradation (Mitchell et al. 2008). Literature suggests a potential paracrine function of circulating miRNAs (Landry et al. 2009, Zampetaki et al. 2010, Diehl et al. 2012, Risitano et al. 2012). Circulating miRNAs show distinctive expression patterns in serum, platelet-poor plasma (PPP), platelet-rich plasma (PRP) and other body fluids. They originate from both circulating cells like leukocytes and from tissues like ECs, liver and cardiomyocytes. In circulation, miRNAs reside in microparticles or in lipoprotein-associated transport molecules and are, thereby, protected from degradation (Weber, Baxter et al. 2010, Zampetaki et al. 2012, Bang et al. 2014). Alterations of miRNA profiles in circulation may reflect disorders and diseases: Fichtlscherer et al. identified a remarkable number of altered miRNAs in plasma of stable CAD patients compared to healthy volunteers, among them miR-126 (Fichtlscherer et al. 2010). The research group of Prof. Mayr previously assessed circulating miRNA profiles in the Bruneck study and found correlations with decreased circulating levels of miR-126-3p and miR-223-3p in patients with type 2 diabetes mellitus (T2DM) (Zampetaki et al. 2010). Moreover, the latter were found to have predictive potential as they showed lower expression years before manifestation of T2DM. Subsequent studies showed a predominant platelet origin of many abundant circulating miRNAs (Kaudewitz et al. 2016, Willeit et al. 2017). Thus, profiling of circulating miRNAs might reveal potentials for their use as biomarkers in CVD (Zampetaki et al. 2010, Meder et al. 2011, Zampetaki et al. 2012, Willeit et al. 2013).

1.8.4.1 Circulating microRNAs and platelets

Besides their function in haemostasis, the understanding of the function of platelets has expanded considerably over the last decades: 50 years ago, Warshaw and collaborators reported on their potential to conduct protein synthesis (Warshaw et al. 1967). Since the pathogenesis of CVD and atherosclerosis seems to be closely linked to platelets, various technical approaches

are taken to analyse the complexity of the processes regulating platelet function and to encrypt the mechanisms leading to atherogenic and thrombotic disorders. Dysregulation of platelet activation in particular is regarded to be a high-risk factor for developing major thrombotic and cardiac events (Gidlof et al. 2013, Thomas et al. 2015). Platelets contribute approximately 41-45% to the blood microparticles, which in turn contain most of the miRNAs abundant in human circulation (Diehl et al. 2012). High throughput sequencing unveiled the large number of over 492 miRNAs residing in platelets (Ple et al. 2012). Furthermore, it was found that microvesicles carrying miRNAs and other ncRNAs – as released by platelets or ECs – enable intercellular communication, a process which may be altered in CVD (Landry et al. 2009, Risitano et al. 2012). Enhanced platelet activity in patients with MI leads to the release of specific miRNAs into circulation, which in turn can be taken up by ECs and, thereby, influence gene expression in the ECs. For example, platelet-derived miR-320b and miR-223 were shown to target expression of intercellular adhesion molecule 1, F-box/WD repeat-containing protein 7, an onco-suppressor protein, and ephrin A1, a glycosylphosphatidyl inositol-anchored receptor tyrosine kinase ligand (Zimmerman and Weyrich 2008, Gidlof et al. 2013, Laffont et al. 2013). Others have shown that miR-223 targets the $P2Y_{12}$ receptor in human platelets, while the Mayr group reported that miR-126 targets ADAM9, which is involved in platelet activation by collagen (Landry et al. 2009, Kaudewitz et al. 2016). It seems conceivable that platelet miRNAs play an important role as a regulator of platelet function and, therefore, contribute to various diseases including CVDs. Thus, platelet-derived miRNAs may serve as a novel class of biomarkers.

1.8.4.2 Circulating microRNAs in stroke patients

In patients suffering from ischaemic stroke, several alterations in circulating miRNAs were found. For example, Jickling et al. showed circulating miR-122, miR-148a and miR-320d to be decreased within 72 h after ischaemic stroke onset compared to control patients with vascular risk factors (Jickling et al. 2014). Others reported altered expression of miR-320d in acute stroke conditions (Wang et al. 2014, Mick et al. 2017). Long and collaborators noticed a decrease of miR-126 and miR-30a in ischaemic stroke patients compared to healthy volunteers for up to 24 weeks after the event (Long et al. 2013). Mick et al. reported incident stroke to be associated with abundance of miR-941. Furthermore, miR-124-3p, miR-320d and miR-877 were differentially expressed in prevalent stroke (Mick et al. 2017). A recent study implicated that the upregulation of miR-125a-5p, miR-125b-5p and miR-143-3p is a marker of acute

ischaemic stroke, offering superior performance compared to multimodal cranial computed tomography and other previously reported protein biomarkers (Tiedt et al. 2017).

1.9 Aim of this work

There are indications that miRNAs play an important role in regulation of cardiac development and function as well as in the pathogenesis of CVD. Besides their potential as a therapeutic target in CVD therapy, alterations in circulating miRNA could be highly useful as novel type of biomarkers for detecting CVD. The aim of the present work is to investigate the association of miRNAs with platelet function, especially, with a focus on passenger strands of miRNAs. Furthermore, the understanding of how miRNAs take part in the complex processes of platelet activation might yield fundamental insights into platelet physiology. To date, no study has been conducted on the effects of thrombolysis on circulating miRNA profiles. At present, there are no advanced clinical parameters that monitor clot breakdown and reduction of thrombotic material. The author hypothesises that thrombolysis might alter levels of miRNAs in circulation. Additionally, the author aims to investigate and refine modern methods of RNA analysis and to extend the knowledge of associations between transcriptomic data and functional phenotypes. The author expects that this field in cardiovascular basic research offers potentially novel types of highly sensitive and specific diagnostic and preventive information and, furthermore, provides necessary information about future targets for (pharmacological) CVD therapies.

2 Materials

Vascular Proteomics Laboratory, James Black Centre, King's College London	
Benches	Tecomak, Kent, UK
Pipettes and Tips	
Eppendorf Research plus pipettes	Eppendorf, Hamburg, Germany
TipOne 10, 200, 1000 µL pipette tips	Starlab International, Hamburg, Germany
Disposable transfer pipettes 10 mL, 25 mL	Thermo Fisher Scientific, Waltham, USA
Disposable tips (70 µL) for Agilent Bravo	Agilent Technologies, Santa Clara, USA
Tubes	
50 mL Falcon tube	Thermo Fisher Scientific, Waltham, USA
200 µL RNAse free PCR tubes and caps	Thermo Fisher Scientific, Waltham, USA
1.5mL microcentrifuge tubes, RNAse/ DNase free	Elkay Laboratory Products, Basingstoke, UK
Centrifugal filter units: Amicon Ultracel 3K	Merck Millipore, Darmstadt, Germany
MicroAmp optical 8-cap strip	Thermo Fisher Scientific, Waltham, USA
Tubes and flat cap strips(8x)	Thermo Fisher Scientific, Waltham, USA
Plates	
MicroAmp 96 well plate	Thermo Fisher Scientific, Waltham, USA
MicroAmp optical 384 well reaction plate	Thermo Fisher Scientific, Waltham, USA
MicroAmp optical adhesive film	Thermo Fisher Scientific, Waltham, USA
Pick-&-Mix microRNA PCR Panels, 384-well	Exiqon, Vedbaek, Denmark
Instruments	
Vortex Mixer: VWR-VV3-S42	Madison Dearborn Partners, Chicago, USA
Eppendorf centrifuge 5415r	Eppendorf, Hamburg, Germany
Eppendorf centrifuge 5804r	Eppendorf, Hamburg, Germany
Kinetic energy centrifuge for 0.2 ml tubes)	Madison Dearborn Partners, Chicago, USA
Veriti thermal cycler	Thermo Fisher Scientific, Waltham, USA

Bravo automated liquid handling system (96 ST head)	Agilent Technologies, Santa Clara, USA
ABI ViiA™7 real-time PCR system	Thermo Fisher Scientific, Waltham, USA
VerifyNow $P2Y_{12}$ analyser	Accriva, San Diego, USA
LSRII flow cytometer	Becton Dickinson, Franklin Lakes, USA
Infinite 200 PRO fluorescence microplate reader	Tecan, Maennedorf, Switzerland
Eppendorf Brunswick freezer	Eppendorf, Hamburg, Germany
Chemicals	
RNase ZAP	Thermo Fisher Scientific, Waltham, USA
Prostaglandin E1	Sigma-Aldrich, St. Louis, USA
Indomethacin	Sigma-Aldrich, St. Louis, USA
Phosphate buffered saline (PBS 10x)	Sigma-Aldrich, St. Louis, USA
Ultra-pure DEPC treated water	Thermo Fisher Scientific, Waltham, USA
Electran absolute ethanol for molecular biology	VWR, Radnor, USA
QIAzol lysis reagent 50 mL	Qiagen, Hilden, Germany
Chloroform 99,8% (GLC) 1.489 g/ mL stabilised with 100 ppm amylene for analysis	Thermo Fisher Scientific, Waltham, USA
RT, PreAmp, RT-qPCR materials: TaqMan system	
MS2-carrier	Roche, Basel, Switzerland
MultiScribe reverse transcriptase (50 U/ µL)	Thermo Fisher Scientific, Waltham, USA
$MgCl_2$ (25 mM)	Thermo Fisher Scientific, Waltham, USA
10x RT buffer	Thermo Fisher Scientific, Waltham, USA
Megaplex RT primers (10x) pool A v2.1 P/ N 4399966	Thermo Fisher Scientific, Waltham, USA
Megaplex RT primers (10x) pool B v3.0 P/ N 4444281	Thermo Fisher Scientific, Waltham, USA
dNTPs with dTTP (100 mM)	Thermo Fisher Scientific, Waltham, USA
RNase inhibitor (20 U/ µL)	Thermo Fisher Scientific, Waltham, USA
Megaplex PreAmp master mix (2x)	Thermo Fisher Scientific, Waltham, USA
Megaplex PreAmp primers (10x) pool A v2.1 P/ N 4399233	Thermo Fisher Scientific, Waltham, USA
Megaplex PreAmp primers (10x) pool B v3.0 P/ N 4444303	Thermo Fisher Scientific, Waltham, USA
Nuclease-free H_20 (PCR-H_20)	Thermo Fisher Scientific, Waltham, USA

TaqMan 2x qPCR universal master mix	Thermo Fisher Scientific, Waltham, USA
Individual TaqMan microRNA assays (see below)	Thermo Fisher Scientific, Waltham, USA
RT, RT-qPCR materials: Exiqon	
5x reaction buffer	Exiqon, Vedbaek, Denmark
10x enzyme mix	Exiqon, Vedbaek, Denmark
Nuclease-free water	Exiqon, Vedbaek, Denmark
ExiLent SYBR Green master mix	Exiqon, Vedbaek, Denmark
Pick-&-Mix microRNA PCR panels, 384-well	Exiqon, Vedbaek, Denmark
Kits	
miRNeasy MiniKit	Qiagen, Hilden, Germany
Universal cDNA synthesis kit	Exiqon, Vedbaek, Denmark
DuoSet ELISA development kits (catalogue numbers DY137, DY393, DY795)	R&D Systems, Minneapolis, USA
DuoSet ancillary reagent Kit 2	R&D Systems, Minneapolis, USA
mirCURY LNA microRNA cDNA synthesis kit	Exiqon, Vedbaek, Denmark
ELISA DY137, SELP/ CD62P	R&D Systems, Minneapolis, USA
ELISA DY393, PPBP	R&D Systems, Minneapolis, USA
ELISA DY795, PF4	R&D Systems, Minneapolis, USA
TruSeq RNA library prep kit	Illumina, San Diego, USA
KASP genotyping kit	LGC, Teddington, UK
Software	
ABI ViiA7 Version v1.1(qPCR)	Thermo Fisher Scientific, Waltham, USA
VWorks (Agilent Bravo)	Agilent Technologies, Santa Clara, USA
Trim Galore	Babraham Bioinformatics, Cambridge, UK
Microsoft Word 365	Microsoft, Redmond, USA
Microsoft Excel 365	Microsoft, Redmond, USA
Microsoft PowerPoint 365	Microsoft, Redmond, USA
Inkscape 0.91	Inkscape, Boston, USA

TaqMan individual microRNA assays & primer sequences			
Assay name	**Assay ID**	**Full name (mirbase*)**	**Sequence**
cel-miR-39	000200	cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG
miR-20b	001014	hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG
miR-21	000397	hsa-miR-21-5p	CAACACCAGUCGAUGGGCUGU
miR-21-3p	002438	hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU
miR-24	000402	hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG
miR-27b	000409	hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC
miR-28-3p	002446	hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA
miR-30b	000602	hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU
miR-92a	000431	hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU
miR-93	001090	hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG
miR-106a	002169	hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG
miR-122	002245	hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG
miR-122-3p	002130	hsa-miR-122-3p	AACGCCAUUAUCACACUAAAUA
miR-126-3p	002228	hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
miR-126-5p	000451	hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
miR-146a	000468	hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
miR-146b	001097	hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU
miR-148a	000470	hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
miR-150	000473	hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG

TaqMan individual microRNA assays & primer sequences

Assay name	Assay ID	Full name (mirbase*)	Sequence
miR-191	002299	hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG
miR-191-3p	002678	hsa-miR-191-3p	GCUGCGCUUGGAUUUCGUCCCC
miR-195	000494	hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
miR-197	000497	hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC
miR-223-3p	002295	hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA
miR-223-5p	002098	hsa-miR-223-5p	CGUGUAUUUGACAAGCUGAGUU
miR-320	002277	hsa-miR-320-3p	AAAAGCUGGGUUGAGAGGGCGA
miR-335	000546	hsa-miR-335-5p	UCAAGAGCAAUAACGAAAAAUGU
miR-486	001278	hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG
RNY4 3’	custom-made		CCCCCCCACUGCUAAAUUUGACUGGCU
RNY4 5’	custom-made		GGCUGGUCCGAUGGUAGUGGGUUAUCAGAACU

* full name according to mirbase, URL: “http://www.mirbase.org/”, visited 20.10.2018, 12.15 CET.

Plate design Exiqon custom-made RT-qPCR. This table originates from Kaudewitz et al. (Kaudewitz, Skroblin et al. 2016).

Well	MicroRNA	Sequence	Well	MicroRNA	Sequence
A01	hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU	E01	hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA
A03	hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	E03	hsa-miR-331-3p	GCCCCUGGGCCUAUCCUAGAA
A05	hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	E05	hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU
A07	hsa-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA	E07	hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
A09	hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	E09	hsa-miR-451a	AAACCGUUACCAUUACUGAGUU
A11	hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG	E11	hsa-miR-454-3p	UAGUGCAAUAUUGCUUAUAGGGU
A13	hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	E13	hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU
A15	hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA	E15	hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG
A17	hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU	E17	U6 snRNA	reference gene
A19	hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC	E19	hsa-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU
A21	hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	E21	UniSp6 CP	reference gene
A23	hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	E23	Cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG
C01	hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	G02	hsa-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA
C03	hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	G04	hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
C05	hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG	G06	hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA
C07	hsa-miR-139-5p	UCUACAGUGCACGUGUCUCCAGU	G08	hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
C09	hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU	G10	hsa-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU

C11	hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG	G12	hsa-miR-27a-3p	UUCACAGUGGCUAAGUUCCGC
C13	hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU	G14	hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC
C15	hsa-miR-186-5p	CAAAGAAUUCUCCUUUUGGGCU	G16	hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG
C17	hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	G18	hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
C19	hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC	G20	hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU
C21	hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU	G22	hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA
C23	hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA	G24	hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA
I02	hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	M02	hsa-miR-363-3p	AAUUGCACGGUAUCCAUCUGUA
I04	hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	M04	hsa-miR-374a-5p	UUAUAAUACAACCUGAUAAGUG
I06	hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	M06	hsa-miR-381-3p	UAUACAAGGGCAAGCUCUCUGU
I08	hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	M08	hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
I10	hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	M10	hsa-miR-518b	CAAAGCGCUCCCCUUUAGAGGU
I12	hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA	M12	hsa-miR-518f-3p	GAAAGCGCUUCUCUUUAGAGG
I14	hsa-miR-143-3p	UGAGAUGAAGCACUGUAGCUC	M14	hsa-miR-532-3p	CCUCCCACACCCAAGGCUUGCA
I16	hsa-miR-152	UCAGUGCAUGACAGAACUUGG	M16	hsa-miR-574-3p	CACGCUCAUGCACACACCCACA
I18	hsa-miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	M18	hsa-miR-590-5p	GAGCUUAUUCAUAAAAGUGCAG
I20	hsa-miR-192-5p	CUGACCUAUGAAUUGACAGCC	M20	hsa-miR-628-5p	AUGCUGACAUAUUUACUAGAGG
I22	hsa-miR-193b-3p	AACUGGCCCUCAAAGUCCCGCU	M22	hsa-miR-660-5p	UACCCAUUGCAUAUCGGAGUUG
I24	hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA	M24	hsa-miR-744-5p	UGCGGGGCUAGGGCUAACAGCA

K02	hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	O02	hsa-miR-758-3p	UUUGUGACCUGGUCCACUAACC
K04	hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA	O04	hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA
K06	hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA	O06	hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGU
K08	hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC	O08	hsa-miR-885-5p	UCCAUUACACUACCCUGCCUCU
K10	hsa-miR-18a-5p	UAAGGUGCAUCUAGUGCAGAUAG	O10	hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG
K12	hsa-miR-503-5p	UAGCAGCGGGAACAGUUCUGCAG	O12	hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG
K14	hsa-miR-328	CUGGCCCUCUCUGCCCUUCCGU	O14	hsa-miR-130b-3p	CAGUGCAAUGAUGAAAGGGCAU
K16	hsa-miR-335-5p	UCAAGAGCAAUAACGAAAAAUGU	O16	hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
K18	hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA	O18	hsa-miR-202-3p	AGAGGUAUAGGGCAUGGGAA
K20	hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU	O20	hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
K22	UniSp3 IPC	inter-plate calibrator/ control	O22	hsa-miR-376c-3p	AACAUAGAGGAAAUUCCACGU
K24	hsa-miR-340-5p	UUAUAAAGCAAUGAGACUGAUU	O24	hsa-miR-195-3p	CCAAUAUUGGCUGUGCUGCUCC

3 Methods

3.1 Next-generation sequencing

Next-generation sequencing (NGS) was performed at the biomedical research centre at Guy's campus of King's College, London, in cooperation with Prof. Alka Saxena. In short, this novel method used in genetic research allows determination of the exact order of nucleotides within a small RNA or DNA molecule and is an appropriate tool for sequencing the entire RNA within one sample at once – even with comparatively low input. Samples from two healthy volunteers (1 male, 1 female) were processed to PPP and PRP in order to identify potential influences and differences affected by platelets and, thus, explore their contribution to small ncRNA levels in circulation.

RNA was isolated and Cel-miR-39 was spiked into each sample for normalisation purposes before preparing the libraries with the TruSeq RNA Library Preparation Kit (Illumina). This kit allows high-throughput RNA sequencing with easier handling compared to other commercially available kits: the specific adapters ligate to both ends of the RNA molecules before all samples are reversely transcribed into complementary deoxyribonucleic acid (cDNA). Then, the cDNA was preamplified with 12 cycles of PCR and the library was purified and washed with the Agencourt AMPure XP - PCR Purification system (Beckman-Coulter). Using Qubit (Invitrogen) and Bioanalyzer (Agilent), the libraries were quantified and mixed together before sequenced on a HiSeq2000 (Illumina). After adapter removal at the end of each RNA (adapter sequence TGGAATTCTCGGGTGCCAAGG), the samples were analysed using the software 'Trim Galore' allowing a 10% mismatch. Contamination with white blood cells may confound the analysis of PPP, PRP and serum ncRNAs due to high intracellular RNA concentration. Therefore, the absence of transcripts encoding leukocyte-specific CD45 was confirmed in both PPP and PRP. Additionally, the presence of platelet-derived RNA was assessed in PRP and PPP by real-time quantitative polymerase chain reaction (RT-qPCR). A whole blood sample was used as a positive control.

3.2 RNA isolation

For RNA extraction, a phenol-guanidine-based method was used as introduced by Chomczynski in 1987 (Chomczynski and Sacchi 1987). Importantly, no heparin-treated samples were used for analysis by RT-qPCR, as the group of Prof. Mayr previously noticed interactions between magnesium and calcium in the PCR master mix reagents and heparin (Kaudewitz et al. 2013).

First, 700 µL of QIAzol lysis reagent, a monophasic solution for lysing and denaturing protein complexes and inhibiting RNases, and 4 µL of an exogenous spike-in control RNA (Cel-miR-39) were mixed with 100 µl of the original sample. 1.25 µL MS2 carrier, a bacteriophage RNA, was added in order to maximise the efficiency of extraction by increasing the total RNA amount in the sample. Then, the mixture was vortexed thoroughly and incubated at room temperature for 5 min. 140 µL of Chloroform were added in order to increase efficiency of the process of denaturing proteins in comparison to using phenol. The phenol and chloroform solution reduces the formation of insoluble protein complexes at the interphase and forces sharper separation of the aqueous phase because of higher density of Chloroform (at 20 °C: 1.47 g/ cm^3) compared to Phenol (at 20 °C: 1.07 g/ cm^3). After 15 min of centrifugation at 4 °C at 12,000 rpm, the solution is visibly separated in an upper aqueous phase and a lower organic phase: the negative charge of the DNA becomes neutralised by protonation of the sugar-molecules with decreasing pH-level in this acid. Consequently, it dissolves in the lower organic phase while hydrogen bonds are formed between the nitrogenous bases of the single-stranded RNA with water molecules, thus, miRNAs and other small ncRNAs stay in the inorganic upper phase.

Next, 280 µL of the upper aqueous phase were carefully transferred and dissolved in 420 µL of pure ethanol in order to clean the RNA from remaining contamination and to provide appropriate binding conditions for RNA molecules, especially those with a length starting from 18 nt. Afterwards, this solution was applied into a RNeasy MinElute spin column, which contains a silica membrane filter. While centrifuging at room temperature at 13,200 rpm, the RNA molecules bind to the membrane assisted by guanidine thiocyanate from the Qiazol lysis reagent before the remaining flow-through is discarded. Next, different steps of cleaning with RWT (a guanidine salt- and ethanol-based stringent washing buffer) and RPE buffer (a buffer mainly to remove remaining traces of salt) followed in order to remove contaminating reagents

from the RNA. After the membrane was transferred onto a new Eppendorf tube, 35 µL of nuclease-free water were pipetted onto the membrane, in order to solve and detach the RNA by short centrifuging. The eluted RNA including miRNA was immediately stored at -80 °C for at least 24 h before further use.

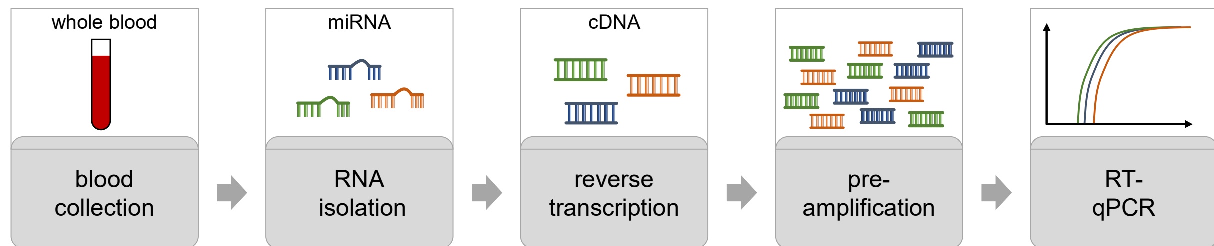


Figure 4 MiRNA analysis workflow

3.3 Reverse transcription (RT)

Since extracted and isolated miRNAs are prone to biological and experimental influences and highly susceptible for degradation, the TaqMan cDNA kits were used in order to transcribe the original miRNA molecules into cDNA templates. The kit contains reverse transcriptase enzyme, a natural catalyst from Moloney murine leukaemia retrovirus for recombinant construction of the cDNA and Megaplex stem-looped reverse transcription primers. These primers are ready to use and available as a set of two predefined pools of up to 380 primers each in order to enable simultaneous cDNA synthesis for a various number of mature miRNAs within one run. In general, an RT primer consists of a stem, a loop and an overhang sequence at the 3' end of 5 to 8 nt length. The stem-loop structure anneals specifically to the 3' end of the mature miRNA and is extended in the presence of enzyme reverse transcriptase. This species of sequence-specific primer evades the problems of the short length of mature miRNAs and enables a nearly conventional performance of RT-qPCR-analysis. Additionally, the stem-loop structure decreases hybridisation of its primer to other protein species, such as genomic DNA and long RNA molecules.

The RT reaction mix was adapted according to the manufacturer's protocol: dependent on the target miRNAs of interest, either Megaplex human pool A (v2.1) or human pool B (v3.0) RT primers were used. The Megaplex RT reaction kit components, $MgCl_2$ and the Cel-miR-39 primer, were slowly defrosted on ice. The reaction mix was prepared as follows:

Reaction Mix Components	**Vol. per sample (µL)**
Enzyme MultiScribe reverse transcriptase (50 U/ µL)	2
$MgCl_2$ (25 mM)	1.2
10 x RT buffer	1
Megaplex RT primers (10x) pool A or B	1
dNTPs with dTTP (100 mM)	0.3
RNase inhibitor (20 U/ µL)	0.2
Cel-miR-39 primer	1.3
Total	**7**

7 µL per sample of the shortly centrifuged RT reaction mix were pipetted into a 96-well plate, a fixed volume of 3 µL of the total extracted 35 µL RNA eluate was added after being defrosted and mixed by pipetting up and down six times. The plates were sealed with caps, shortly centrifuged and incubated on ice for 5 min. The PCR thermal program for the reverse transcription set up was as follows:

Stage	**Temp. (°C)**	**Time**
40 cycles	16	2 min
	42	1 min
	50	1 sec
Hold	85	5 min
Hold	4	∞

3.4 Preamplification (PreAmp)

The amplification of RNA and DNA molecules is an appropriate method for increasing the DNA amount for analysis of gene expression profiles. It has to be conducted when the target RNA for a RT-qPCR reaction is at low expression level as otherwise stochastic effects may distort the analysis (Weis, Tan et al. 1992, Zampetaki and Mayr 2012). As miRNAs are at relatively low expression levels in circulation, the amount of cDNA after RT is low as well. Therefore, 12 cycles of amplification were performed before loading the miRNA arrays. The Megaplex Taqman PreAmp system was used. The amplification reaction was performed

according to the manufacturer's protocol: either Megaplex human pool A (v2.1) or pool B (v3.0) PreAmp primers were used. These primer sets contain predefined pools of forward primers – specifically binding to a single strand of the target cDNA – and a reverse primer, which anneals to the unfolded stem-loop sequence present in every cDNA. All reagents were defrosted on ice, mixed by inverting the tube six times and shortly centrifuged. Then, the reagents were pipetted as follows into a 1.5 mL Eppendorf tube, vortexed and briefly spun down.

Components	**Vol. per sample (µL)**
TaqMan PreAmp master mix (2x)	5
Megaplex PreAmp primers pool A or B(10x)	1
Nuclease-free H_20 (PCR-H_20)	3
Total	**9**

Next, 9 µL of reaction mix were pipetted onto a 96-well plate before 1 µL of the cDNA was added and thoroughly mixed by pipetting up and down six times. After sealing the plates with adhesive film and incubation on ice for 5 min, the Thermal cycler program was set up as follows:

Stage	**Temp. (°C)**	**Time**
Hold	95	10 min
Hold	55	2 min
Hold	72	2 min
12 cycles	95	15 sec
	60	4 min
Hold*	99.9	10 min
Hold	4	∞

* enzyme inactivation

Heating the reaction mix to 95 °C causes the unwinding of the cDNA strands. The following controlled cooling down to 55 °C in the first and 60 °C in the following cycles enables the antisense primer to anneal to the template cDNA strand. The next stage at 72 °C in the first and

60 °C in the remaining cycles facilitates optimal activity for DNA polymerase, which becomes deactivated after 12 cycles by heating the reaction mix up to 99.9 °C. Then, the samples were cooled down to 4 °C, before 30 µL of nuclease-free water were added in order to solve and dilute the amplified cDNA product. Finally, the RNA product was either directly processed or stored at -20 °C for up to one week.

3.5 Polymerase chain reaction

The polymerase chain reaction – as invented in the mid-1980's – is a highly specific and sensitive method for detecting and analysing DNA and RNA. Currently, it is available in various types and is a frequently used method for profiling of miRNAs (Zampetaki and Mayr 2012). For screening experiments, the Exiqon system was used. For the study of bigger cohorts and individual miRNA assessment, RT-qPCR was performed using the TaqMan Universal PCR system. Basically, a hot-start DNA polymerase enzyme in the PCR master mix amplifies the target cDNA using sequence-specific probes and primers and, thereby, detects the presence of the targets in real time. The TaqMan probe contains a minor groove binder – which increases the melting temperature and, thus, allows a shorter probe design – a reporter dye at the 5' end and a non-fluorescent quencher at the 3' end of the probe. After every cycle, difference and absolute fluorescence is measured and indicated in an amplification curve.

First, the diluted, preamplified cDNA and all necessary reaction reagents were slowly defrosted on ice. 5 µL of the sample were further diluted with 85 µL of nuclease-free water and shortly spun down. The PCR reaction was conducted according to the manufacturer's protocol. 9 parts of TaqMan 2x universal master mix and 1 part of a specific TaqMan miRNA assay were mixed and briefly spun down. Then, 2.75 µL of the master mix/ probe solution and 2.25 µL of the diluted cDNA were pipetted (in duplicates) onto an optical 384-well reaction plate. For bigger sample numbers, the Bravo pipet robot was used. After sealing the plate with an adhesive film and centrifuging at room temperature at 1000 rpm for 1 min, RT-qPCR was performed on a ViiA7 cycler with settings baseline start 3, baseline end 15 and Reporter/ Quencher dye FAM/ MGB-NFQ. The measurements of YRNA fragments were performed with the cDNA directly from the RT product (without PreAmp). The ViiA7 RT-qPCR was set up as follows:

Stage	Temp (°C)	Time (min)
Hold	50	2
Hold	95	10
40 cycles	95	0.15
	60	1

Once the protocol is started, the specific TaqMan probe binds to its complementary sequence within the cDNA. The reporter dye emits light energy, when it is excited by specific radiation, e.g. when using FAM, excitement with about 494 nm absorbance wavelength provokes emission wavelength of 518 nm. Close distance between the reporter and the quencher causes the emitted light energy from the reporter to be absorbed by the quencher due to Förster resonance energy transfer mechanism between light sensitive molecules. This is the situation at the beginning of the RT-qPCR. However, as it progresses, DNA polymerase extends the primers' bounds and cleaves probes, which are hybridised to the target sequence. Thus, the distance between quencher and reporter increases and the quencher is no longer able to absorb the emitted reporter energy. Subsequently, there is a fluorescence signal, e.g. 518 nm for FAM, which is technically detected. The intensity of this signal indicates the number of copies and, thus, reflects the relative gene expression level within the sample.

3.6 Custom-made microRNA screening panels

Screening experiments in the thrombolysis cohort were performed using Exiqon custom-made RT-qPCR arrays. This system allows quick assessment of multiple miRNAs on a ready-to-use PCR plate. 92 miRNAs were measured using the miRCURY LNA universal RT miRNA PCR kit according to the manufacturer's protocol.

Components RT	Vol. per sample (µL)
5 x RT buffer	2
10 x enzyme	1
Spike-in control	0.5
RNA	2
RNAse free H_2O	4.5
Total	**10**

The RT reaction on the Verity Thermal cycler was performed as follows:

Stage	Temp. (°C)	Time (min)
Hold	42	60
Hold	95	5
Hold	4	∞

The RT-qPCR amplification was performed using 2x SYBR green master mix and diluted cDNA products. The RT-qPCR cycler conditions were as follows:

Stage	Temp. (°C)	Time (min)
Hold	50	2
Hold	95	10
40 cycles	95	0.15
	60	1

3.7 Comparative CT method

For the miRNA RT-qPCR analysis, the comparative cycle threshold (CT) method was used. The CT value indicates the PCR cycle number, at which the fluorescence signal passes the defined threshold. Next, the difference between the miRNA of interest (e.g. miR-126-3p) and the normalisation control (e.g. Cel-miR-39 or CT average) is calculated for each sample in order to account for differences in the input amount of cDNA. Then, the value of each sample is subtracted from the reference sample before this value is transformed using 2 to the power of (- delta delta CT), which is the fold change between the sample and the reference sample.

3.8 Platelet function tests

Platelet function tests were performed at the University of Sheffield, Department of Cardiovascular Science, by Prof. Rob Storey and collaborators: Light transmittance aggregometry (LTA), VerifyNow $P2Y_{12}$ and Vasodilator-stimulated phosphoprotein phosphorylation (VASP) assay. To date, 4 platelet function tests seem most likely to provide useful and reliable results for monitoring platelet reactivity: LTA, Multiplate, VerifyNow $P2Y_{12}$ and VASP. These are, therefore, capable of controlling $P2Y_{12}$ therapy (Aradi et al. 2014). Over

the years, LTA became the most frequently used clinical tests, but recently the introduction of the VerifyNow $P2Y_{12}$ system has created new possibilities for clinicians in terms of monitoring platelet responsiveness. Currently, there exists no recommendation – neither in the European Society of Cardiology nor in the guidelines of the American Heart Association – for performing platelet function tests in CVD patients under therapy of platelet inhibitors. However, modern test methods could provide a useful tool for monitoring and, if necessary, enable immediate adapting of antiplatelet therapy regimes (Grove et al. 2012, Aradi et al. 2014).

3.8.1 Light transmittance aggregometry

Light transmittance aggregometry is an established test method for assessing platelet reactivity and, thus, controlling the effects of antiplatelet therapy: platelet suspension is stimulated by an activator of aggregation and the dynamic light transmittance reflects the process of clotting of platelets in an aggregation curve by a photometer. PRP and PPP were extracted from citrate-anticoagulated blood. If necessary, PRP was diluted with PPP to a platelet count of 400×10^9/ L. Maximum platelet activation was assessed after 6 min using either agonist arachidonic acid (AA) (20 µM max) – in order to investigate platelet activation via thromboxane pathway – or ADP (2mM max) – in order to activate them via the ADP-receptor expressed on platelets' surface (Gum et al. 2001, Aradi et al. 2014). Analysis was performed on a BioData PAP-8E optical aggregometer.

3.8.2 VerifyNow $P2Y_{12}$ assay

The VerifyNow $P2Y_{12}$ system is a commercially available point-of-care platelet function test for assessing the response to different types of antiplatelet agents. It uses readily-prepared cartridges containing agents like arachidonic acid, ADP, thrombin receptor-activating peptide and prostaglandin E1 and measures platelet aggregation to fibrinogen-coated beads by optical turbidimetry. These data are digitally transformed into the platelet reactivity index (PRI). A major concern is its dependency on platelet count and erythrocyte volume fraction, but overall it is the most specific clinical test method for the identification of $P2Y_{12}$ inhibition to date. Samples were analysed on a VerifyNow $P2Y_{12}$ analyser by Accriva according to the manufacturer's protocol for assessing platelet reactivity in citrate-anticoagulated blood samples within the Sheffield cohort (Gurbel et al. 2007, Aradi et al. 2014).

3.8.3 Vasodilator stimulated phosphoprotein phosphorylation

The platelet vasodilator-stimulated phosphoprotein phosphorylation assay is a commercially available flow cytometry test, which assesses platelet function in response to thienopyridine drugs, such as clopidogrel, prasugrel and ticagrelor. An enormous advantage compared to other antiplatelet-testing methods is that there is nearly no interference with other drugs like GPIIb/IIIa antagonists or ASA; thus, it specifically assays the ADP dependent $P2Y_{12}$ receptor pathway. However, there exists no point-of-care test version.

Intracellular cyclic adenosine monophosphate – abundant at higher levels in resting platelets – activates protein kinases, which in turn phosphorylate the second messenger VASP. Then, monoclonal antibodies bind to the VASP, which in turn can be quantified by flow cytometry. Some studies describe the VASP method as the most accurate assay for monitoring platelet inhibition by targeting $P2Y_{12}$ receptor on platelets surface. Currently, this is the only method not being affected by concomitant $P2Y_1$ receptor activation. However, currently there is no platelet function test specifically assessing a single pathway of platelet activation (Gurbel et al. 2007, Bouman et al. 2010, Kim et al. 2013, Aradi et al. 2014, Kerneis et al. 2015). For the VASP assay, blood was collected in tubes containing citrate; the first few millilitres were discarded in order to prevent activation of platelets in the sample. The samples were then analysed on a LSRII flow cytometer (Becton Dickinson) using a VASP assay kit by BioCytex, France. The cytometer calculates the PRI: a lower percentage indicates a good response to $P2Y_{12}$ inhibition, a higher percentage shows a hypo-responsiveness; healthy volunteers normally reach values bigger than 69%, sufficient anti-$P2Y_{12}$ therapy is reflected by a PRI below 50%.

3.9 Spike-in experiment

3.9.1 Isolation of platelets

Acid-Citrate-Dextrose stock solution was made up of 100 mL water, 1.5 g citric acid, 2.0 g glucose, and 2.5 g sodium citrate. Platelets were isolated from 16 mL of peripheral venous whole blood into a tube containing 4 mL of anticoagulant Acid-Citrate-Dextrose. After centrifuging at 190 x g_n for 30 min at room temperature, the supernatant was transferred into a new tube and 20 µM indomethacin and 1 nM prostaglandin E1 were added to avoid platelet activation. Another centrifugation step of the supernatant at 180 x g_n for 10 min at room temperature followed in order to deplete remaining leukocytes before transferring 100 µL of the supernatant into to a new 1.5 mL Eppendorf tube.

3.9.2 PRP and PPP

Like all nucleated body cells, white blood cells contain a high number of RNAs, which would influence ncRNA measurements in serum and plasma**.** For the purpose of depleting such cell remnants, samples were centrifuged at room temperature at 280 × g_n for 10 min. 100 µL of the supernatant, the PRP, were transferred into a new 1.5 mL tube. Furthermore, in order to separate the PRP from platelet remnant and to prepare PPP, the sample was centrifuged at room temperature at 1180 × g_n for 10 min. For the spike-in experiment, the remnant platelet pellet was washed twice in modified Tyrode's buffer (134 mM NaCl, 20 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), 12 mM $NaHCO_3$, 2.9 mM KCl, 1mM $MgCl_2$, 0.34 mM Na_2HPO_4, Glucose 45 mg/ 50 mL). Between washing, prostaglandin E1 and indomethacin were added and centrifuged at room temperature at 1180 × g_n for 10 min. Subsequently, the platelet pellet was re-suspended in 5% of the original PRP volume to get a 20x stock solution per each sample and this solution was spiked into PPP of the same individual in increasing amounts corresponding to 5, 50, 100 and 200% platelet count of PRP. Then, RNA was extracted immediately from whole blood, PPP, PRP, platelets, serum and plasma. For normalisation control purposes, an exogenous spike-in-Control, synthetic Cel-miR-39 mimic, was used.

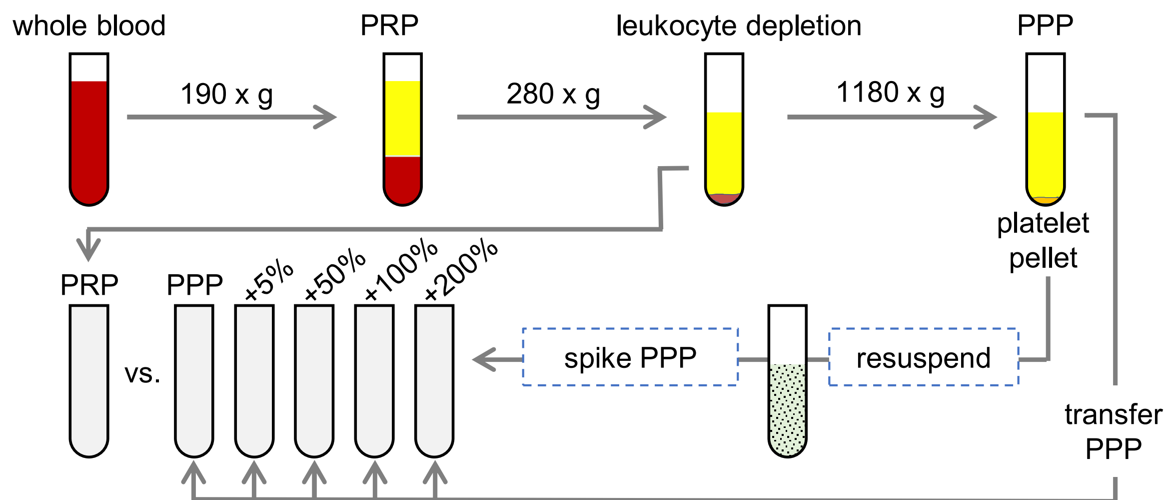


Figure 5 Workflow of the platelet spike-in experiment
This figure originates from Kaudewitz et al. (Kaudewitz, Skroblin et al. 2016).

3.10 ELISA

The enzyme-linked immunosorbent assay (ELISA) is an established method for protein analysis. In the present work, it was used to quantify levels of platelet factor 4 (PF4), pro-platelet basic protein (PPBP) and P-selectin (SELP) within plasma samples of the Bruneck population cohort. The ELISA development kits by R&D Systems were used according to the manufacturer's protocol. Light transmittance was measured at 450 nm wavelength on a Tecan Infinite 200 reader using 570 nm as a reference wavelength. Statistical calculation is based on the four-parameter logistic fit nonlinear regression model.

3.11 SNP genotyping

The competitive allele-specific PCR (KASP) method is a relatively simple PCR-based method for single nucleotide polymorphism (SNP) discovery. In the experiments conducted for this thesis, the KASP kit by LGC was used for analysing the abundance of the rs4636297 SNP in the Bruneck samples according to the manufacturer's protocol. Basically, the KASP assay mix contains two allele-specific primers with different fluorescence dyes. In case of a heterozygous genotype at the investigated SNP, two different fluorescent signals are detectable. Only one emittance signal is measurable if the genotype at this SNP is homozygous.

3.12 Bruneck population study

The Bruneck ischaemic heart disease and stroke prevention study is a prospective, cross-sectional population-based investigation on CVDs, especially on ACS and stroke and its risk factors, hosted at the hospital of Northern Italian town Bruneck in cooperation with the University Hospital of Innsbruck. The study was approved by the research ethics committees of Bolzano and Verona. Recruitment and baseline investigations started in 1990 (n=1000) and were followed-up in a 5-year cycle. After study recruitment, exclusion criteria were insulin treatment, incomplete data collection or insufficient sample collection. Altogether, 909 individuals were selected at baseline in 1990. For the work at hand, venous serum and plasma samples from the year 2000 follow-up were used (n=669). The standard procedure was as follows: all patients had to be abstinent from smoking for at least 12 hours, then, peripheral blood was drawn by venepuncture. Serum and citrate plasma samples were divided into aliquots and immediately stored at -80 °C as previously described by Kiechl et al. (Kiechl et al. 2002). Plasma samples from the Bruneck study cohort have previously been used for miRNA profiling in the Mayr group (Zampetaki et al. 2010, Zampetaki et al. 2012, Willeit et al. 2013). For the work at hand, RT-qPCR Screening (Exiqon), individual RT-qPCR (Taqman), ELISA, the expression of different platelet activation proteins and the distribution of a specific SNP were assessed.

3.13 Sheffield ACS patients

The cohort comprises 125 ACS patients recruited from the Northern General Hospital, Sheffield, UK, enrolled in a retrospective observational study. The study was approved by the local research ethics committee and the participants provided written consent. Exclusion criteria were less than 18 years of age, suspected or confirmed pregnancy, end-stage renal failure or serious co-morbidities. One month after diagnosis of ACS, venous blood was collected from patients suffering from STEMI, NSTEMI or unstable angina pectoris and prevented from clotting by 3.13% trisodium citrate dihydrate and processed to plasma. According to clinical standard of care, patients were treated with dual antiplatelet therapy as described by Joshi et al. (Joshi et al. 2014). RT-qPCR Screening (Exiqon), individual RT-qPCR (Taqman) assessments and different platelet function tests were performed in these samples.

3.14 Healthy volunteers taking antiplatelet agents

Samples were provided from 9 male healthy volunteers (age 18-40 years), participating in a study at St Thomas Hospital, London, UK. All study subjects gave written consent and the study was approved by the local research ethics committee (Ref. 07/Q0702/24). The study protocol is described elsewhere (Leadbeater et al. 2011). After a loading dose of 60 mg Prasugrel on day 1 and a maintenance dose of 10 mg OD, 75 mg OD ASA were added on day 8 and increased to the amount of 300 mg until day 21. Blood was drawn on baseline and on days 7, 14 and 21 by peripheral venepuncture into tubes containing trisodium citrate (3.2%, 1:9 v/v; Sigma, Poole, Dorset, UK).

3.15 Radial injury cohort

The radial injury experiment was conducted to investigate the effects of local endothelial denudation by wire rotation in the radial artery on levels of selected circulating miRNAs. Healthy volunteers (n=15) who underwent local endothelial injury participated in a study at King's College London, UK. All study subjects gave written consent and the study was approved by the local research ethics committee (REC 07/Q0702/62). After access to the radial artery through a cannula, endothelial denudation was induced by wire rotation. Venous blood samples were drawn from 5 healthy volunteers at both the injured and uninjured arm and from the others (n=10) at the injured site only.

3.16 Thrombolysis cohort

The thrombolysis cohort comprises 21 patients with symptoms and radiological appearance of ischaemic stroke treated at the University Hospital of Innsbruck, Austria. These patients underwent thrombolysis therapy with intravenously applied rtPA. Venous blood samples were collected immediately before, 6 h post and 24 h post thrombolysis and coagulation was preventes. In order to identify changes in miRNA patterns, a screening experiment using Exiqon custom-made RT-qPCR plates was performed. For validation purposes, individual TaqMan RT-qPCR assays were used. Patients' characteristics are shown in Table 2.

Table 2 Characteristics of stroke patients
Clinical characteristics of 21 ischaemic stroke patients who underwent thrombolysis therapy at the University Hospital of Innsbruck, Austria.

Number	**21**
Age, years (SD)	**72** (12)
Diabetes mellitus, n (%)	**2** (10)
Smoker (%)	**5** (24)
NIHSS (SD)	
before treatment	**12** (6)
24 h after treatment	**6** (6)
at discharge	**4** (5)
Infarction visible on CT or MRI, n (%)	**18** (86)
Infarction location, n (%)	
MCA	**12** (57)
Basal ganglia	**3** (14)
PCA	**1** (5)
Thalamus	**1** (5)
Pons	**1** (5)
N/A	**3** (14)
Responder, n (%)	**17** (81)
Aetiology, n (%)	
atherothrombotic	**9** (43)
cardioembolic, AF	**5** (24)
lacunar	**3** (14)
cryptogenic	**3** (14)
arterial dissection	**1** (5)

4 Results

4.1 Next-generation sequencing

NGS was performed in order to analyse the general abundance of small ncRNAs in peripheral blood components. For the identification of influences of platelets, PPP and PRP of two healthy volunteers was used. Figure 6 shows the NGS results of the PPP samples: a first peak appears at a sequence length of 21-24 nt, which contains miRNAs, and a second one at about 32-33 nt lengths, which corresponds to fragments of YRNAs.

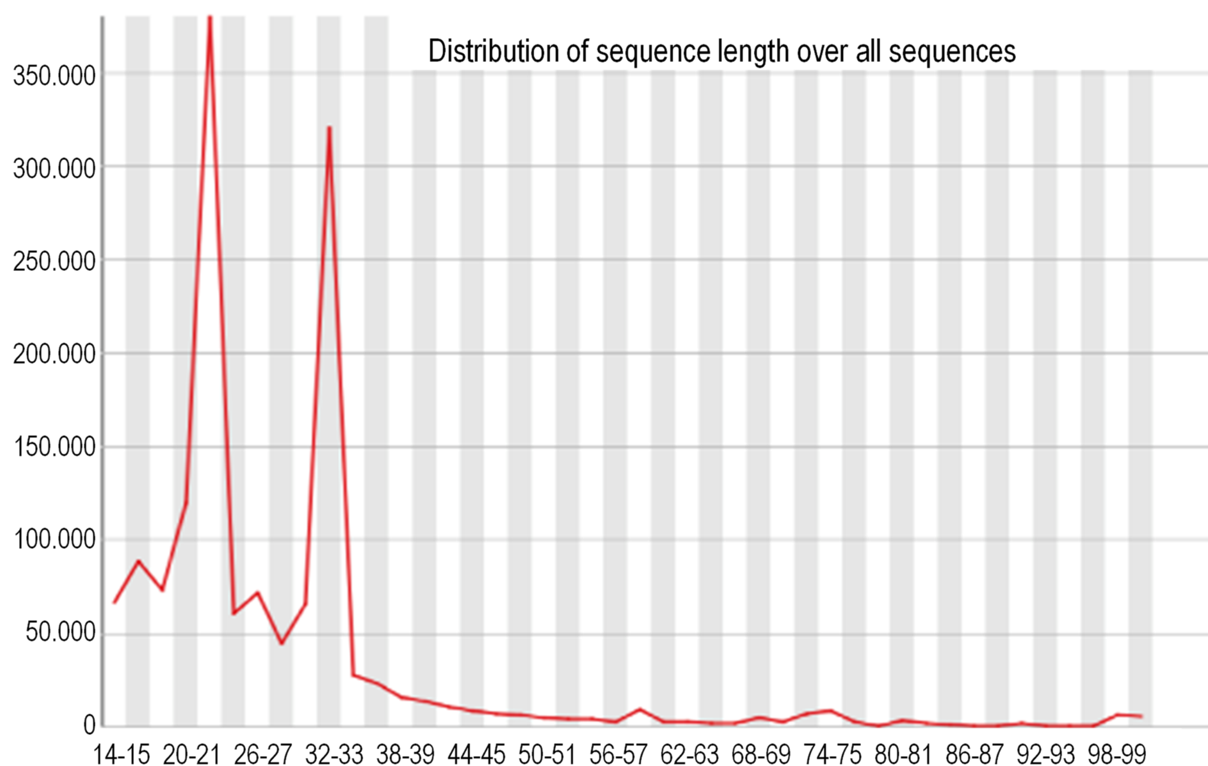


Figure 6 Next-generation sequencing
Histogram showing results of NGS of PPP samples (n=2) after adapter removal. The axis of abscissae shows the sequence length in bp, the axis of ordinates the read counts. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

4.1.1 Exclusion of contamination

The author sought to identify circulating miRNAs. To exclude the possibility of cellular contamination, in particular with leukocyte RNA, RT-qPCR for CD45 was performed. Results are shown in Table 3. CD45 was not detected in any of the samples. Integrin alpha-IIb (ITGA2B) was detectable in PRP but not in PPP samples, which demonstrates that these samples differ in their platelet content.

Table 3 RT-qPCR confirmation of leukocyte depletion
Integrin alpha IIb (ITGA2B), beta-actin (Actb) and CD45 were assessed with individual TaqMan assays in four samples of two healthy volunteers (same as used for NGS). Whole blood was used as a positive control. CT values are shown as mean, RT-qPCR was performed in duplicates. This table is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

Sample	**CT ITGA2B**	**CT CD45**	**CT Actb**
PPP 1	not detectable	not detectable	37.21
PPP 2	not detectable	not detectable	37.40
PRP 3	33.56	not detectable	32.81
PRP 4	36.89	not detectable	33.86
whole blood	not assessed	22.75	24.60

4.1.2 NGS reveals YRNA fragments

The sequencing results revealed two distinct groups of small ncRNAs: in addition to miRNAs, RNA fragments of 31-33 nt length corresponding to fragments of YRNAs were found. YRNAs are ncRNAs of 84-112 nt length that are mainly aligned to Chromosome 7 148660407-148660502 and are ubiquitously expressed in mammalian cells (Dhahbi et al. 2013). Nonetheless, the abundance of circulating YRNA fragments in circulation was surprising. While analysing the distribution of YRNA fragments, the sequencing reads showed mainly RNY4 derived fragments – predominantly from the 5' and less from the 3' end. This was also the case for RNY1- and RNY5-derived fragments. Furthermore, the abundance of YRNA fragments was notably higher in PRP compared to PPP (see Figure 7).

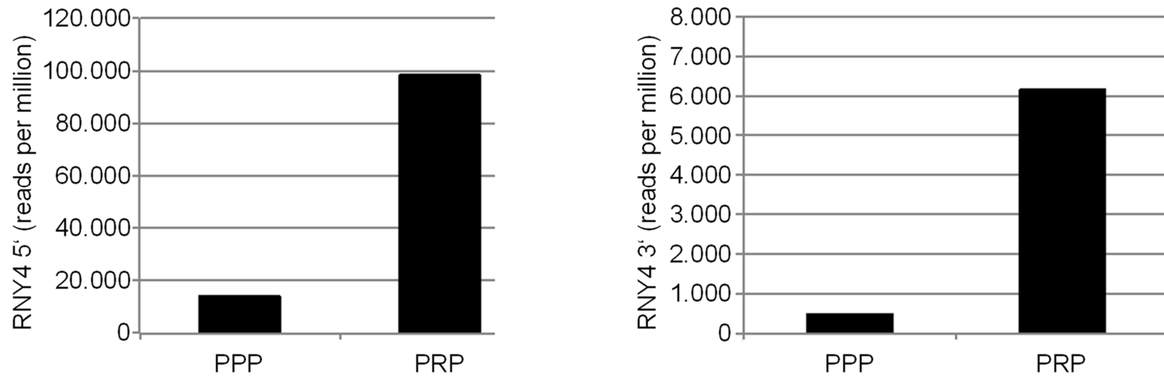


Figure 7 NGS reveals YRNA fragments
Fragments of YRNAs were expressed at higher levels in PRP compared to PPP (n=2; 1 male, 1 female healthy volunteer). This figure originates from Kaudewitz et al. (Kaudewitz et al. 2016).

4.1.3 The 25 most abundant small ncRNAs in NGS

NGS of small ncRNAs identified a total of 224 miRNAs in both PPP and PRP after adapter removal. Table 4 displays the 25 most abundant small ncRNAs across all 4 samples. Interestingly, not only RNY4-derived fragments were detected by NGS but also both strands of certain miRNAs, formerly termed “sense” and “antisense”, “star” and “non-star” or “guide” and “passenger” strands, such as miR-126-3p and miR-126-5p, miR-223-3p and miR-223-5p, miR-21-3p and miR-21-5p (see Figure 8). For the following miRNAs, public database mirbase[5] contains no definition as to which of the two strands is listed as the passenger and the guide strand: miR-151a, miR-338, miR-339, miR-361 and miR-423.

[5] URL: “http://www.mirbase.org/”, visited 20.10.2018, 12.15 CET.

Table 4 Small ncRNAs detected by NGS
Ranking of the 25 most abundant small ncRNAs in both PPP and PRP of two healthy volunteers. "N.-Cnt" denotes normalisation count, reads in million total reads. Fragments of YRNAs are highlighted in bold. This table originates from Kaudewitz et al. (Kaudewitz et al. 2016).

	PPP				PRP			
	Sample 1		2		1		2	
	Name	N.-Cnt	Name	N.-Cnt	Name	N.-Cnt	Name	N.-Cnt
1	**RNY4 5'**	**22308**	miR-486	15392	**RNY4 5'**	**97325**	**RNY4 5'**	**101280**
2	miR-486	20832	**RNY4 5'**	**5957**	miR-486	22977	miR-486	22038
3	miR-92a	2865	miR-92a	2417	miR-26a	15454	miR-191	16986
4	miR-191	1651	miR-22	1168	miR-191	14051	miR-26a	14734
5	miR-423-5p	1428	miR-191	897	miR-92a	10121	miR-92a	9482
6	miR-10b	1336	miR-423-5p	894	let-7f	8248	let-7f	8999
7	**RNY5 5'**	**1213**	miR-16	772	miR-22	8222	miR-22	8306
8	miR-22	1191	miR-26a	689	**RNY4 3'**	**7183**	let-7a	6179
9	let-7f	813	miR-10b	668	let-7a	5986	miR-423-5p	5803
10	miR-142	810	miR-142	586	miR-423-5p	4367	**RNY4 3'**	**5091**
11	miR-26a	808	miR-451a	553	miR-181a	3911	miR-126-5p	4900
12	**RNY4 3'**	**735**	**RNY5 5'**	**521**	miR-30d	2943	miR-181a	3965
13	miR-181a	726	miR-181a	515	miR-151a-5p	2846	miR-21-5p	3658
14	miR-16	528	let-7f	463	miR-16	2813	miR-30d	3526
15	miR-10a	520	miR-25	341	miR-126-5p	2661	miR-16d	3069
16	let-7a	409	miR-126-5p	327	miR-423-3p	2642	miR-142	2659
17	miR-126-5p	397	miR-10a	326	miR-222	2349	miR-151a-5p	2425
18	miR-25	390	let-7a	257	miR-142	2293	miR-30e	2088
19	miR-192	357	miR-27b	241	miR-21-5p	2221	miR-151a-3p	2076
20	miR-27b	331	miR-30e	239	**RNY5 5'**	**1892**	miR-423-3p	1976
21	miR-30d-5p	293	**RNY4 3'**	**238**	miR-30e	1845	miR-27b	1928
22	**RNY1 3'**	**255**	miR-30d	235	miR-151a-3p	1724	miR-222	1837
23	miR-21-5p	249	miR-192	210	miR-409	1438	**RNY5 5'**	**1679**
24	miR-148a-3p	240	miR-21-5p	172	miR-28-3p	1373	miR-28-3p	1667
25	miR-423-3p	239	miR-186	172	miR-27b	1252	let-7i	1425

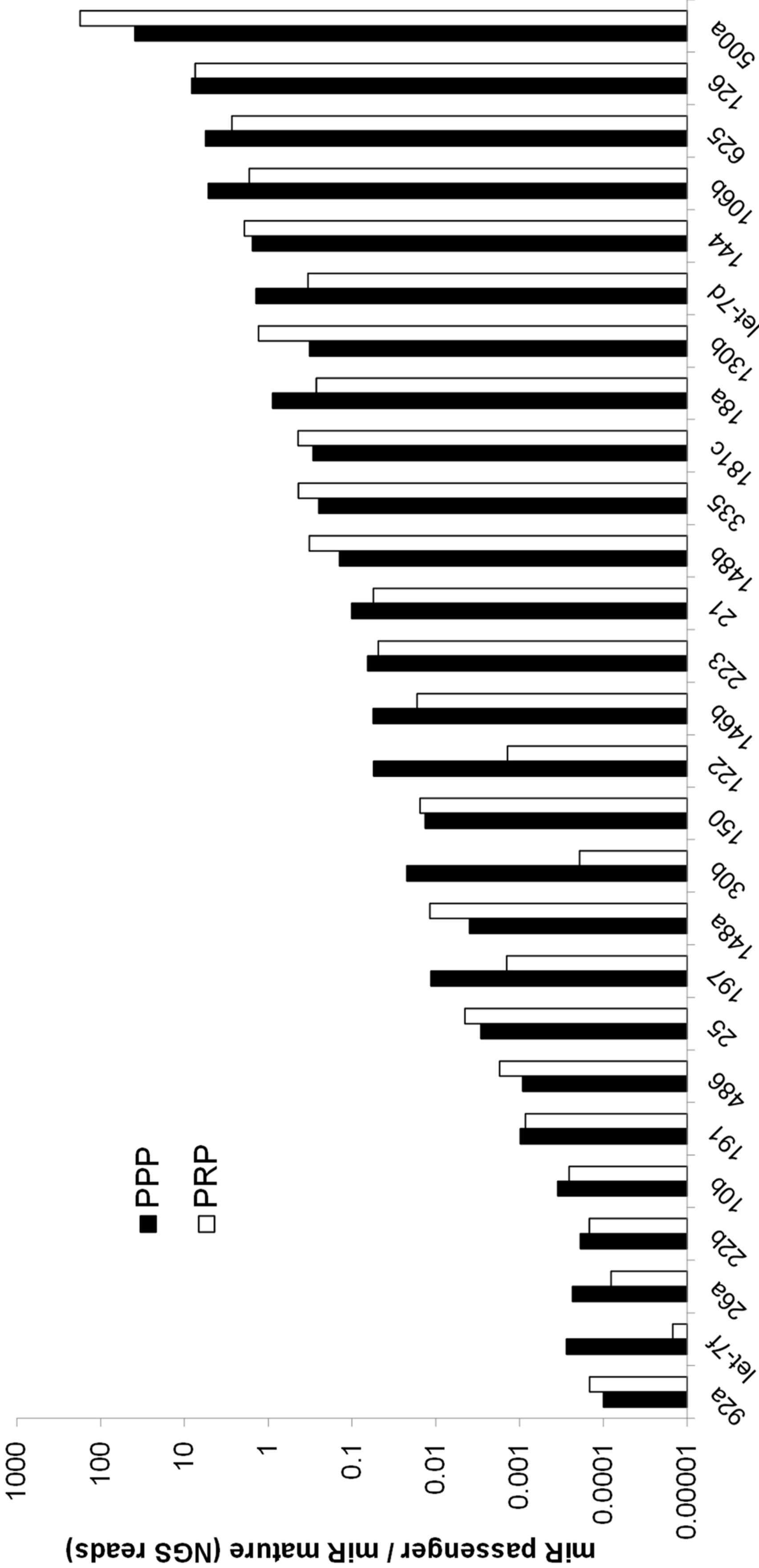


Figure 8 NGS analysis
Proportion of reads for the passenger/ star strands relative to the corresponding guide/ mature strand in PPP and PRP (n=2 per group).

4.2 Confirmation of YRNA fragments by RT-qPCR

To validate the NGS results regarding abundant YRNA fragments in circulation, individual RT-qPCR analysis was performed using TaqMan custom-made primers for RNY4 5' and RNY4 3' in platelets, PPP and PRP from three healthy volunteers. Notably, RNY4 5' and 3' fragments were abundant in platelets, suggesting a potential contribution of platelets on levels of YRNA fragments in peripheral blood (see Figure 9).

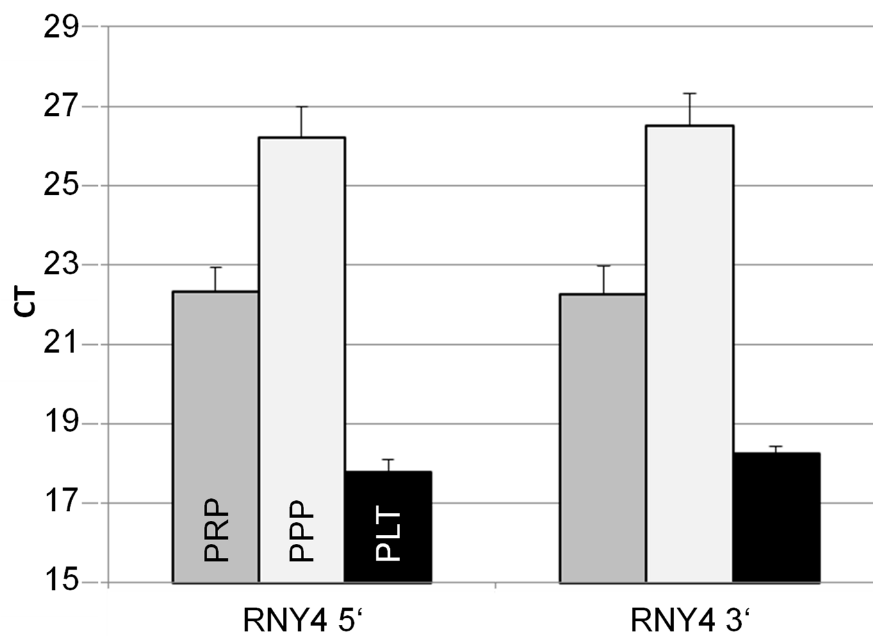


Figure 9 RT-qPCR assessing YRNA fragments
Individual TaqMan RT-qPCR detecting YRNA fragments in PPP, PRP and platelets (PLT). CT values are shown as CT mean, RT-qPCR was performed in duplicates, n=3, x-axis=CT value, error bar=SD.

4.3 RT-qPCR measurements of miR-126-5p and miR-223-5p

For evaluation of the corresponding expression of 3p and 5p strands from the same miRNAs, two platelet-related miRNAs (miR-126 and miR-223) were screened by individual RT-qPCR. Although the TaqMan system allows multiple assessments of miRNAs within one sample, there is a necessity to choose between two different sets of RT and PreAmp primer pools (pool A and pool B). Therefore, RT-qPCR for both corresponding 3p and 5p strands of miR-126 and miR-223 cannot be performed in the same cDNA product as the primers for the 3p and 5p strands are part of different pools. Initially, 10 randomly chosen samples from the Bruneck cohort were analysed. The mean CT value was 29.44 and 30.27 for miR-126-5p and miR-223-5p. Thus, the 5p strands were readily detectable (see Figure 10). Their presence was confirmed by individual RT-qPCR. Later, these miRNAs were analysed among others in the entire Bruneck cohort.

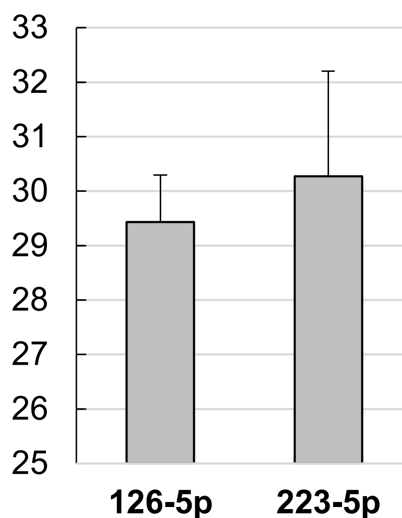
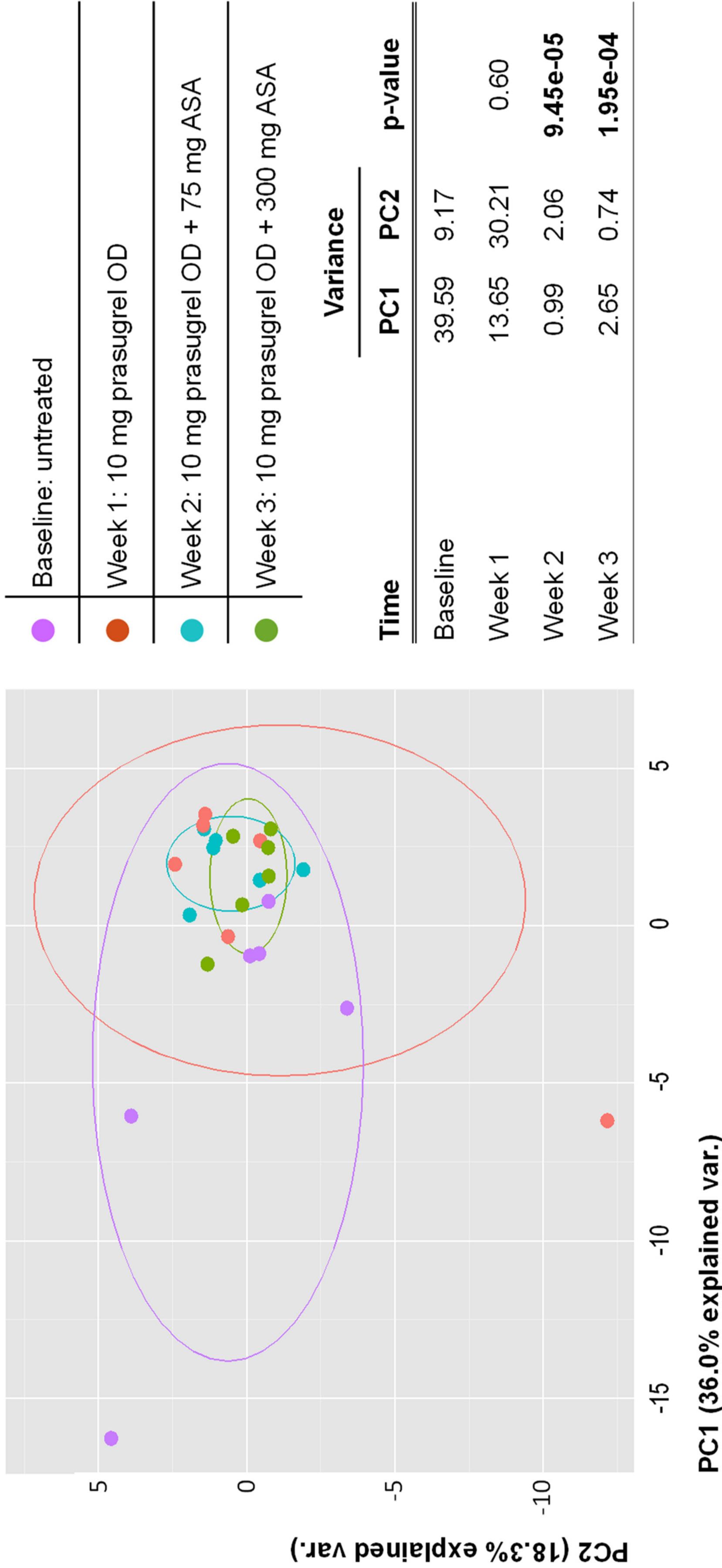


Figure 10 RT-qPCR screening with primer pool B
RT-qPCR performed in 10 plasma samples of the Bruneck cohort, x-axis=CT mean, error bar=SD.

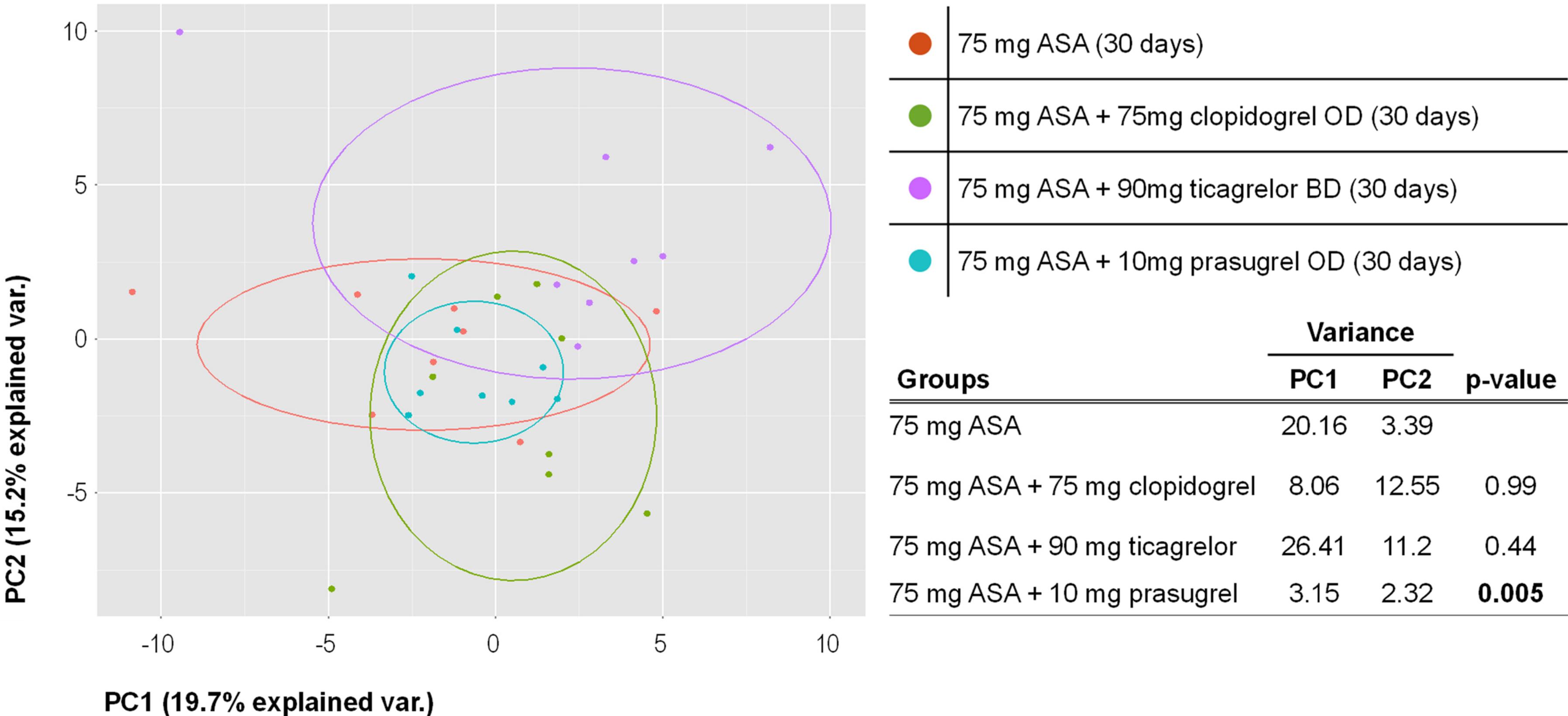
4.4 Antiplatelet therapy affects microRNA concentration

Next, experiments focussed on platelet-related miRNAs as previous work by the Mayr lab and others had shown a connection between platelet inhibition and miRNA abundance in the circulation (de Boer et al. 2013, Willeit et al. 2013). In samples from 6 healthy volunteers another screening for 92 miRNAs using custom-made Exiqon RT-qPCR plates was performed. Baseline measurements before medication were compared to measurements after one week of administration of 10 mg prasugrel and another week of administration of 10 mg prasugrel + 75 mg ASA and, finally, 10 mg prasugrel + 300 mg ASA. The miRNA profile revealed decreasing inter-individual variability in levels of platelet-related circulating miRNAs with prolonged antiplatelet medication. The principal component analysis (PCA, see Figure 11) based on 92 miRNAs shows circulating miRNA patterns to be affected by antiplatelet medication and, thus, presumably by platelet activity. Similarly, the same 92 miRNAs were measured in 32 closely matched ACS-patients treated with different antiplatelet medication (see Figure 12). Patients received either 75 mg ASA only (n=8), 75 mg ASA + 5 mg clopidogrel OD (n=8), 75 mg ASA + 90 mg ticagrelor BD (n=8) or 75 mg ASA + 10 mg prasugrel OD (n=8). MiRNAs were measured using the same custom-made RT-qPCR plates (Exiqon). The time for collecting plasma samples was chosen to be as late as 30 days after the event in order to avoid confounding by acute inflammation. Among the four treatment groups, the lowest inter-individual differences in miRNA patterns were observed in the ASA + prasugrel cohort as shown in the PCA (see Figure 12).



Time	Variance PC1	Variance PC2	p-value
Baseline	39.59	9.17	
Week 1	13.65	30.21	0.60
Week 2	0.99	2.06	**9.45e-05**
Week 3	2.65	0.74	**1.95e-04**

Figure 11 PCA healthy volunteers
6 male, healthy volunteers were chosen for screening for 92 miRNAs by RT-qPCR using custom-made Exiqon miRNA RT-qPCR plates. This figure originates from Kaudewitz et al. (Kaudewitz et al. 2016).



Groups	Variance PC1	Variance PC2	p-value
75 mg ASA	20.16	3.39	
75 mg ASA + 75 mg clopidogrel	8.06	12.55	0.99
75 mg ASA + 90 mg ticagrelor	26.41	11.2	0.44
75 mg ASA + 10 mg prasugrel	3.15	2.32	**0.005**

Figure 12 PCA ACS patients
32 ACS patients undergoing different regimens of antiplatelet therapy for 30 days were assessed for 92 miRNAs by RT-qPCR using Exiqon custom-made RT-qPCR plates. This figure originates from Kaudewitz et al. (Kaudewitz et al. 2016).

4.5 5p strands of miR-126 and miR-223

Next, 5p strands of miR-126 and miR-223 were analysed in platelets, serum and plasma of healthy volunteers. The ratio of miR-126-5p to miR-126-3p was higher in platelets compared to serum and plasma, while the ratio of miR-223-5p to miR-223-3p was lower in platelets compared to serum and plasma (see Figure 13). The influences of different antiplatelet agents on circulating levels of the 5p strands of miR-126 and miR-223 were further investigated. Individual TaqMan RT-qPCR analysis of platelet-related miRNAs was performed in healthy volunteers (n=23) and in ACS patients (n=155). Patients were treated with ASA only or ASA in combination with a $P2Y_{12}$ inhibitor. The distribution of both 3p and 5p strands of miR-126 and miR-223 differed among the treatment groups. Especially in the ticagrelor group, an increase of miR-126-5p but not of miR-223-5p was observed. When levels of miR-126-3p and miR-223-3p were compared to their respective passenger strands, miR-126-5p was detected at lower mean Ct values in patients on ASA + ticagrelor and amounted to 6.48% of miR-126-3p compared to 3.79-3.96% in all other treatment groups. The proportion of miR-223-5p to miR-223-3p remained unaltered (see Figure 14).

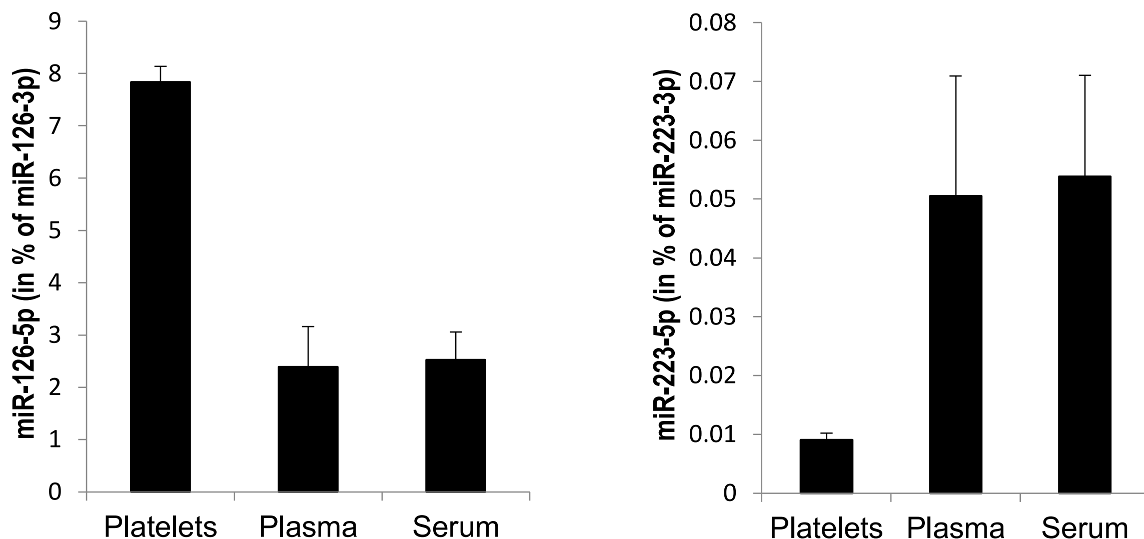


Figure 13 Distribution of miR-126-5p & miR-223-5p in platelets, serum & plasma
Proportion of 5p strands of miR-126 and miR-223 in platelets (n=4), plasma (n=23) and serum (n=23) of healthy volunteers. RT-qPCR analysis was performed using individual TaqMan assays. Error bar=SD.

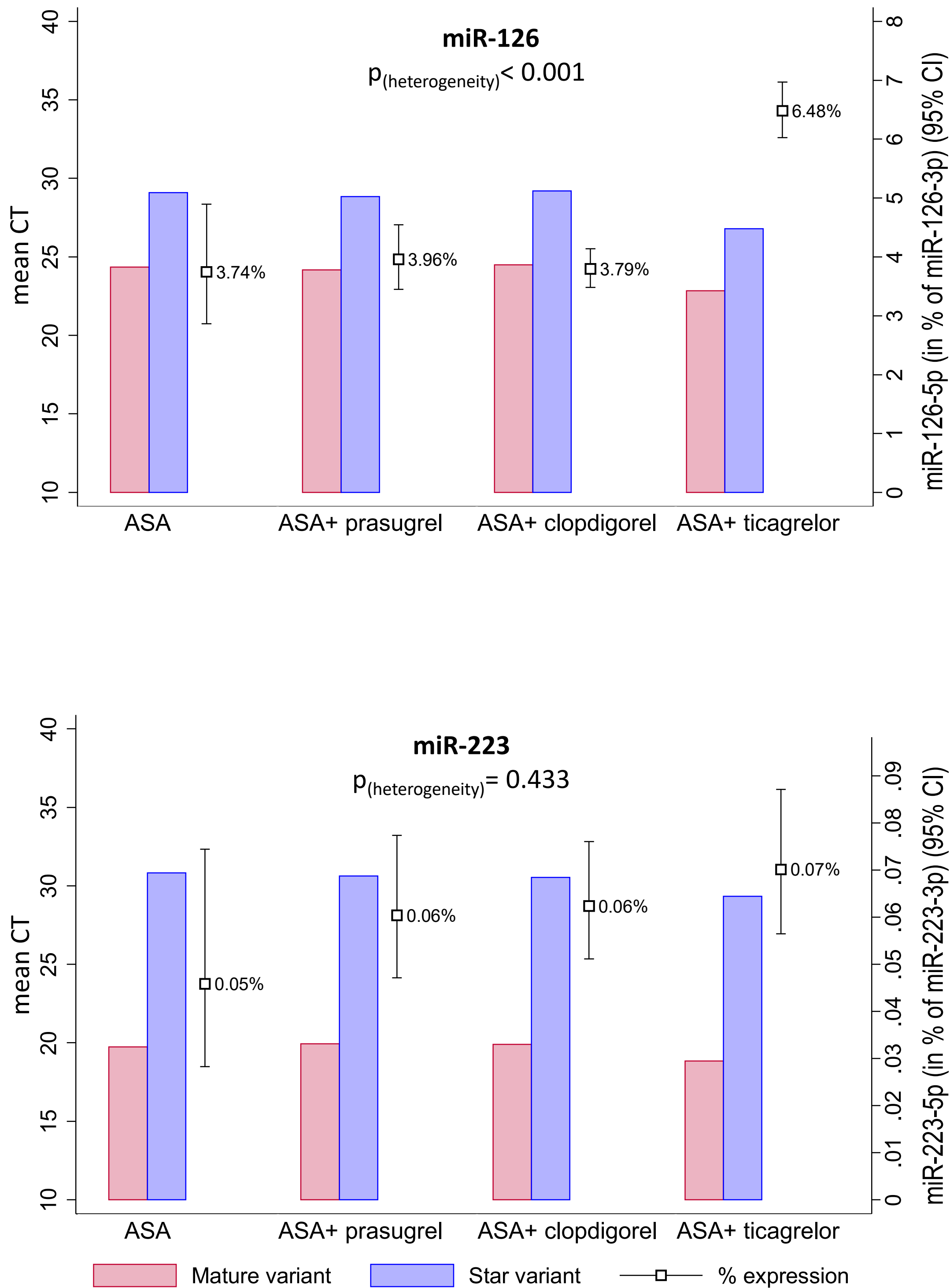


Figure 14 MiR-126 and miR-223 in response to antiplatelet agents
Alterations of the mean Ct value (left axis) of the 3p and 5p strands for miR-126 and miR-223 in patients with different antiplatelet therapies are shown (total n=155; ASA only=13; ASA + prasugrel=33, ASA + clopidogrel=77, ASA + ticagrelor=32). Note the proportional increase of miR-126-5p (right axis, % expression of guide strand) but not of miR-223-5p in plasma of ACS patients treated with ASA + ticagrelor. P-values refer to the heterogeneity between treatment groups.

4.6 MicroRNAs and platelet function tests

Next, the author aimed to identify correlations between platelet function and circulating small ncRNAs. The platelet activity was assessed using three different platelet function tests, LTA, VerifyNow and VASP. The results are shown in Figure 15. LTA did not correlate with any of the selected miRNAs, however, a statistically significant association with the VerifyNow $P2Y_{12}$ and the VASP assay was found (see Table 5). The VerifyNow $P2Y_{12}$ assay showed a significant positive association with miR-126-3p, the VASP assay showed a significant positive association with miR-24, miR-126-5p, miR-126-3p, miR-191, miR-223-3p and fragments of YRNAs, RNY4 3' and RNY4 5'. Notably, a similar correlation was observed for both 3p and 5p strands of miR-126 and platelet reactivity. All assessed miRNAs are part of the platelet transcriptome (Ple et al. 2012). Some of the analysed miRNAs, e.g. miR-126 and miR-223, showed a high correlation with platelet function whereas others correlated less or not at all, e.g. miR-93, miR-106a, miR-146b and miR-150 (see Table 5).

Table 5 Platelet function tests
Correlation between selected miRNAs and platelet function tests (VerifyNow $P2Y_{12}$ and VASP) in ACS patients. Significant values ($p<0.05$) are marked in bold. "rp" denotes partial correlation.

	VerifyNow n=40		VASP n=121	
small ncRNA	**rp**	**p-value**	**rp**	**p-value**
miR-20b	0.26	0.116	0.22	**0.014**
miR-24	0.26	0.119	0.25	**0.006**
miR-126-3p	0.35	**0.033**	0.22	**0.016**
miR-126-5p	0.27	0.117	0.31	**0.001**
miR-191	0.24	0.145	0.24	**0.009**
miR-197	0.17	0.310	0.23	**0.012**
miR-223-3p	0.24	0.139	0.28	**0.002**
miR-223-5p	0.20	0.247	0.10	0.279
RNY4 3'	0.25	0.128	0.23	**0.012**
RNY4 5'	0.18	0.292	0.21	**0.025**

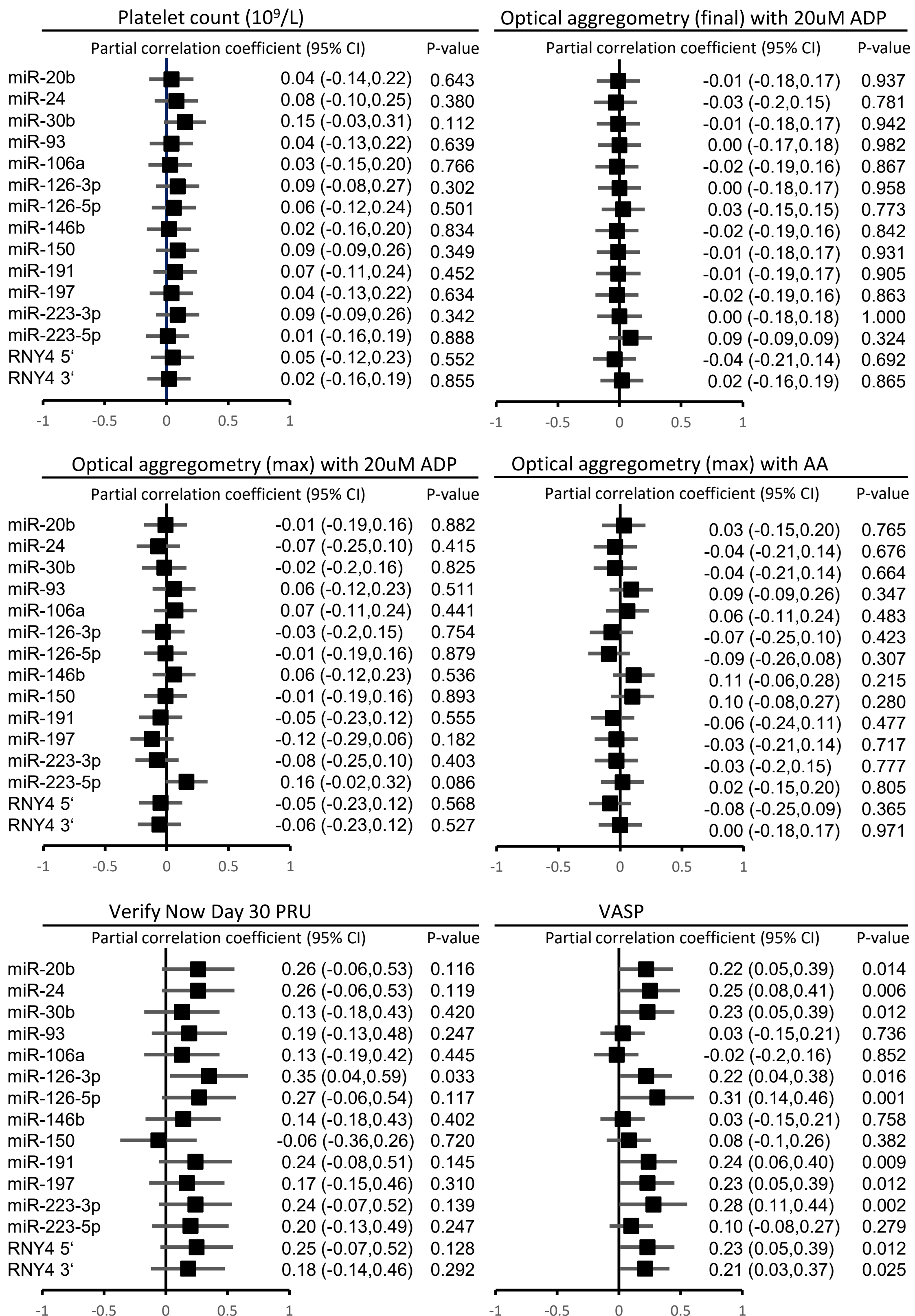


Figure 15 Pearson correlation forest plot

Correlations of small ncRNAs to platelet count and function tests in 125 ACS patients undergoing antiplatelet therapy were analysed. Sample numbers were lower for the VASP (n=121) and Verify Now $P2Y_{12}$ (n=40) assays. 11 miRNAs and two fragments of YRNAs were assessed by individual RT-qPCR, normalised to Cel-miR-39. AA denotes arachidonic acid. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

4.6.1 Platelet spike-in experiment

In order to verify whether selected miRNAs and YRNA fragments derive from platelets, isolated and washed platelets from PRP were re-suspended into PPP in increasing fractions of the initial volume. RT-qPCR was performed using individual TaqMan assays in order to analyse the abundance of both selected miRNAs and YRNA fragments, RNY4 3' and RNY4 5' (see Figure 16). MiR-223-3p as well as miR-223-5p showed a linear increase with platelet spike-in, even though the latter to a lesser extent. MiR-126-5p and miR-126-3p showed a similar increase. Circulating miR-126 was formerly believed to be derived mostly from ECs (Fish, Santoro et al. 2008, Wang, Aurora et al. 2008). The obtained results highlight that both strands of miR-126 reside within platelets at considerable amounts. Strong associations were also observed for RNY4 3' and RNY4 5' as well as miR-191 and miR-21. No dependency on platelets was observed for miR-122: this miRNA is liver specific and, thus, the platelet spike-in did not affect miR-122 levels (see Figure 16 and 17). Similar results were observed for miR-486, which is highly expressed in red blood cells (Pritchard et al. 2012).

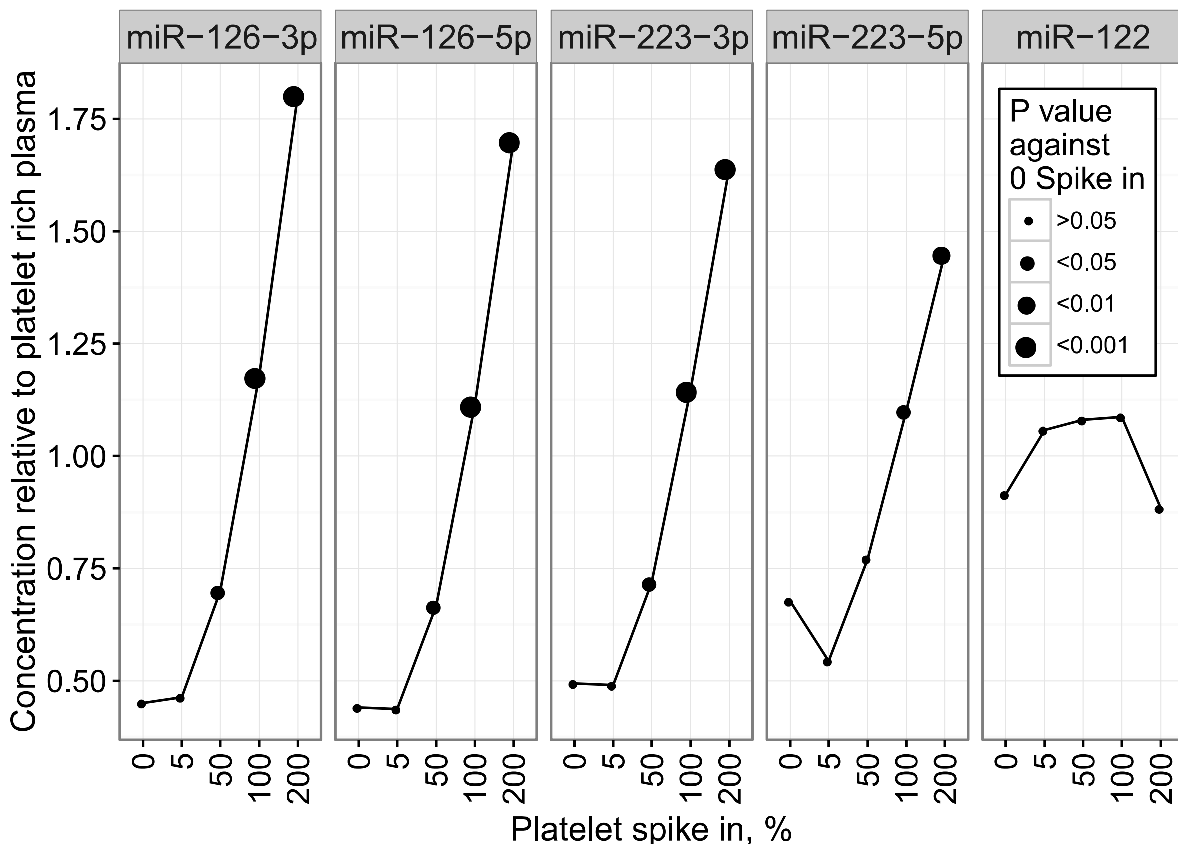


Figure 16 Platelet spike-in experiment in PPP
Levels of miR-126-3p, miR-126-5p, miR-223-3p, miR-223-5p and miR-122 relative to PRP as assessed in the platelet spike-in experiment in PPP. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

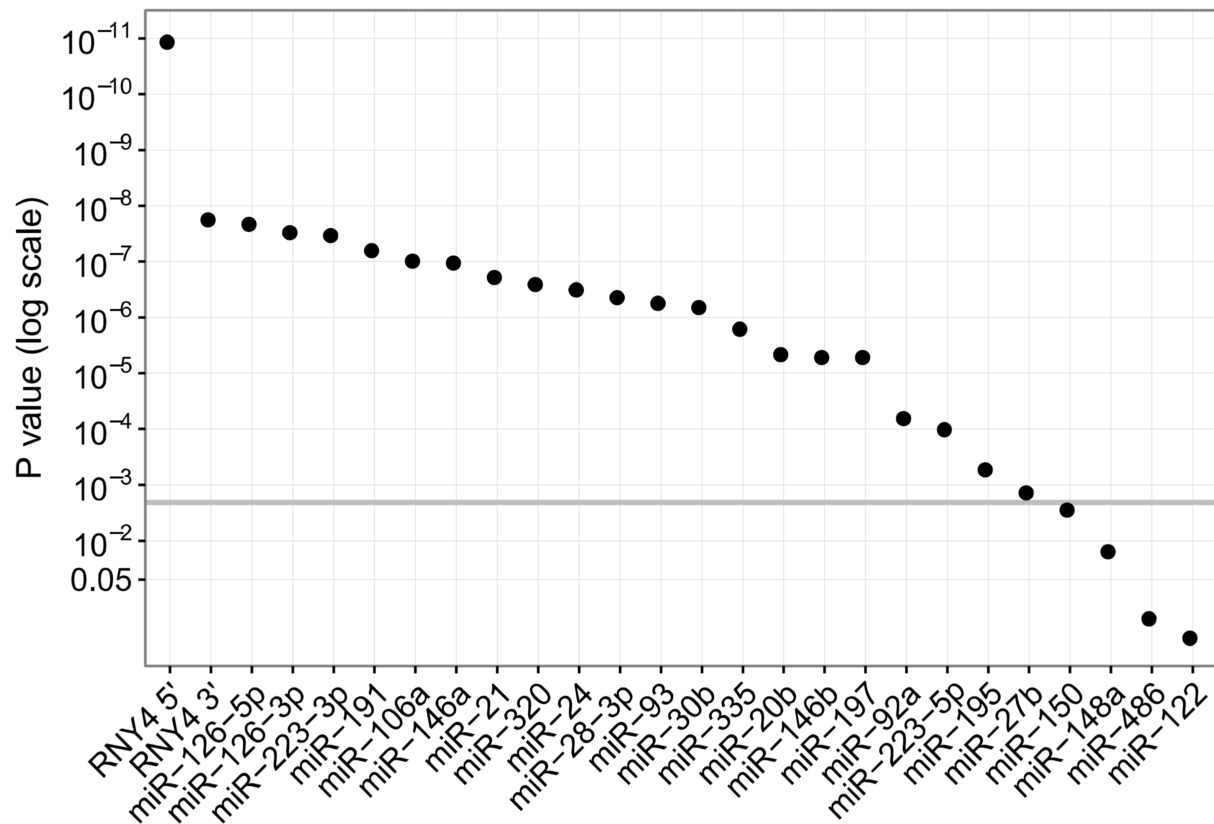


Figure 17 Platelet spike-in experiment and dependency of small ncRNA levels
Representation of the statistical significance of associations of levels of 24 miRNAs and two YRNA fragments with the relative amount of platelet spike-in. P-values were calculated by linear mixed models. The grey horizontal line indicates the significance level after adjustment for multiple testing. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

4.6.2 Platelet activation markers

In order to further investigate the relationship between circulating miRNAs, YRNAs and platelets, measurements in samples from the Bruneck study were performed. Plasma samples of 669 participants were analysed: selected miRNAs and YRNA fragments were measured by individual RT-qPCR. The platelet activation markers platelet factor 4 (PF4), pro-platelet basic protein (PPBP) and P-selectin (SELP) were quantified by ELISA. All three platelet activation markers showed remarkable correlation with most of the assessed miRNAs and the two YRNA fragments: RNY4 5’ (PPBP rSp=0.65; PF4 rSp=0.66; SELP rSp=0.54) and RNY4 3’ (PPBP rSp=0.69; PF4 rSp=0.71; SELP rSp=0.55). Compared to PF4 and PPBP, SELP showed weaker correlations. Again, there were no associations with the liver-derived miR-122; similarly, some miRNAs failed to show significant correlations or showed remarkably weaker correlations to platelet proteins, e.g. miR-150. Notably, strong correlations were found inter alia for RNY4 3’, RNY4 5’, miR-126-3p and miR-223-3p (see Figure 18, 19 and 20).

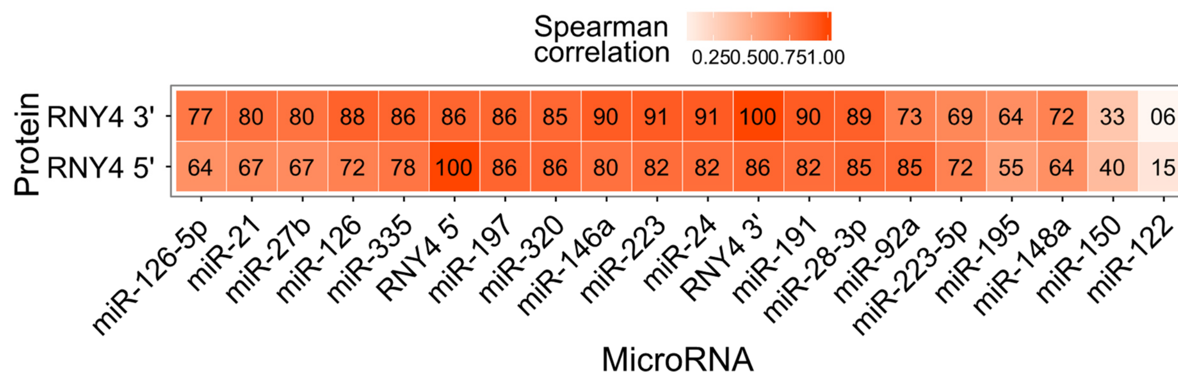


Figure 18 Correlation of plasma miRNAs and YRNA fragments
Expression of 20 small ncRNAs (18 miRNAs, 2 YRNAs) measured in the Bruneck population (n=669), several of which correlated with levels of plasma YRNA fragments. Numbers represent the first two decimal digits of the magnitude of Spearman correlation, the colour indicates magnitude and direction of correlation. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

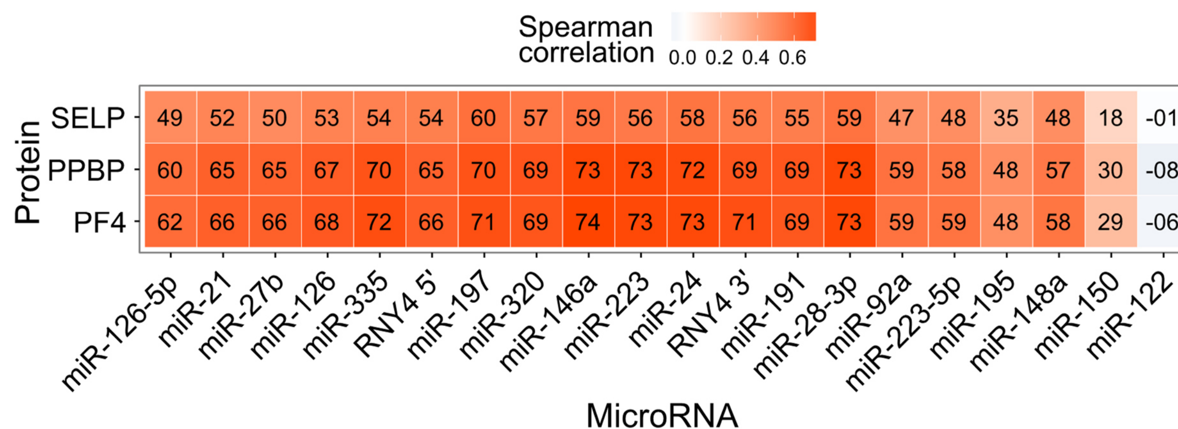


Figure 19 Correlation of small ncRNAs and markers of platelet activation
Results of the RT-qPCR measurements in the entire Bruneck cohort (n=669) correlated with results of the ELISA tests assessing PF4, PPBP and SELP. Numbers represent the first two decimal digits of the magnitude of Spearman correlation, the colour indicates magnitude and direction of correlation. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

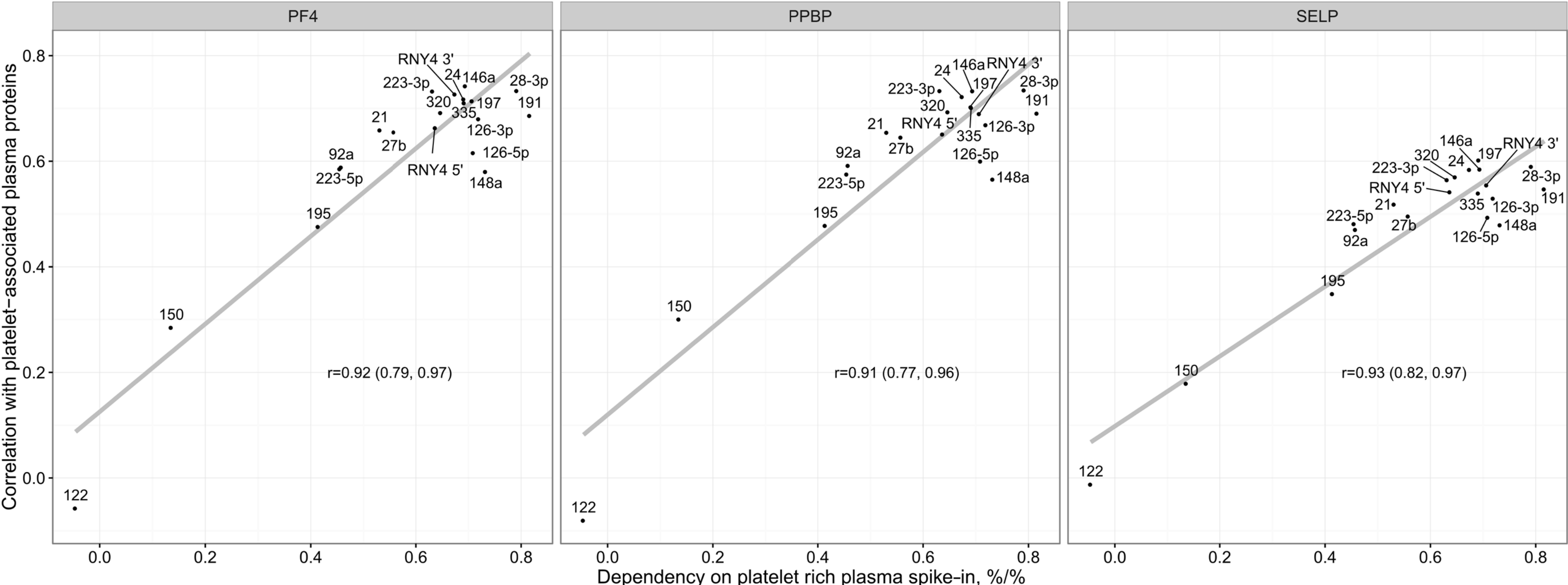


Figure 20 Dependency of small ncRNAs on platelets
Relationship between small ncRNA expression in platelets in the spike-in experiment and correlation of those with platelet activation markers PF4, SELP and PPBP concentration in the Bruneck cohort. Grey lines represent Deming regression lines, r=Pearson correlation with 95% confidence interval. This figure is adapted from Kaudewitz et al. (Kaudewitz et al. 2016).

4.7 Genetic influence on miR-126

Over the last years, it became apparent that single nucleotide polymorphisms (SNP) of the human genome are, at least partly, altered in several disease conditions. One functional SNP has been described for miR-126 as a nucleotide variant in the pri-miR-126 sequence downstream of the Drosha/ DGCR8 cleavage site: the rs4636297 SNP as listed in the single nucleotide polymorphism database (dbSNP). This SNP was previously analysed in the 1000 genomes project (Genomes Project, Abecasis et al. 2012). Within the European population (n=503), the allele distribution was 36.4% for the A allele and 63.6% for the G allele.[6] In the Bruneck cohort, similar distributions were identified: 39.0% of the population are carrier of the minor allele, 61.0% of the major allele (see Table 6). It is yet unclear, whether these allele contributions are responsible for variations in levels of circulating miRNAs such as miR-126-3p, although this rs4636297 SNP has been found to influence the cleavage process of pri-miR-126 and, thus, the expression of miR-126. Pri-miR-126 with the minor A allele is more likely to be transformed into mature miR-126 than the major G allele variant (Harnprasopwat et al. 2010). Genotyping for this SNP was performed in the entire Bruneck cohort.

Table 6 Genotype and allele distribution for the rs4636297 SNP
Analysis of the genotype and allele frequencies in the entire Bruneck cohort (n=628) yielded similar frequency distribution as observed in the European population due to the 1000 genomes project.[7] This table originates from Kaudewitz et al. (Kaudewitz et al. 2016).

	Bruneck (n=628)		European Population (n=503)	
	count	frequency	count	frequency
Allele				
A	490	0.390	366	0.364
G	766	0.610	640	0.636
Genotype				
AA	105	0.167	70	0.139
GA	280	0.446	226	0.449
GG	243	0.387	207	0.412

[6] URL:"http://grch37.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=9:139564650-139565650;v=rs4636297;vdb=variation;vf =2807655", visited 22.03.2017, 16.59 CET.
[7] Ibid.

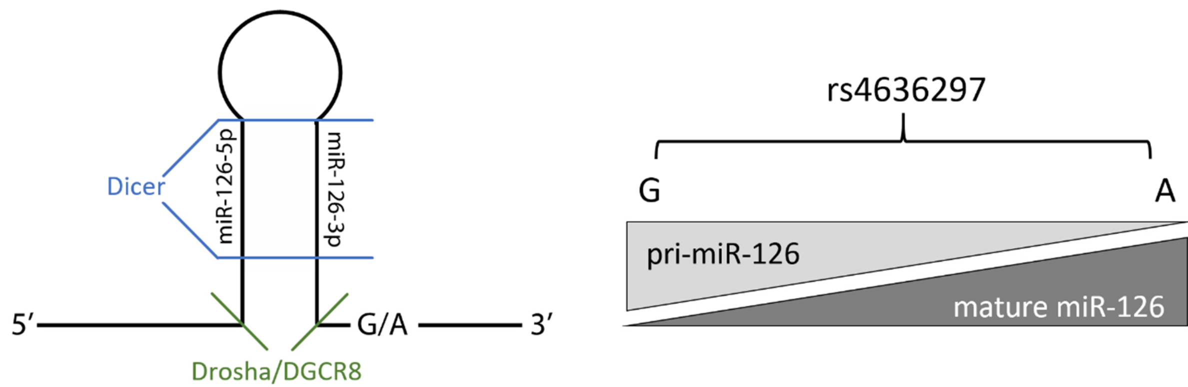


Figure 21 The rs4636297 SNP cleavage site and effect on miR-126
The rs4636297 SNP affects expression levels of mature miR-126. Left: schematic representation of the cleavage sites for enzyme Drosha downstream of the stem-loop on the pri-miR-126. Right: pri-miR-126 with the minor A allele is more likely processed to mature miR-126 compared to the major G variant. This figure originates from Kaudewitz et al. (Kaudewitz et al. 2016).

Individuals carrying homozygous alleles for the AA (minor) genotype showed higher levels of mature miR-126-3p in both serum (+4.4%, p=0.050) and plasma (+6.9%, p=0.099) compared to the GG (homozygous for major allele) and GA genotype (see Table 7). For the 5p strand the results failed to reach significance although a similar pattern was observed in individuals carrying the AA (+7.1%, p=0.203) and the GA alleles (+7.4%, p=0.208). Interestingly, the AA genotype showed positive associations with plasma levels of markers that reflect platelet activation, such as PF4 (p=0.002), PPBP (p<0.001) and SELP (p=0.099) (see Figure 22).

Table 7 MiR-126 dependency on the rs4636297 SNP
Associations of plasma and serum levels of miR-126-3p and miR-126-5p in the Bruneck cohort (n=669) with the rs4636297 SNP. “A” indicates the minor allele, “G” the major allele. This table originates from Kaudewitz et al. (Kaudewitz et al. 2016).

MiRNA	Model	Mean difference	P-value
Serum miR-126-3p	dominant	1.6% (-1.7, 4.9%)	0.356
	additive	3.9% (-0.7, 8.6%)	0.097
	recessive	4.4% (-0.0, 8.9%)	0.050
Plasma miR-126-3p	dominant	1.2% (-4.8, 7.6%)	0.697
	additive	4.9% (-3.5, 14.1%)	0.258
	recessive	6.9% (-1.3, 15.8%)	0.099
Plasma miR-126-5p	dominant	3.6% (-4.5, 12.4%)	0.387
	additive	7.4% (-3.9, 20.1%)	0.208
	recessive	7.1% (-3.7, 19.1%)	0.203
	additive (per A-allele), dominant (AA+GA vs. GG), recessive (AA vs. GA+GG), Genotype (n): GG (243), GA (280), AA (105)		

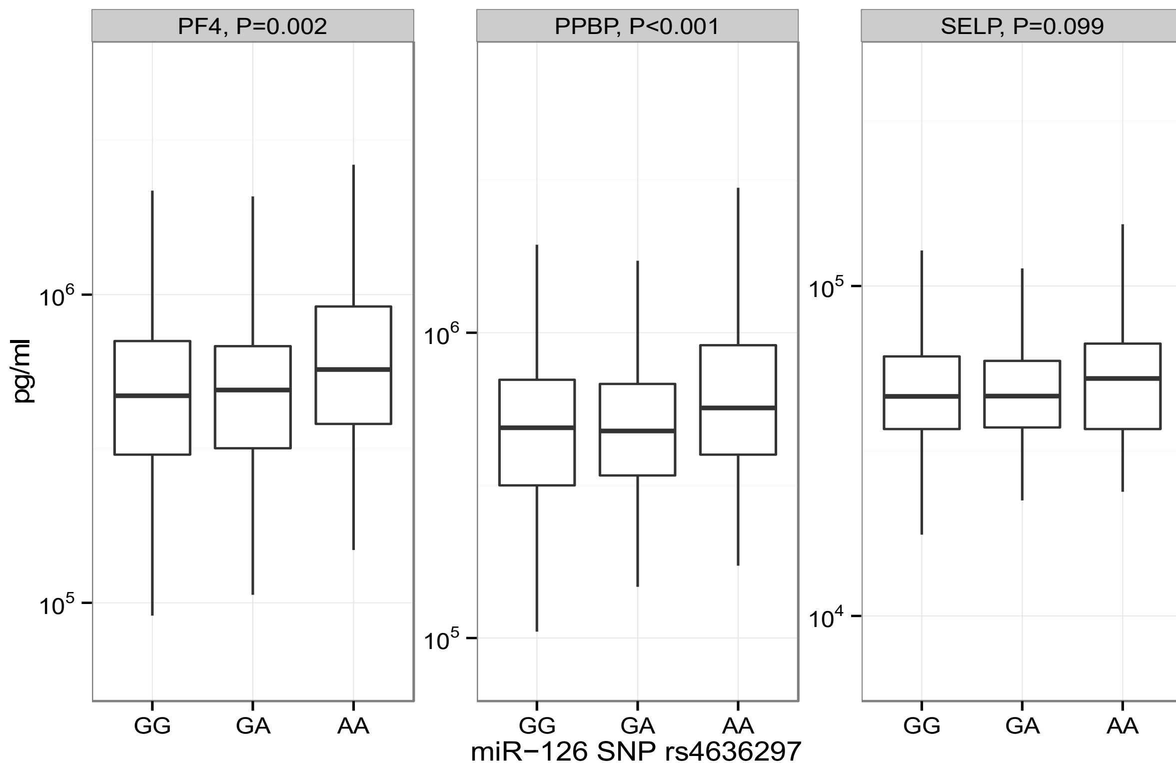


Figure 22 Genetic impact of the rs4636297SNP on platelet activation proteins
Associations between the rs4636297 SNP genotype and levels of platelet activation proteins PF4, PPBP and SELP in the Bruneck population (n=669). This figure originates from Kaudewitz et al. (Kaudewitz et al. 2016).

4.8 Radial injury experiment

In order to investigate reactions to acute arterial injury, early effects of endothelial denudation on circulating miRNAs in five healthy volunteers were analysed. No significant changes were found in the early reaction to vascular injury after 10 min, 60 min and 5 h. Interestingly, 7 days after intervention, levels of miR-126-3p and miR-126-5p were higher in plasma taken from the injured arm compared to the uninjured arm. For miR-126-3p, this difference remained significant after adjustment for multiple testing. Levels of miR-223-3p were also higher at day 7 compared to baseline in the injured arm. No significant changes were observed for miR-223-5p (see Figure 23). Overall, the ratio of both strands of miR-126 and miR-223 remained similar in plasma.

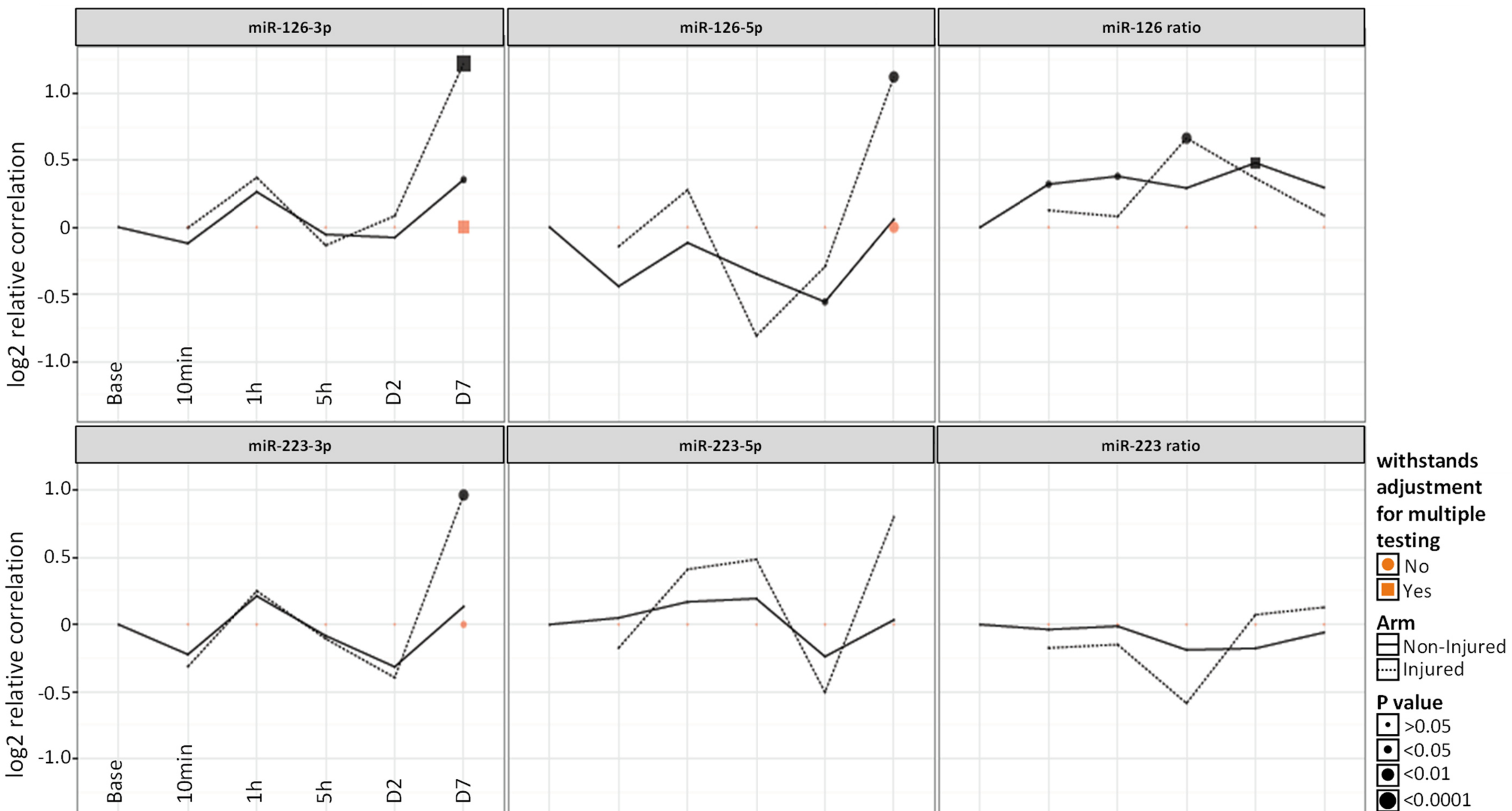


Figure 23 Radial injury experiment
Endothelial denudation was performed in 15 healthy volunteers by wire rotation. MiRNAs were measured at baseline, 10 min, 1 h, 5 h, 2 days and 7 days after intervention by individual TaqMan RT-qPCR. Black symbols=test against baseline, non-injured arms; red symbols=tests for effect modification by arm within time points.

4.9 Effects of thrombolysis on circulating microRNAs

In order to determine influences of thrombolysis therapy with rtPA on circulating miRNAs, miRNAs were screened using custom-made RT-qPCR plates (see

Figure 24). Results were then validated by individual TaqMan RT-qPCR (see Figure 25). Blood samples were drawn at baseline (immediately before infusion of rtPA), 6 h and 24 h after thrombolysis by peripheral venepuncture. All patients (n=21) presented clinical symptoms and radiologic signs of an ischaemic stroke. Significantly lower levels were observed for miR-122, miR-125b, miR-192, miR-214 and miR-375 at 24 h after rtPA administration. For miR-122 and miR-375, this reduction was already significant at 6 h. In contrast, a significant increase was observed for miR-26b, miR-28-5p and miR-223-5p 6 h after thrombolysis therapy using Cel-miR-39 for normalisation. When normalising to CT average and, thus, adjusting for platelet-associated alterations of circulating miRNAs, significance of downregulation of miR-122, miR-125b, miR-214 and miR-375 at both 6 h and 24 h post injection was observed. The drop of miR-126-5p and miR-192 was only significant at 6 h, but not at 24 h compared to baseline. Interestingly, miR-126-5p returned to baseline levels 24 h after the onset of therapy. Significantly higher levels were observed for miR-191 and miR-223-5p at both points in time. For miR-26b, miR-106a and miR-223-3p, the increase was significant at 24 h but not at 6 h. Overall, the levels of miR-122 and miR-375 were significantly downregulated at both points in time after thrombolysis using either normalisation method (see Figure 25 and 26).

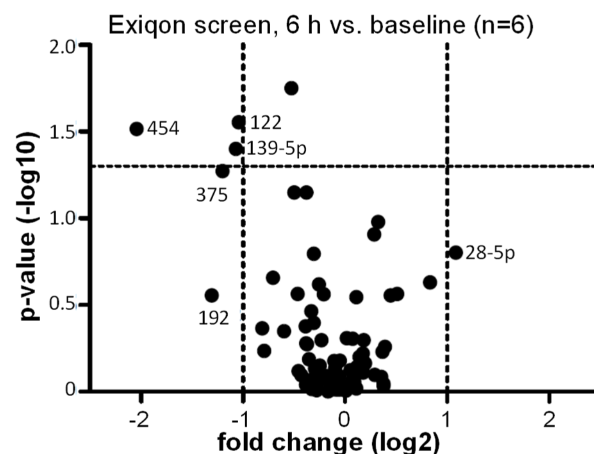


Figure 24 MiRNA screening after thrombolysis
Plasma samples from 6 ischaemic stroke patients were screened at baseline and 6 h after thrombolysis. 92 miRNAs were assessed by custom-made RT-qPCR plates. The labelled miRNAs were found up- or downregulated at 6 h at least twofold compared to baseline (vertical dotted lines). Data points above the horizontal dotted line (p-value in paired t-test < 0.5) are significant changes.

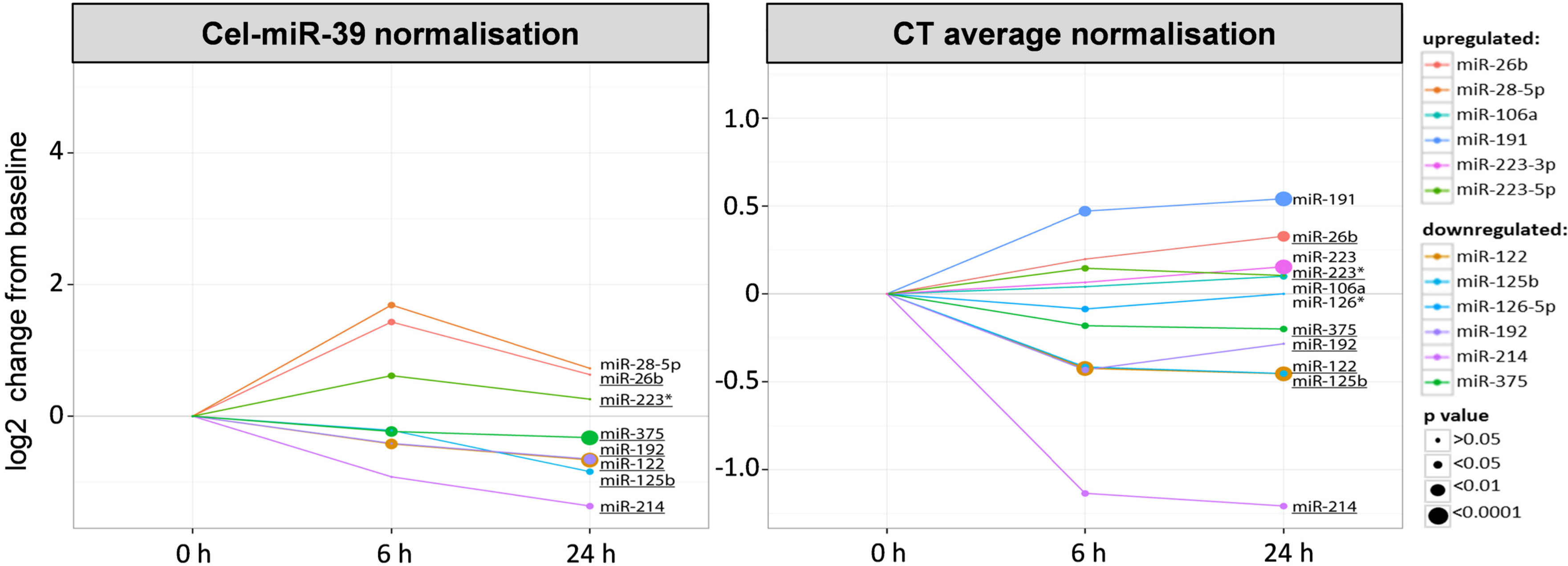


Figure 25 Temporal miRNA alterations in stroke patients upon thrombolysis
The miRNAs displayed in the graphs were selected from the screening experiment and analysed by individual RT-qPCR. The graph on the left shows RT-qPCR data normalised to Cel-miR-39, on the right several unaffected endogenous miRNAs were used for CT average normalisation. The miRNAs, which show consistently significant alterations with both normalisation methods, are underlined.

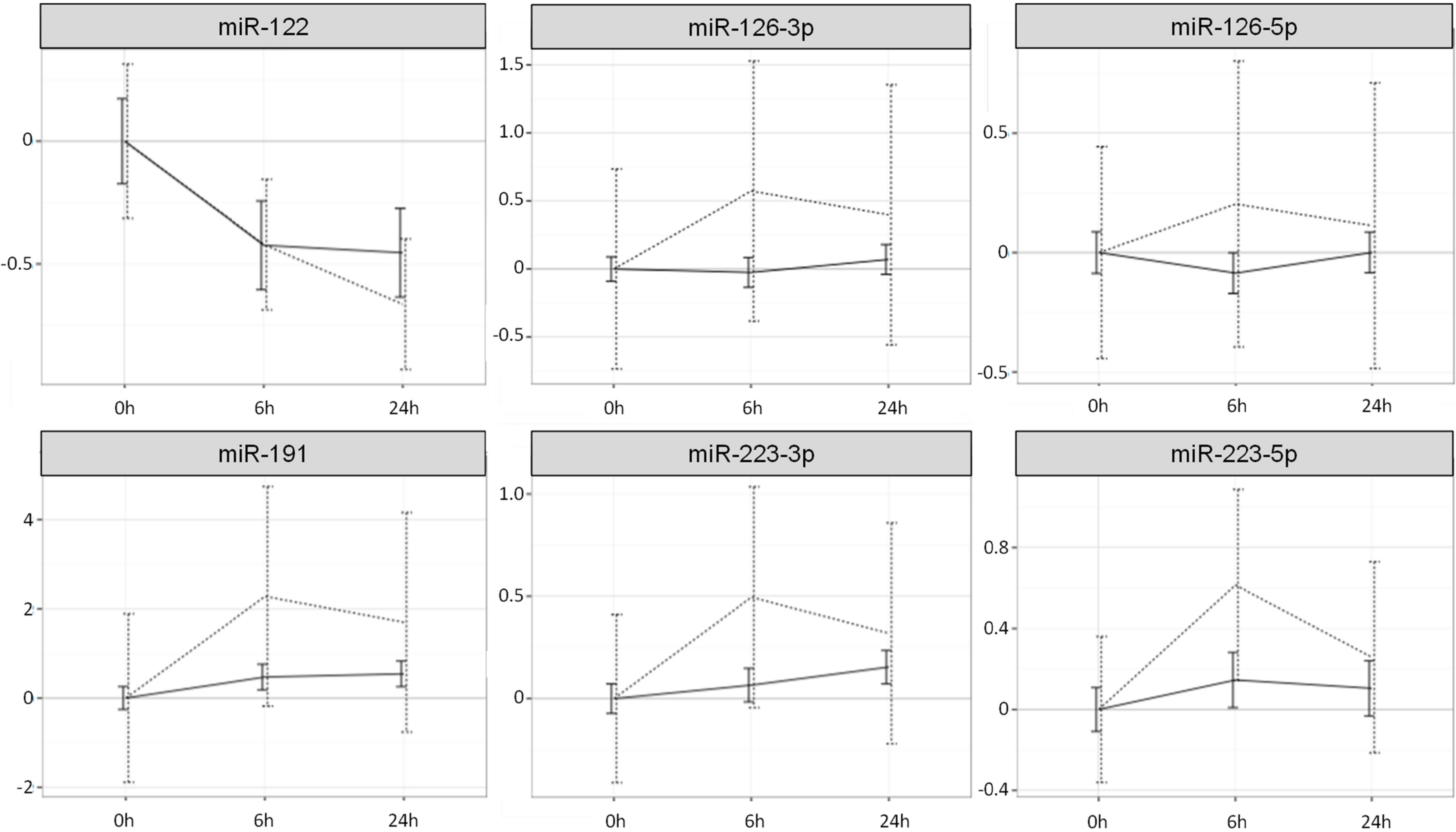


Figure 26 MiRNA changes upon thrombolysis
This graph displays selected miRNAs analysed by individual RT-qPCR. Dotted line indicates data normalised to Cel-miR-39, the solid line indicates CT average normalisation. Y-axis=average change from baseline (log2).

5 Discussion

5.1 Overview

According to the WHO, about one third of annual global deaths are caused by CVDs and, thus, there is a high demand for improvement of preventive, diagnostic and therapeutic strategies.[8] Especially gene regulation by several small ncRNAs like miRNAs has previously been shown to be linked to CVD (Zampetaki et al. 2010, Zampetaki et al. 2012, Olson 2014, Zampetaki et al. 2014). In this work, associations between circulating miRNAs and platelets were further investigated. State-of-the-art NGS was used for screening of the overall abundance of small ncRNAs in PPP and PRP of healthy volunteers. Platelet function was assessed using LTA, VASP and VerifyNow. Samples from healthy volunteers participating in the Bruneck study and from patients with ACS in the Sheffield cohort were analysed: the Bruneck cohort was used in order to investigate miRNA correlations with platelet activation markers in the general population and to identify aberrations by a functional SNP for miR-126. Subsequently, a spike-in experiment of washed platelets in PPP was conducted in order to demonstrate the dependency of several miRNAs upon platelets. The Sheffield cohort was used to identify miRNA correlations with platelet function tests in ACS patients under dual antiplatelet therapy. In order to identify acute effects of endothelial denudation on levels of platelet-related circulating miRNAs, repeated measurements were obtained from a small cohort where injury in the radial artery was provoked. Finally, circulating miRNAs were investigated in ischaemic stroke patients in order to explore influences of therapeutic lysis with alteplase.

5.2 Platelet contribution to small ncRNAs in plasma

In the NGS experiment, the 5p strands of miR-223 miR-126 and, interestingly, fragments of YRNA were found to be present in plasma of healthy volunteers. This is in line with recent findings by Dhahbi et al., who also found high levels of YRNAs in human serum and plasma (Dhahbi et al. 2013). Data obtained by conducting individual RT-qPCR confirm the presence

[8] URL: “https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)”, visited 02.09.2017, 10.35 CET.

of RNY4 3’ and RNY4 5’ fragments as well as the 5p strands of miR-126 and miR-223 in circulation. In humans, YRNAs were first detected in the cytoplasm of systemic lupus erythematosus patients: the pre-letter “Y” indicates its cytoplasmic origin and, thus, makes it distinct from nuclear URNAs (Lerner et al. 1981). Other groups have shown the enrichment of YRNAs in exosomes (Tosar et al. 2015, van Balkom et al. 2015).

The data presented above revealed the relative abundance of RNY4 5’ and RNY4 3’ in descending order in platelets, PRP and PPP, and, thus, the author proposes an additional platelet origin of YRNA fragments in circulation. In circulation, fragments of YRNA have been shown – together with transfer RNAs – to be packed in vesicles or in cell-free ribonucleoprotein complexes in mammals. However, variations between species were observed: for example, expression levels in humans are significantly higher compared to those in mice (Dhahbi et al. 2013, Dhahbi et al. 2014). To date, the function of YRNAs is poorly understood: 4 types of human YRNAs – RNY1, RNY3, RNY4 and RNY5 – were detected. The formerly termed RNY2 is suspected to be a modified type of RNY1. YRNAs originate from several genes encoded on one locus on chromosome 7 148660407-148660502 and other pseudogenes, which are responsible for type-specific biogenesis happening before they are being released into circulation (Pruijn et al. 1993, Maraia et al. 1994, Verhagen and Pruijn 2011, Nicolas et al. 2012). YRNAs might be released due to cellular stress and apoptosis (Rutjes et al. 1999).

When excluding contamination from other circulating cells in the platelet spike-in experiment, the platelet origin of several small ncRNAs, including miR-126-5p, miR-223-5p, RNY4 3’ and RNY4 5’ has been validated. Platelets are capable of releasing active exosomes and microvesicles, possibly for the purpose of intercellular communication with surrounding cells like ECs. At present, there is no evidence that platelet-derived YRNA fragments are taken up by other cells and mediate paracrine functions. Thus far, such a mechanism was only described for certain platelet miRNAs (Landry et al. 2009, Pan et al. 2014). Moreover, transcriptomic alterations in platelets influence these processes and promote pathogenesis as has been, for instance, proposed in chronic inflammation (Heijnen et al. 1999, Risitano et al. 2012). In 2006, Christov et al. showed YRNAs to be an essential factor required for an initiation step of chromosomal S1 phase DNA-replication in human cells (Christov et al. 2006). Others reported an interaction of RNAs with La/ SSB antibody and Ro protein (e.g. Ro60) – both of which are necessary proteins for the physiological degradation and elimination of incorrectly folded

proteins (Stein et al. 2005, Verhagen and Pruijn 2011). YRNA fragments were found to be altered in solid tumour and breast cancer cells. Thus, it seems conceivable that their dysregulation might contribute to tumorigenesis (Meiri et al. 2010, Nicolas et al. 2012, Dhahbi et al. 2014).

However, Repetto and collaborators have proposed YRNAs as biomarkers for detecting CVD: they identified apoptotic macrophages in humans and mice as a source of circulating YRNA fragments – especially RNY1-5' – and noticed altered levels in atherosclerosis. Furthermore, they reported that RNY1 5' and RNY4 5' levels are higher in serum of CAD patients than in healthy controls. Hence, they suggest YRNAs like RNY1 5' derived from macrophages to be a suitable biomarker for CAD (Repetto et al. 2015). This work, in contrast, demonstrates that platelets contribute to YRNA fragments in circulation. This must be taken into consideration when implicating YRNA fragments as potential biomarkers for CVD.

5.3 ACS patients and effects of antiplatelet agents

Apart from their aggregation response, the involvement of platelets in CVD is poorly understood. This work highlights that platelets contain and release miRNAs into circulation – a process which may be altered in patients with CVD (Heijnen et al. 1999, Risitano et al. 2012, Zampetaki et al. 2012).

Platelet function tests were performed in plasma of patients 30 days after diagnosis of an ACS in order to extenuate the impact of acute inflammation. Significant correlations between platelet function and several platelet related miRNAs and YRNAs, including miR-126 and miR-223, have been noted. Both miRNAs were shown to be reliably detectable in plasma, serum and platelets. The ratio of miR-126-5p to miR-126-3p was higher in platelets compared to plasma and serum. Levels of miR-126-3p were higher in plasma and serum compared to platelets. VerifyNow $P2Y_{12}$ – used for analysing platelet aggregation in whole blood – only showed significant correlation with miR-126-3p (p=0.033). The VASP assay positively correlated with several miRNAs, including miR-126-3p (p=0.016) and miR-223-3p (p=0.002). Interestingly, miR-126-5p (p=0.001) also reached statistical significance, whereas miR-223-5p (p=0.279) did not. Additionally, levels of RNY4 3' (p=0.012) and RNY4 5' (p=0.025) showed a positive correlation with the VASP assay. Potentially, the relatively low number of samples (n=40)

analysed with the VerifyNow system is, at least partly, responsible for the non-significant results for the investigated miRNAs. For the VASP assay, measurements in 121 samples were available. LTA did not show correlation with any of the measured miRNAs and YRNAs, neither in response to arachidonic acid nor to ADP. LTA – using arachidonic acid or ADP as an agonist reagent – was performed in PRP. VerifyNow $P2Y_{12}$ and the VASP assay was used in order to evaluate platelet activation, which is provoked by targeting receptors of the $P2Y_{12}$ family in whole blood (Gum et al. 2001, Grove et al. 2012).

Currently, the VASP assay seems to be the most specific test method for assessing platelet function in comparison to other platforms because it does, for example, not interfere with ADP-mediated co-activation of $P2Y_1$ receptors and, thus, enables more specific monitoring of pharmacological treatment with clopidogrel, prasugrel and ticagrelor. However, this method is not yet eligible for wider clinical use – it is only available in specialised laboratories (Bouman et al. 2010, Tantry et al. 2010, Grove et al. 2012). Furthermore, the results of this work partially correspond to a study by Shi et al., who investigated patients with coronary heart disease: LTA in response to ADP did not show significant correlation with detected miRNAs, whereas VASP-detected PRI did (Shi, R. et al. 2013). Potentially, these differences could be, at least partly, caused by an impact of Aspirin on the aggregometry analysis. In the ACS cohort, all patients were treated with Aspirin. Aspirin was shown to inhibit platelets' response to arachidonic acid to such an extent that it disguises inter-patient variability and results in a loss of sensitivity (de Boer et al. 2013). Aspirin does not only obliterate the platelet response to arachidonic acid but also raises the threshold concentration of ADP that triggers platelet aggregation in LTA (Capurro et al. 1980). In plasma of stable CAD patients undergoing dual antiplatelet therapy, Fichtlscherer and collaborators reported a significant reduction of vasculo-protective miR-126-3p. This study, however, failed to consider the confounding effects of several administered antiplatelet drugs (Fichtlscherer et al. 2010). The obtained data show influences of different antiplatelet agents being reflected in levels of circulating miRNAs in ACS patients: least inter-individual variability of 92 screened miRNAs was identified in the patient group having been administered 75 mg ASA and 10 mg prasugrel OD. These findings are consistent with previous publications by the Mayr lab having shown that antiplatelet agents affect the abundance of platelet-related miRNAs in circulation, among them miR-126 and miR-223 (Zampetaki et al. 2012, Willeit et al. 2013). The data presented in this thesis show that both strands of miR-126 and miR-223 are affected. There are different classes of $P2Y_{12}$ inhibitors

with different properties: clopidogrel and prasugrel are thienopyridines, whilst ticagrelor is a cyclopentyl-triazolopyrimidine. Clopidogrel demands two sequential enzymatic oxidative steps by CYP450 isoenzymes for activation; prasugrel is activated by one enzymatic processing step and ticagrelor is an initially active oral agent (Cannon et al. 2010).

In the ACS cohort, the author made the novel observation that plasma levels of miR-126-3p and miR-126-5p were increased in patients having been administered ASA + ticagrelor. Recently, Carino and collaborators observed a decrease of miR-126-3p after therapeutic switch from clopidogrel to ticagrelor (Carino et al. 2016). It should be considered whether these observations at 24 h after switching might be biased by after-effects of clopidogrel and, thus, not specifically attributable to ticagrelor. The results at hand, in contrast, were obtained at 30 days of dual antiplatelet therapy and provide evidence for a long-term impact of ticagrelor. A proportional increase of miR-126-5p but not of miR-223-5p in the ASA + ticagrelor group was found. Thus, it seems conceivable that these alterations of miR-126-5p are not exclusively attributable to the degree of platelet inhibition. Storey et al. reported a more sufficient platelet inhibition measured by LTA in ticagrelor versus clopidogrel treatment. Paradoxically, levels of inflammation markers C-reactive protein and IL-6, were increased in ticagrelor patients although ticagrelor has been shown to be more effective than clopidogrel in the PLATO study. However, these alterations might be unrelated to $P2Y_{12}$ inhibition and platelet function (Storey et al. 2011, Storey et al. 2014). Ticagrelor seems to influence adenosine metabolism by regulating the reuptake and, thus, has positive side effects besides platelet inhibition (van Giezen et al. 2012). Furthermore, ticagrelor showed an additional effect by targeting equilibrative nucleoside transporter 1, which inhibits adenosine intake by platelets. Thus, extracellular adenosine levels are increased, which in turn leads to greater probability of adenosine receptor activation and, therefore, could influence platelet activity (Armstrong et al. 2014). Future studies need to solve how $P2Y_{12}$ inhibitors influence miRNA release by platelets and other cells as well as their physiological functions.

5.4 The rs4636297 SNP affects expression of miR-126

The data presented in this work provide further evidence for a SNP in the MIR126 gene that affects levels of miR-126 in circulation. Over the last century research revealed numerous influences of genetic and epigenetic dysfunctions in various pathophysiologies and, exemplary, even the biogenesis of several miRNAs was shown to be dependent on genotypic differences. SNPs are a main source of genotypic variations in humans. Harnprasopwat reported that the rs4636297 SNP is differently expressed throughout ethnic groups. Cells containing the ancestral allele (G) of the rs4636297 SNP – the pri-126-24G variant – inhibit the processing of pri-miR-126 to pre-miR-126 and, thus, the expression of mature miR-126. This has an suppressing effect on the target genes of miR-126 (Harnprasopwat et al. 2010). Deleting the MIR126 gene in zebrafish and mice caused major angiogenetic problems and vascular dysintegrity (Fish et al. 2008, Wang et al. 2008). McAuley and collaborators confirmed similar effects of this SNP in another CVD context when they found associations with diabetic retinopathy, a microvascular sight-threatening complication of diabetes (McAuley et al. 2015). Likewise, miR-126 synthesis was shown to be affected by epigenetic influences in the EGFL7 T-2 promoter region during breast tumour growth: methylation of the T-2 region was associated with lower expression of miR-126 and vice versa, which shows that differences in the EGFL7 host gene are associated with altered mature miR-126 levels in tumour cells (Zhang et al. 2013).

To the author's knowledge, effects of this SNP on endogenous expression of circulating miR-126 have not been reported yet. In the general population (Bruneck cohort, n=669), attenuated levels of miR-126 in individuals carrying the GG and GA allele compared to those carrying AA have been identified. The G allele inhibits the processing of pri-miR-126 to pre-miR-126 and, thus, AA carriers express higher levels of the mature strands in both serum (+4.4%, $p=0.050$) and plasma (+6.9%, $p=0.099$). The higher plasma concentrations of miR-126 in AA carriers in turn correlate with levels of markers that reflect platelet activation, PF4 ($p=0.002$), PPBP ($p<0.001$) and SELP ($p=0.099$). Correlation was more distinct for PPBP and PF4 compared to SELP. PPBP and PF4 originate exclusively from platelets, while SELP is present on both platelets and endothelial cells (Fijnheer et al. 1997). Taken together, the rs4636297 SNP might affect platelet activation. In the case of miR-126, it seems conceivable that it has an impact on platelet function, although the rs4636297 SNP per se may not be sufficient for a contribution to pathological disorders with dysregulated thrombocyte function.

Furthermore, the author observed correlations between several platelet-derived miRNAs, YRNA fragments and platelet activation markers in the Bruneck study. Platelets were found to play a significant role in the development of CVD, but their transcriptomic regulations, especially mediated by small ncRNAs, and their defined mechanistic roles remain unclear. Neither miR-126 nor miR-223 are exclusively present in platelets. For example, miR-223 is abundant in inflammatory cells and miR-126 was initially thought to be specific for ECs (Fish et al. 2008, Diehl et al. 2012, Willeit et al. 2013). The latter is not the case. MiR-126 is also present in megakaryocytes and the spike-in experiment demonstrates the dependency of circulating miR-126 levels on platelets. Thus, changes in circulating levels of miR-126 cannot be attributed to ECs exclusively. Likewise, therapeutic strategies targeting miR-126 will not only have an impact on ECs but also affect megakaryocytes. A better understanding of miR-126 function in megakaryocytes and platelets is a prerequisite for designing novel diagnostic and therapeutic concepts based on miR-126 (Ple et al. 2012, Willeit et al. 2013, Coupland and Parish 2014). Conclusively, the author observed an influence of the rs4636297 SNP on levels of circulating miR-126 and an impact of this genotype on plasma concentrations of platelet activation proteins PF4, SELP and PPBP. This implies effects of miR-126 on platelet function.

5.5 Radial injury

Besides, effects of acute arterial injury and endothelial denudation on levels of circulating miRNAs have been investigated in healthy volunteers. Both platelets and ECs contribute to circulating miRNAs (Fish et al. 2008, Zampetaki et al. 2010). The author detected increased levels of circulating miR-126-3p and miR-126-5p in the injured arm 7 days after intervention. This complements earlier work by Prof. Mayr’s group: in a limb ischaemia-reperfusion experiment in healthy volunteers, miR-126 was elevated 7 days after intervention (Zampetaki et al. 2012).

As described earlier in the context of antiplatelet therapy, the data obtained show a similar alteration regarding both strands of miR-126 and miR-223. No changes were observed in the early phase after intervention, however, results reached statistical significance for the upregulation of miR-126 at day 7. Thus, the initial miRNA release in response to arterial injury was apparently too small to result in a significant change in circulating miRNA levels. However, higher miR-126-3p levels were observed at day 7 during the vascular healing response in the injured but not the uninjured arm. Previously, it has been described that both

mature strands of miR-126 are consistently detectable in ECs and, moreover, both have distinct functions (Fish et al. 2008, Zhang et al. 2013, Schober et al. 2014). MiR-126-3p and miR-126-5p seem to be expressed in a site- and function-specific manner in the vessel wall: in aortic tissue miR-126-5p levels are higher at non-predilection sites compared to the 3p strand and, thus, have potential athero-protective function as a suppressor of notch 1 inhibitor delta-like 1 homolog (DLK1), which is a protein that has antiproliferative effects after endothelial injury. Overexpression of miR-126-5p decreases levels of DLK1, which in turn increases endothelium layer proliferation rates. While miR-126-3p delivered by apoptotic ECs seems to enhance plaque stability at predilection sites and, thereby, limits atherosclerosis, the 5p strand does so by promoting EC proliferation at non-predilection sites (Zernecke et al. 2009, Weber and Noels 2011, Schober et al. 2014).

This work shows that both miR-126-3p and miR-126-5p are upregulated in circulation at day 7 after endothelial denudation. Given the evidence of the importance of miR-126 for endothelial integrity, this is an interesting observation. Potentially, EC proliferation as a late reaction to injury contributes to the higher expression of miR-126 in circulation. However, this cannot offer a satisfying explanation for the upregulation of miR-223. The latter association achieved nominal significance but did not withstand correction for multiple testing. An alternative explanation could be the activation of platelets at the former site of injury. However, one would expect platelet activation to be an acute rather than a delayed response to vascular injury. Nonetheless, as described earlier, platelet-derived miRNAs such as miR-126 may stimulate proliferation of ECs at the injury site (Schober et al. 2014). This shows exemplary how small RNA molecules might impact both physiological and pathological functions. More work is needed to address whether the paracrine release of platelet miRNAs is necessary for endothelial maintenance and response to endothelial disintegration.

5.6 Thrombolysis

Several studies have been performed investigating the effects of stroke on miRNA expression (Long et al. 2013, Jickling et al. 2014, Wang et al. 2014, Liu et al. 2015, Mick et al. 2017). However, to the author's knowledge, this work is the first that compares circulating miRNA profiles before and after thrombolysis in circulation of ischaemic stroke patients. In an ischaemic stroke mouse model, Zhao et al. recently reported that 31 miRNAs were upregulated and 11 downregulated upon rtPA thrombolysis (Zhao et al. 2015). The data presented in this thesis show that circulating miRNAs are altered upon thrombolysis therapy in humans.

After an initial screening experiment, several miRNAs were selected for detailed assessment by individual RT-qPCR in samples of 21 stroke patients. A significant rise of miR-26b, miR-106a, miR-191, miR-223-3p and miR-223-5p after thrombolysis was observed. To the extent of the author's knowledge, none of these miRNAs have been described to be affected by rtPA treatment in humans before. MiR-26b was reported to decrease the size of cardiac hypertrophy in mouse cardiomyocytes (Han et al. 2012). Upregulation of miR-26b was shown to be linked to hypoxia and hypoxia-related apoptosis in rat cardiomyocytes (Wang et al. 2015). To this date, no study has been published about circulating miR-106a in CVD. MiR-106a is reported to be associated with different types of cancer (Landais et al. 2007). Hackl et al. reported a decrease of miR-106a transcription in cellular models of ageing (Hackl et al. 2010). MiR-191 was previously shown to be decreased in patients with prediabetes and manifest T2DM (Zampetaki et al. 2010). As described earlier, the data given here show that circulating miR-191 also derives from platelets. Of the selected miRNAs validated by RT-qPCR, miR-191 showed the highest fold change upon thrombolysis. In the spike-in experiment, the author shows both mature strands of miR-223 to be expressed in platelets and to undergo similar changes upon antiplatelet therapy. The group of Prof. Mayr previously found that miR-223, alongside miR-126 and miR-197, is predictive of major cardiac events (Zampetaki et al. 2012). Furthermore, as described earlier, miR-223 correlates with platelet function in healthy volunteers as well as ACS patients. Activated platelets are structural parts of the thrombus and thrombolysis with rtPA enhances clot breakdown. The increase of platelet-related miRNAs (miR-191, miR-223) upon thrombolysis might be explained by a release of platelet material trapped in the thrombus. Future studies need to find out, why these two platelet-related miRNAs but not others are differently altered in stroke patients and affected by rtPA therapy.

The author did not observe any significant alteration of levels of miR-126-3p during the course of thrombolytic therapy. Potentially, this is due to its expression in various cell types and/or the effects of clot breakdown being too small to be represented in peripheral plasma. For example, miR126-3p and miR-126-5p are highly expressed in both ECs and platelets (Harris et al. 2008, Kaudewitz et al. 2016). Long et al. also reported a general lower abundance of miR-126 in stroke patients compared to healthy controls (Long et al. 2013). Interestingly, a decrease of miR-126-5p at 6 h after thrombolysis that reverted to baseline at 24 h has been observed. It needs to be taken into consideration that thrombolysis does not target the thrombus specifically and has effects on other cell and tissue types. For example, data in this thesis show that levels of miR-122 decrease after thrombolysis. The expression of the liver-specific miR-122 was found to be altered in acute stroke patients and decreased 72 h after stroke onset (Jickling et al. 2014, Wang et al. 2014). The development of miR-122 levels in stroke patients compared to healthy control samples could, however, not be confirmed in the thrombolysis cohort as no reference samples from healthy controls or samples from the time before stroke onset have been available. Besides, the Mayr group recently reported that circulating levels of miR-122 strongly correlate with the risk of developing metabolic syndrome and T2DM, which in turn are risk factors for stroke (Willeit et al. 2017). In the spike-in experiment, it has been proved that levels of miR-122 are not affected by platelets. It is plausible that rtPA, which is metabolised hepatically, influences the abundance of liver-derived miRNAs such as miR-122. Alternatively, the decrease of miR-122 might be caused by a previous upregulation by the acute event instead of being induced by rtPA.

Several other circulating miRNAs showed a decrease upon the course of thrombolysis. Levels of miR-125b-5p, miR-192, miR-214 and miR-375 were lower at both 6 h and 24 h after rtPA administration. Interestingly, in the literature only miR-125b-5p was reported to be abundant in platelets, although platelets are the main contributor to many other circulating miRNAs (Ple et al. 2012, Tiedt et al. 2017). Van Balkom et al. reported high expression of miR-214 in ECs due to hypoxia and a release of miR-214-packed exosomes, which stimulate angiogenesis (van Balkom et al. 2013). MiR-214 application in mice reduced the myocardial cell damage, injury size and apoptosis rates induced by experimental hypoxemia or hypoperfusion (Wang et al. 2016). It seems possible that miR-214 is released into circulation during ischaemia either actively by ECs or due to endothelial damage in the hypoxic stroke area. MiR-125b-5p, which is described to be abundant in the platelet transcriptome at moderate levels, was also decreased

at both points in time (Ple et al. 2012). Sempere et al. found a high neuronal expression of miR-125b-5p in human and mouse brains (Sempere et al. 2004). Recent work by the Mayr group unveiled a significant inverse correlation of aortic diameter size and miR-125b in aortic aneurysm patients (Zampetaki et al. 2014). Recently, Tiedt and collaborators reported that miR-125a-5p, miR-125b-5p and miR-143-3p are elevated after stroke onset and, thus, potentially offer diagnostic utility as biomarkers for ischaemic stroke (Tiedt et al. 2017). In this work, the author shows that levels of miR-125b-5p – besides miR-122, miR-192 and miR-375 – were higher before thrombolysis. This might be due to the effects of alteplase therapy on levels of circulating miR-125b-5p. Pharmacological impact on levels of miR-125b-5p must be taken into consideration when discussing its biomarker potential. MiR-125b, miR-275 and miR-122 have also been shown to be upregulated in liver biopsies of simple steatosis or non-alcoholic steatohepatitis (Pirola et al. 2015). Similarly, patients with unstable angina pectoris presented higher levels of miR-192 and miR-375 than healthy volunteers (Ren et al. 2013). Furthermore, miR-375 is positively associated with heart failure, diabetes, myocardial infarction and cardiac hypertrophy but reduced in different types of cancer in animal studies. Furthermore, Garikipati and collaborators found an upregulation of miR-375 in the rate of human heart failure. Silencing of miR-375 in turn enhanced angiogenesis and reduced inflammation after MI (Garikipati et al. 2015). Thus, alterations in circulating miRNA patterns after thrombolysis are not exclusively attributable to rtPA effects or clot breakdown. As a consequence of the stroke, the ischaemia might trigger the release of several miRNAs from the injury site, which might explain their relapse to baseline levels after some time. However, it seems probable that thrombolysis also triggers the release of miRNAs into circulation. After intervention, serum from stroke patients showed a modest upregulation of platelet-derived miRNAs. These effects were less pronounced for miRNAs that are abundant in multiple cell and tissue types such as miR-126-3p. While the expression of miR-126-5p decreased 6 h after thrombolysis but recovers to baseline levels after 24 h, levels of miR-126-3p did not change. This might be due to influences of EC damage or activity at the ischaemic damage site and the release of miRNAs upon thrombolysis. However, it needs to be taken into account that the investigated cohort comprises three patients with cryptogenic stroke. In these patients, the effect of thrombolysis might not be comparable to that in patients with ischaemic stroke since thrombotic occlusion was not clinically confirmed.

Furthermore, the author observed analytical differences dependent on the applied normalisation methods. Especially the use of endogenous miRNAs as a normalisation control in patients after

thrombolysis might be problematic, as effects of rtPA on circulating miRNAs are poorly investigated yet. Therefore, care must be taken when using endogenous miRNAs in the context of ischaemic diseases such as stroke. However, the presented data support the conclusion that thrombolysis in stroke patients leads to specific miRNA changes in circulation.

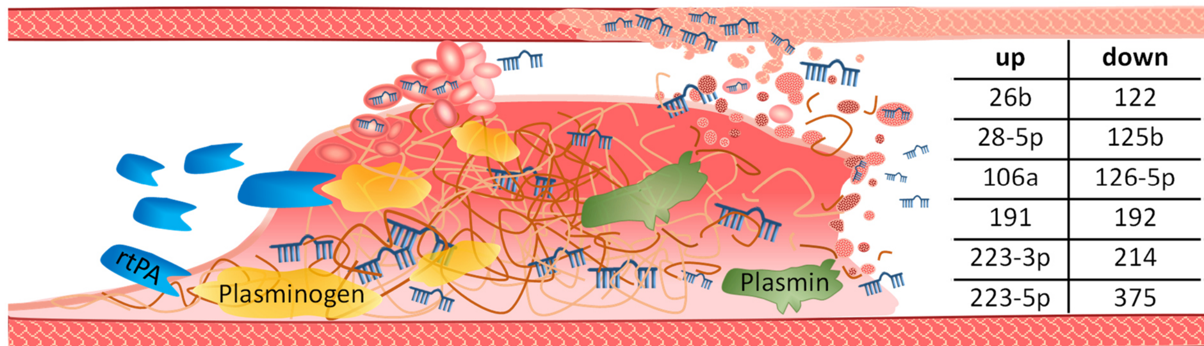


Figure 27 Effects of thrombolysis
Several miRNAs were up- or downregulated in plasma of ischaemic stroke patients after thrombolysis therapy.

5.7 Normalisation problems

Throughout this work, differences were observed between normalisation methods. Normalisation of miRNA measurements is key in miRNA research due to inter- and intrapersonal variability in sample preparation and RNA-extraction efficiency. In analogy to protein measurements by enzyme-linked immunosorbent assays, a fixed volume of serum or plasma is used. No correction is performed for the RNA content within a sample. Given the minute contribution of miRNAs, the total RNA amount of plasma would also not be suitable as a normalisation control. Similarly, the small nuclear RNA U6 is less suitable as an endogenous control. It is an intracellular RNA with low circulating levels and high interindividual variability that is strongly affected by cell damage or apoptosis (Benz et al. 2013). However, normalisation of the data is essential in order to limit influences of technical variabilities and to ensure comparability of data. For the work at hand, two different normalisation methods were used: firstly, a synthetic non-mammalian miRNA was used (Cel-miR-39), which was described earlier as an exogenous normalisation control. A concern when using synthetic miRNAs e.g. manually spiked-in Cel-miR-39 is that they are more likely to be degraded by ribonucleases compared to protected endogenous miRNAs packed in protein complexes or residing in microparticles. Secondly, CT average values calculated using panels of unaffected abundant

miRNAs such as the liver-specific miR-122 – which must not show a disease association – were used as an endogenous normalisation control (Lagos-Quintana et al. 2002, Zampetaki and Mayr 2012).

However, no single appropriate control for circulating miRNAs is available so far. CT average seems suitable when a broader miRNA panel is analysed. Furthermore, it has been shown that several circulating miRNAs are co-expressed in clusters and highly correlated. Thus, analysing miRNA patterns, so called miRNA signatures, can offer more comprehensive information compared to alterations of single miRNAs. However, all applied normalisation methods are prone to analytical errors and, therefore, it is recommended to use different normalisation methods in order to gain reliable and consistent data (Zampetaki and Mayr 2012, Benz et al. 2013, Fiedler et al. 2018).

5.8 Clinical relevance

In the era of precision medicine, soluble biomarkers become ever more important for predicting or diagnosing a wide range of diseases and treatment responses. For instance in cardiology, altered levels of troponin indicate an acute MI, elevated BNP reflects heart failure and increased D-dimer, a fibrin degradation product, points towards a thrombotic event while none of the parameters offer a hundred percent sensitivity. Small ncRNAs have the potential to offer a new entity of soluble biomarkers to diagnose diseases, to predict progression, to monitor therapeutic interventions and to prevent complications in order to improve clinical outcome and reduce overall mortality.

5.8.1 Antiplatelet therapy

The importance of effective antiplatelet therapy becomes clear when studying high-on-treatment platelet reactivity patients, which are at a significantly higher risk of major athero-thrombotic and cardiac events (Breet et al. 2011). This effect was first described in patients who suffered from thrombotic stent occlusion after PCI stenting followed by immediate dual platelet inhibition with ASA and clopidogrel. Such patients were then called clopidogrel non-responders. Nowadays, an estimate of one third of patients suffers from high-on-treatment platelet reactivity, despite seemingly adequate dual platelet inhibition with ASA and a $P2Y_{12}$ inhibitor (Gurbel et al. 2003, Tantry et al. 2013). There is a need for an ideal platelet agent, which would show quick onset of efficacy without the necessity of activation of a prodrug, fewer side effects and interactions (other drugs, genetic influence), a wide therapeutic window and the possibility of antagonism. To date, several SNPs have been found to have an impact on the effectiveness of CYP enzymes and, thus, affect the therapeutic success of antiplatelet therapy (Preissner et al. 2013).

$P2Y_{12}$ inhibitors act by targeting the seven-transmembrane domain receptor of type $P2Y_{12}$, which triggers platelet aggregation via G_i and $G_{12/13}$ protein (Hochtl and Huber 2014, Joshi et al. 2014). Clinical studies have described the varying effectiveness of $P2Y_{12}$ inhibition. Clopidogrel, in particular, does not work well in individuals who have low levels of the CYP2C19 liver enzyme needed to activate the prodrug. Prasugrel shows a more efficient platelet inhibition compared to clopidogrel and significantly improves clinical outcome with decreased rates of ischaemic events – as shown for stent thrombosis in ACS patients with planned PCI – but is accompanied by a higher risk of major bleeding complications (Brandt et al. 2007, Wiviott et al. 2007). Others report that ACS patients benefit more from treatment with ticagrelor compared to clopidogrel administration (Storey et al. 2007, Cannon et al. 2010). However, in a recently published large clinical review of antiplatelet therapy in ACS patients (n=16,098), Larmore and collaborators concluded that prasugrel (n=13,134) is the most widely administered drug and preferable to ticagrelor (n=2,964) in terms of unintended side effects while showing similar effectiveness in platelet inhibition (Larmore et al. 2015). In PCI patients, the highest efficacy was achieved by prasugrel in contrast to high-dose clopidogrel or ticagrelor treatment. However, the former was accompanied by higher risk of major side effects like bleeding compared to ticagrelor (Singh et al. 2015). In a small clinical trial of cardiac arrest ACS patients (n=40) undergoing PCI, prasugrel and ticagrelor showed better therapeutic

success compared to clopidogrel, in particular with regards to a reduction of re-thrombosis (Bednar et al. 2015). Furthermore, in the PLATO trial, Storey et al. reported fewer adverse events of sepsis and pulmonary complications in the ticagrelor versus the clopidogrel group, but this benefit was independent of platelet function (Storey et al. 2011, Storey et al. 2014).

Altogether, modern antiplatelet therapy has fundamentally improved clinical outcome of CVD patients' treatment and, thus, lowered overall mortality rates. Platelet-related miRNAs are, amongst others, influenced by antiplatelet medication. Furthermore, circulating miRNAs might reflect processes leading to platelet formation and, thereby, offer useful information regarding the state of platelet and megakaryocyte activity. Thus, customised antiplatelet drugs and sufficient monitoring of platelet function – potentially by miRNA-based methods – could be a further step towards improving CVD therapy in the future (Hochtl and Huber 2014, Bednar et al. 2015, Larmore et al. 2015, Thomas et al. 2015, Levine et al. 2016, Ibanez et al. 2018).

5.8.2 Platelet function tests

Since William Duke started to explore the physiological function of platelets in 1910, platelet activity became an expanding topic in research and clinical settings (Duke 1983). Approximately 50 years later, Gustav Born invented the light transmittance aggregometry method and, since then, platelet function tests have become an important tool for detecting haemostatic disorders (Born 1962). However, currently, there is no function test with a wide clinical utility, which is capable of assessing the diverse signalling events leading to platelet activation in physiological as well as pathological conditions (Gurbel et al. 2007, Grove et al. 2012). Classical test methods like bleeding time and platelet count are prone to biological and artificial influences and only highly specialised laboratories provide useful results for advanced coagulation tests. However, the latest platelet function tests, such as VASP and VerifyNow $P2Y_{12}$, are more specifically monitoring $P2Y_{12}$ activation in platelets but lack an assessment of other agonists and processes triggering platelet activation (Grove et al. 2012, Thomas et al. 2015). To date, it is unclear to what extent miRNA biomarkers might improve clinical diagnostic strategies.

Novel miRNA-based platelet function tests could offer possibilities for monitoring and adapting antiplatelet therapy. However, one must be careful when interpreting experimentally assessed platelet function data. For instance, inter-laboratory comparability of platelet function tests by

LTA is poor. Even commercially available platelet function tests are only comparable to a limited extent and do not capture the diverse mechanisms, which can lead to platelet activation and aggregation (Bouman et al. 2010, Tantry et al. 2010). Recently, Kerneis and collaborators compared STEMI-patients 30 days after undergoing PCI and consecutive treatment with either prasugrel 10 mg OD (n=60) or ticagrelor 90 mg BD (n=58) and performed LTA, VerifyNow $P2Y_{12}$ and VASP. Notably, they found remarkable differences in on-treatment platelet reactivity using the different platforms (Kerneis et al. 2015).

The group of Prof. Mayr as well as others analysed circulating platelet miRNAs in both physiological and pathophysiological processes: platelet miRNAs might contain information on the functionality of platelet activation and aggregation but also on the dysfunctionality leading to various disorders and diseases like thrombosis, MI or stroke (Zimmerman and Weyrich 2008, Nagalla et al. 2011, Zampetaki et al. 2012, Laffont et al. 2013, Willeit et al. 2013). Ward and collaborators described cell-specific expression profiles of circulating miRNAs and, particularly in MI patients, differences between high thrombus burden STEMI (n=9) and NSTEMI (n=4), which could, at least partly, result from the presence of activated and aggregated platelets in the thrombotic and ischaemic tissue (Ward et al. 2013). It is yet unclear to what extent these miRNAs also influence platelet function. For example, Landry and co-workers propose that the regulation of mRNAs of $P2Y_{12}$ receptor proteins and the receptor expression itself on the surface of platelets is, at least partially, controlled by argonaute-2-miR-223 complexes (Landry et al. 2009). Altogether, the data presented in this thesis show that miR-223-3p, miR-223-5p, miR-126-3p, miR-126-5p and a few other miRNAs as well as YRNA fragments reside in platelets, are influenced by antiplatelet agents and correlate with platelet function as well as platelet activation proteins. Future studies need to ascertain, whether these molecules modulate platelet function and are, therefore, suitable for monitoring platelet function and platelet inhibition.

5.8.3 Technical challenges for small ncRNAs as biomarkers

Small ncRNAs, miRNAs in particular, have characteristics desirable for serving as a novel class of biomarkers. They are involved in protein regulation via mRNA interaction, reflect intracellular signal pathways, genetic processes and have regulatory functions in various physiological and pathophysiological conditions. Moreover, some miRNAs seem to be synthesised and released in a cell and tissue-specific manner such as miR-122 exclusively being produced by hepatic cells (Lagos-Quintana et al. 2002). Furthermore, miRNAs are detectable in all types of body fluids – such as urine, saliva, ejaculate or breast milk – they are packed in microparticles, exosomes or lipoproteins and, thus, relatively stable. Circulating miRNAs originate from various circulating cells like erythrocytes, leukocytes or platelets but also from tissues, including ECs that line the blood vessels. They are secreted in an active or passive manner. Although miRNAs only occur at low levels in circulation, about 200 miNRAs can be readily detected by RT-qPCR-analysis even in small volumes (100-200 µl) of either fresh blood or even frozen plasma and serum samples. This is advantageous in comparison to many other test methods, for example platelet function tests, which require fresh samples or larger sample volumes (Mitchell et al. 2008, Weber et al. 2010, Zampetaki and Mayr 2012, Laffont et al. 2013, Willeit et al. 2013, Bang et al. 2014, Jickling et al. 2014, Bertoia et al. 2015). Nonetheless, there are several reasons that still limit the suitability of miRNA biomarkers, especially in clinical practice. The process of RNA purification, RT, PreAmp and RT-qPCR is time-consuming and at present the workflow for assessing miRNA expression in body fluids cannot be fully automated. Thus, it is not an appropriate point of care method in emergency situations (Shiu et al. 2014). Another major concern is the relative rather than absolute quantification and a lack of a general normalisation method for miRNA measurements, which accounts for variability in sample preparation and is a source of methodological error. Standardised reference values are essential for comparability and eligibility as an appropriate tool for routine diagnostics. However, these absolute reference values have yet to be established.

Novel technologies might overcome some of the problems miRNA research faces to date: NGS enables highly specific detection of a wide range of RNA molecules and, thus, allows a more sensitive identification of small ncRNAs including miRNAs and their isomirs within one run. Few experiments have shown variations of bases preferably at the 3p or 5p end of mature miRNAs, for which conventional RT-qPCR primer might not account for; this is supposedly caused by alternative cleavage by enzymes Dicer and Drosha and further posttranscriptional

modifications (Yang et al. 2006, Wu et al. 2009). Moreover, several NGS findings of miRNAs were not coherent with the sequence list on mirbase[9], as this database is mainly based on analyses by RT-qPCR (Ebhardt et al. 2009, Lee et al. 2010). Still, NGS is time-consuming and expensive and also not yet suitable for clinical routine application in CVD diagnosis. Therefore, miRNA research needs more consistent technologies and platforms, common standards and accepted normalisation methods for better comparability. However, the accepted paradigms in miRNA research are still shifting. Exemplary, over the last decade there was, more or less, a consensus in this field that only one strand of a pre-miRNA duplex becomes biologically active while its corresponding star strand is degraded. The recent discovery regarding the biological function of some star strands claim the opposite and, finally, it was shown that both strands of miRNAs are expressed in various cell and tissue types with specific biological functions (Shin 2008, Bang et al. 2014, Schober et al. 2014). Future research might overcome the current limitations and help to uncover the potential of miRNAs as biomarkers for CVD.

5.8.3.1 The biomarker potential of miR-223

Encoded on X chromosome locus q12, miR-223 has its highest expression in the bone marrow and is inter alia involved in regulation of haematopoietic cell development (Chen et al. 2004, Johnnidis et al. 2008). The work at hand confirms miR-223-3p to be expressed at high levels in both PPP and PRP as detected by NGS and validated by individual RT-qPCR. Similarly, others and the Mayr group identified miR-223-3p to be among the highly abundant miRNAs in platelet-derived microparticles, PRP and platelets itself and proposed an association with miRNA patterns that correlate with platelet aggregation and are predictive of CVD. Additionally, changes in levels of miR-223-3p were observed in circulation of healthy volunteers upon antiplatelet therapy (Nagalla et al. 2011, Diehl et al. 2012, de Boer et al. 2013, Willeit et al. 2013, Pan et al. 2014). In the experiments conducted for this thesis, associations between circulating levels of miR-223, platelet activation markers and platelet activity were found. Exemplary, during the course of antiplatelet therapy, levels of circulating miR-223 were reduced in both healthy volunteers and ACS patients. In ACS patients, it has been identified that platelet reactivity, which was assessed by VASP, significantly correlates with levels of

[9] URL: “http://www.mirbase.org/”, visited 20.10.2018, 12.15 CET.

circulating miR-223-3p. In line with the presented data, others have also shown that antiplatelet therapy influences the expression of miR-223-3p in circulation. For instance, decreased circulating miR-223-3p levels were detected in both healthy volunteers and patients taking ASA (de Boer et al. 2013, Willeit et al. 2013). In contrast, activation of platelets promotes the secretion of miR-223-3p packed microparticles (Gidlof et al. 2013, Laffont et al. 2013).

In CVD patients identified as clopidogrel non-responders, platelets showed a lower expression of miR-223-3p accompanied by attenuated platelet function compared to healthy volunteers. Similarly, associations between low miR-223-3p levels and decreased responsiveness to clopidogrel were observed in NSTEMI-patients. Altogether, these findings led to the suggestion that miR-223-3p might be a parameter for monitoring platelet reactivity and $P2Y_{12}$ inhibition as well as predicting responsiveness to clopidogrel (Shi,R. et al. 2013, Zhang et al. 2014). Interestingly, Leierseder et al. reported findings regarding miR-223-3p and platelet physiology in mice: compared to the wild-type mice, miR-223-3p deficient mice neither showed differences in platelet volume, number, lifespan and platelet surface receptors nor performed worse in ADP-induced platelet aggregation tests (Leierseder et al. 2013). Mice compared to humans, however, lack a binding site in the 3'-UTR of the mRNA coding for purinergic receptor $P2Y_{12}$. This might, at least partly, explain the distinct effects that miR-223-3p exerts on platelet genesis and function in mice and humans (Landry et al. 2009, Shi et al. 2015). Conclusively, it emphasises the necessity for being careful when extrapolating knowledge gained from animal studies to humans.

In recent years, miR-223 has generated substantial interest in platelet physiology and, concomitantly, in CVD. Previous work of the Mayr group identified miR-223-3p – alongside miR-126-3p and miR-197 – to be predictive of major cardiac events and, thereby, improved the risk re-classification based on the Framingham risk score in a primary preventive setting (Zampetaki et al. 2012). Similarly, Schulte et al. recently revealed that miR-197 and miR-223-3p in serum of CAD patients (n=873) are predictive of cardiovascular death (Schulte et al. 2015). In other diseases such as chronic kidney disease, dysfunction of haemostasis and platelet aggregation seem to be closely linked to disease pathogenesis. A fundamental role of miR-223-3p was observed in a mouse model of chronic kidney disease, where regulation of miR-223-3p correlated with disease stage to a high degree and had predictive power for disease

progression (Taibi et al. 2014). A general association of altered miR-223-3p with endothelial and platelet dysfunction and chronic inflammation is probable (Pan et al. 2014).

Another interesting aspect is the transfer of microparticles containing miR-223-3p, as described by Laffont and collaborators, for mediation between human platelets and ECs, thereby, influencing gene expression in human umbilical vein ECs and targeting regulation of mRNA and protein levels of few endothelial genes (Laffont et al. 2013). Others reported on the process of endothelial uptake of HDL-complexes containing platelet-derived miR-223. This in turn altered the expression of intercellular adhesion molecule 1, an important cellular adhesion molecule, which is expressed on ECs (Tabet et al. 2014). Pan et al. found that platelet-derived microparticles containing miR-223-3p induce apoptosis in ECs by targeting insulin like growth factor 1 receptor (Pan et al. 2014). Shi and collaborators reported attenuated proliferation rates of ECs mediated by overexpression of miR-223-3p in vitro and, thereby, inhibition of angiogenesis. Importantly, they observed a potential experimental source of error: levels of miR-223-3p in ECs increased in vitro after conventional isolation and culture (Shi, L. et al. 2013).

The work at hand corroborates that platelets are a major source of circulating miR-223 and that miR-223 is among the most abundant miRNAs of platelet origin (Landry et al. 2009, Ple et al. 2012). Moreover, it demonstrates the presence of the 5p strand of miR-223 – former called star or passenger strand – in circulation, which, similarly to its corresponding strand, changes upon therapeutic platelet inhibition, endothelial denudation and thrombolysis. Therefore, it could potentially serve as part of a biomarker for reflecting platelet function, vascular injury or ischaemic events. Overall, there is increasing evidence for a role of miR-223 in angiogenesis and maintenance of vascular integrity, which might offer opportunities for establishing novel preventive, diagnostic and therapeutic approaches in CVD.

5.8.3.2 The biomarker potential of miR-126

Substantial evidence points towards a participation of miR-126 in CVD. MiR-126 is encoded on chromosome 9, Egfl7, an intron region of the epidermal-growth factor-like domain gene, and is important for the development of the vascular system. It is proposed to be involved in the control of the vascular endothelial growth factor pathway via DLK1 but also in CVD pathophysiology – for instance, by modulating the vascular cell adhesion molecule 1 (Fish et al. 2008, Wang et al. 2008, Nichol and Stuhlmann 2012). Wang and collaborators were among the first to describe its functions in angiogenesis in vivo: mice with experimental deletion of miR-126-3p suffered from cardiac diseases, haemorrhaging and embryonic lethality. Moreover, they observed pro-angiogenic effects of miR-126-3p on the regulation of MAP kinase signal pathway by repressing sprout-related EVH1 domain-containing protein 1 (Wang et al. 2008). The experimental overexpression of the pro-inflammatory miR-126-3p attenuates levels of vascular cell adhesion molecule 1 and, thereby, reduces cell adherence for instance by leukocytes. Thus, miR-126-3p regulates adhesion molecule expression in ECs and vascular inflammation processes (Merhi-Soussi et al. 2005, Harris et al. 2008).

Another trigger of atherosclerosis, laminar shear stress, accounts for upregulation of miR-126-3p in human umbilical vein ECs in vitro, which coincides with modulations of vascular cell adhesion molecule 1 and Syndecan 4. Similarly, in vivo experiments in Apo-E knockout mice models for chronic kidney disease revealed significant associations of miR-126-3p and expression of Syndecan 4 and C-X-C chemokine receptor type 4. Both molecules were previously shown to be linked to atherosclerosis (Taibi et al. 2014, Mondadori dos Santos et al. 2015). Likewise, the stability of atherosclerotic plaques seems to be affected by levels of miR-126-3p: miR-126-3p is enriched in apoptotic bodies, upregulates the synthesis of C-X-C motif chemokine 12, which enhances plaque stability via EC communication and, in turn, attenuates endothelial damage and, thereby, inhibits atherosclerosis progression. This process is for example disturbed in T2DM, where high blood glucose damages ECs and disturbs vascular maintenance (Zernecke et al. 2009, Jansen et al. 2013). The Mayr group and others revealed associations between low levels of circulating miR-126-3p and dysregulated glucose tolerance in T2DM (Fichtlscherer et al. 2010, Zampetaki et al. 2010, Olivieri et al. 2015). MiR-126-3p was suggested, besides miR-197 and miR-223-3p, to be predictive of major cardiac events (Zampetaki et al. 2012).

Previous studies implicated the existence of circulating mature miR-126-3p of specific EC origin (Fish et al. 2008, Wang et al. 2008, Zampetaki et al. 2010). However, the data in this work complement recent findings and show miR-126-3p to be also expressed in platelets (Diehl et al. 2012, Willeit et al. 2013). MiR-126-5p has just recently been shown to contribute to atherosclerosis development (Schober et al. 2014). The author confirmed the abundance of miR-126-5p in both serum and plasma. Furthermore, significant associations of miR-126-3p and miR-126-5p with platelet function as well as antiplatelet therapy in ACS patients have been noted. Further studies need to investigate to what extent platelet-derived and endothelial miR-126 contribute to the pathogenesis of CVD.

5.8.4 MicroRNAs as therapeutics

The important role of miRNAs in various physiological and pathological mechanisms might reveal novel pharmacological targets. The first miRNA-based drug was tested in hepatitis C patients. Antagonising the liver-specific miR-122 by Miravirsen, which is a modified antisense locked nucleic acid, inhibited viral replication and reduced the hepatitis C viral load. It was well tolerated in a Phase II clinical trial (Janssen et al. 2013). However, clinical trials for miRNA-based therapy of CVD are not yet in sight. Nevertheless, some experimental studies promise potential novel options: inhibition of miR-24 decreased the myocardial infarction area in mice, limited apoptosis and improved cardiac maintenance (Fiedler et al. 2011). In a swine model undergoing cardiac ischaemia, cell-protective and anti-inflammatory benefits were demonstrated by administering antisense miR-92a (locked nucleic acid-92a). Inhibiting expression of miR-92a, surprisingly, had a positive effect on the size of the infarction area. Therefore, an anti-miR-92a based therapy is proposed to reduce ischaemic areas post MI with concomitantly improved cardiac ejection fraction (Hinkel et al. 2013). Similarly, overexpression of miR-126-3p in mesenchymal stem cells and subsequent injections into ischaemic mouse myocardium led to improved angiogenetic and cardiac function (Huang et al. 2013). Schober and collaborators discussed systemic application of miR-126-5p in atherosclerosis patients in order to improve healing of injured ECs by promoting EC proliferation via DLK1 pathway (Schober et al. 2014). Santulli and collaborators designed a miRNA-based drug-eluting stent and inserted it during PCI in a rat balloon injury model: with an adenoviral vector construct with miR-126-3p binding sites (Ad-p27-126TS) they induced overexpression of p27 – a cyclin-dependent kinase inhibitor involved in cell cycle control – in vascular smooth muscle and ECs. This overexpression of p27 showed protective effects on ECs

and significantly reduced rates of hypercoagulability, complete re-endothelialisation and, most importantly, stent thrombosis (Santulli et al. 2014).

While promising concepts emerge from these preclinical studies for deciphering basic pathophysiological mechanisms in CVD, the successful translation of miRNA therapeutics faces several hurdles. Inhibition of one miRNA might not only influence the target of interest but also show side effects in other cellular processes as a single miRNA can lead to multiple effects on gene expression and acts differently in various tissues. For instance, inhibition of miR-34a might on the one hand offer a possible therapeutic approach for reducing cardiomyocyte cell death. However, on the other hand, it has been found to contribute to induction of tumourigenesis, such as in neuroblastoma or hepatocellular carcinoma (Hermeking 2010, Boon et al. 2013). Simultaneously, an interventional phase I study is currently recruiting patients with unresectable liver cell carcinoma in order to test therapeutic use of miR-34 (clinical trial: NCT01829971). Another example is the recent study by Flierl et al., who showed that the phosphorothioate-modified RNA backbone of longer anti-miR-agents – widely used for experimental purposes in laboratories – induces platelet activation and aggregation via glycoprotein VI (Flierl et al. 2015). Thus, systemic miRNA therapeutics may lead to unexpected complications, unless more tissue-specific targeted engagement can be achieved.

6 Conclusion

The work at hand elucidates the role of platelets in contributing to circulating ncRNAs, in particular miRNAs and YRNA fragments, in human physiology and pathology. Small ncRNAs, such as miRNAs and YRNAs, could be important regulators of signal cascades in platelets and, thereby, contribute to CVD pathogenesis. To the author's knowledge, the data presented in this thesis show for the first time that miRNAs like miR-126-3p, miR-126-5p, miR-223-3p and miR-223-5p as well as fragments of YRNA significantly correlate with platelet activity and function and are affected by antiplatelet therapy. Ticagrelor in particular seems to have an impact on circulating levels of miR-126-5p in ACS patients. Additionally, it has been confirmed that the rs4636297 SNP affects miR-126 expression and correlates with markers of platelet activation and, thereby, miR-126 is likely to participate in platelet function. Additionally, this thesis first provides evidence that circulating miRNAs are altered upon thrombolysis therapy in stroke patients, among them miR-214 and miR-125b-5p.

These insights are essential for the development of miRNA-based preventive, diagnostic and therapeutic approaches in CVD research. Future work needs to address whether miRNAs or YRNAs offer clinical utility for platelet-related disorders and CVDs, for example, by enabling individually customised antiplatelet therapy and improvement of monitoring tools for assessment of bleeding risk and by offering a novel class of biomarkers for identifying CVD predisposition. This thesis shall encourage further studies, help to shed light on the role of miRNAs in cardiovascular research and improve prevention, diagnosis and treatment of CVD.

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8 Publications

Kaudewitz, D., P. Skroblin, **L. H. Bender**, T. Barwari, P. Willeit, R. Pechlaner, N. P. Sunderland, K. Willeit, A. C. Morton, P. C. Armstrong, M. V. Chan, R. Lu, X. Yin, F. Gracio, K. Dudek, S. R. Langley, A. Zampetaki, E. de Rinaldis, S. Ye, T. D. Warner, A. Saxena, S. Kiechl, R. F. Storey and M. Mayr (2016). "Association of MicroRNAs and YRNAs with Platelet Function." Circulation Research 118(3): 420-432

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This work is dedicated to my parents.

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05/ 2014 – 12/ 2014	Study abroad internship, BHF Centre of Research Excellence, Cardiovascular Division, King's College London, UK
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10/ 2009 – 09/ 2011	Medical studies, preclinical courses, LMU/ TU München
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