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PO Box 117
221 00 Lund
+46 46-222 00 00

Clinical and Biological Aspects of Cardiovascular microRNA

Olof Gidlöf

LUND
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DOCTORAL DISSERTATION
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To be defended at Segerfalksalen, BMC, 13.00 September 20th

Faculty opponent

Niels Heegaard

Statens Serum Institut, Copenhagen

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Abstract

Ischemic heart disease is the leading cause of death in high-income parts of the world and is caused mainly by atherosclerosis in the coronary arteries. The rupture of an atherosclerotic plaque with subsequent platelet activation and clot formation can lead to myocardial infarction (MI). Atherosclerosis is a complex process, which involves the accumulation and oxidation of low-density lipoprotein in the vessel wall followed by endothelial inflammation, infiltration of monocytes and proliferation and migration of vascular smooth muscle cells towards the vessel lumen. microRNA (miRNA) is a class of short non-coding RNA which regulate gene expression through part-complimentary binding to target sites preferably within the 3'-UTR of specific mRNAs. miRNAs have pervasive roles in animal biology and aberrant expression of miRNAs have been linked with a wide spectrum of human disease. Additionally, the tissue specific manner of miRNA expression together with remarkable stability in plasma and evidence of release of miRNA from stressed or apoptotic cells have made miRNAs interesting as biomarkers for various diseases.

The aim of this thesis was (1) to assess the diagnostic and prognostic value of cardiac-enriched miRNAs in the context of coronary artery disease, (2) to screen for differences in miRNA content in platelets from myocardial infarction patients and elucidate a potential paracrine function for platelet miRNA and (3) to investigate the role of miRNAs in regulating endothelial inflammation in response to extracellular ATP/UTP.

In Study I and II, we found that cardiac-enriched miRNAs are increased 100-3000 fold within 12 h following onset of symptoms in patients with myocardial infarction and that the levels of two specific miRNAs, miR-208b and-499-5p, could be used to discriminate MI-patients from non-MI patients and were associated with increased risk of death and development of heart failure. In Study III, we found differentially expressed platelet miRNAs in MI patients compared to healthy controls using RNA-sequencing. Release of miRNAs upon platelet activation, as well as microparticle-dependent transfer of functional platelet-derived miRNA to endothelial cells was also shown *in vitro*. In Study IV, we showed that the effects of ATP and UTP on cell surface expression of intercellular adhesion molecule 1 (ICAM-1) and leukocyte adhesion is mediated in part by miR-22 in endothelial cells.

In conclusion, the levels of circulating cardiac-enriched miRNA have diagnostic and prognostic value in the context of coronary artery disease, platelet-derived miRNA can act as paracrine mediators in endothelial cells and miR-22 plays an anti-inflammatory role in the endothelium.

Key words Cardiovascular, Coronary Artery Disease, Biomarker, microRNA, Platelet, endothelium, UTP, ATP		
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Clinical and Biological Aspects of Cardiovascular microRNA

Olof Gidlöf

LUND UNIVERSITY

Doctoral Thesis
2013

Department of Cardiology
Faculty of Medicine
Lund University

Cover image: Low-magnification micrograph of the distal right coronary artery with complex atherosclerosis and luminal narrowing. The image is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license.

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The heart is deceitful above all things

J.T. Leroy

It's the little details that are vital. Little things make big things happen.

John Wooden

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This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. Cardiospecific microRNA plasma levels correlate with troponin and cardiac function in patients with ST elevation myocardial infarction, are selectively dependent on renal elimination, and can be detected in urine samples. Gidlöf O, Anderrson P, van der Pals J, Götberg M, Erlinge D. **Cardiology**, 2011;118(4):217-26.

II. Circulating cardio-enriched microRNAs are associated with long-term prognosis following myocardial infarction. Gidlöf O, Smith JG, Miyazu K, Gilje P, Spencer A, Blomquist S, Erlinge D. **BMC Cardiovascular Disorders**, 2013 Feb 28;13:12.

III. Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. Gidlöf O, van der Brug M, Öhman J, Gilje P, Olde B, Wahlestedt C, Erlinge D. **Blood**, 2013 May 9;121(19):3908-17.

IV. Extracellular UTP and ATP attenuate endothelial inflammation through miR-22 mediated ICAM-1 inhibition. Gidlöf O, Magistri M, Faghihi M, Wahlestedt C, Olde B, Erlinge D. Manuscript 2013.

Additional peer-reviewed papers, not included in the thesis

- *Complete discrimination of six individuals based on high-resolution melting of hypervariable regions I and II of the mitochondrial genome.* Gidlöf O, Burvall S, Edvinsson L, Montelius M, Allen M, Molin M. **Biotechniques**, 2009 Aug;47(2):671-2, 674, 676.
- *Succinate independently stimulates full platelet activation via cAMP and phosphoinositide 3-kinase-β signaling.* Högberg C, Gidlöf O, Tan C, Svensson S, Nilsson-Öhman J, Erlinge D, Olde B. **Journal of Thrombosis and Haemostasis**, 2011 Feb;9(2):361-72
- *5'UTR variants of ribosomal protein S19 transcript determine translational efficiency: implications for Diamond-Blackfan anemia and tissue variability.* Badhai J, Schuster J, Gidlöf O, Dahl N. **PLoS One**, 2011 Mar 11;6(3):e17672.
- *Plasma levels of liver-specific miR-122 is massively increased in a porcine cardiogenic shock model and attenuated by hypothermia*. Andersson P, Gidlöf O, Braun OO, Götberg M, van der Pals J, Olde B, Erlinge D. **Shock**, 2012 Feb;37(2):234-8
- *Farnesyl pyrophosphate is an endogenous antagonist to ADP-stimulated $P2Y_{12}$ receptor-mediated platelet aggregation.* Högberg C, Gidlöf O, Deflorian F, Jacobson KA, Abdelrahman A, Müller CE, Olde B, Erlinge D. **Thrombosis and Haemostasis**, 2012 Jul;108(1):119-32.
- *A common missense variant in the ATP receptor P2X7 is associated with reduced risk of cardiovascular events.* Gidlöf O, Smith JG, Melander O, Lövkvist H, Hedblad B, Engström G, Nilsson P, Carlson J, Berglund G, Olsson S, Jood K, Jern C, Norrving B, Lindgren A, Erlinge D. **PLoS One**, 2012;7(5):e37491.
- *Altered serum miRNA profiles during acute rejection after heart transplantation: potential for non-invasive allograft surveillance.* Sukma Dewi I, Torngren K, Gidlöf O, Kornhall B, Öhman J. **Journal of Heart and Lung Transplantation**, 2013 Apr;32(4):463-6.

- *The Antimicrobial Peptide LL-37 Alters Human Osteoblast Ca(2+) Handling and Induces Ca(2+)-Independent Apoptosis*. Säll J, Carlsson M, Gidlöf O, Holm A, Humlén J, Öhman J, Svensson D, Nilsson BO, Jönsson D. **Journal of Innate Immunity**, 2013;5(3):290-300.
- *Development of an MRM assay panel with application to biobank samples from patients with myocardial infarction.* Rezeli M, Végvári A, Donnarumma F, Gidlöf O, Smith JG, Erlinge D, Marko-Varga G. **Journal of Proteomics**, 2013 May 23.

Abbreviations

ACS – Acute Coronary Syndrome

ADP – Adenosine Diphosphate

ALT – Alanine Aminotransferase

APC - Allophycocyanin

AST – Aspartate Aminotransferase

ATP – Adenosine Triphosphate

AUC – Area Under the Curve

CABG – Coronary Artery Bypass Graft

CAD – Coronary Artery Disease

CKMB – Creatine Kinase, isoform MB

CVD – Cardiovascular Disease

ECG – Electrocardiogram

EGF – Epidermal Growth Factor

FACS – Fluorescence-Activated Cell Sorting

FITC – Fluorescein Isothiocyanate

GPR30 – G-Protein Coupled Receptor 30

HDL – High-Density Lipoprotein

HEK293 – Human Embryonic Kidney 293 cells

HMEC-1 – Human Microvascular Endothelial Cell-1

ICAM-1 – Intercellular Adhesion Molecule-1

IL – Interleukin

KLF2 – Krüppel-Like Factor 2

LDL – Low-Density Lipoprotein

LVEF – Left-Ventricular Ejection Fraction

M-CSF – Macrophage Colony-Stimulating Factor

MAPK – Mitogen-Activated Protein Kinase

MCP-1 – Monocyte Chemoattractant Protein-1

MI – Myocardial Infarction

miRNA – MicroRNA

NO – Nitric Oxide

NSTEMI – Non-ST Segment Elevation Myocardial Infarction

OxLDL – Oxidized Low-Density Lipoprotein

PAI-1 – Plasminogen Activator Inhibitor-1

PBMC – Peripheral Blood Mononuclear Cell

PCI – Percutaneous Coronary Intervention

PDGF – Platelet-Derived Growth Factor

PECAM-1 – Platelet Endothelial Cell Adhesion Molecule-1

PMP – Platelet Microparticle

qRT-PCR – Quantitative Real Time-Polymerase Chain Reaction

ROC – Receiver Operating Characteristic

STEMI – ST-Elevation Myocardial Infarction

TRAP – Thrombin Receptor Activating Peptide

TnI – Troponin I

TnT – Troponin T

UDP – Uridine Diphosphate

UTP – Uridine Triphosphate

UTR – Untranslated Region

VAMP8 – Vessicle-Associated Membrane Protein 8

VCAM-1 – Vascular Cell Adhesion Molecule-1

VLDL – Very Low-Density Lipoprotein

VSMC – Vascular Smooth Muscle Cell

Introduction

Cardiovascular disease

The common denominator for the research presented in this thesis is cardiovascular disease (CVD); what molecular and cellular mechanisms drive or attenuate it and how to come up with better diagnostic and prognostic tools for it.

CVD is a term for diseases of the heart and blood vessels, including peripheral arterial disease, stroke and myocardial infarction, and is the leading cause of death in high-income parts of the world[1]. Although many different factors affect the probability of contracting CVD, such as life style, genetics and concomitant disease[2-6] it is mainly driven by the same pathophysiological process: atherosclerosis[7-9].

Atherosclerosis

The vessel wall of large arteries consists of three morphologically distinct layers. The intima is the innermost layer, consisting of a monolayer of endothelial cells facing the lumen and a sheet of elastic fibers, the internal elastic lamina, on the peripheral side. The middle layer, i.e. the media, is made up by vascular smooth muscle cells (VSMC) while the outer layer, the adventitia, consists of connective tissue. The atherosclerotic process is initiated by the uptake of low-density lipoprotein (LDL) molecules from the circulation, which is accumulated beneath the endothelial cells of the intima[10] (Figure 1). Endothelial cell permeability is increased in areas of the vasculature where blood flow is disturbed, such as bifurcations or curvature, thus making them more susceptible to atherosclerotic lesions[11]. A significant step in the atherosclerotic process is the oxidation of LDL by reactive oxygen species formed in the intima. Oxidized LDL (oxLDL) stimulates and accelerates inflammatory processes in the overlying endothelial cells, causing release of pro-inflammatory molecules (e.g MCP-1) as well as increased expression of adhesion molecules (e.g. VCAM-1 and ICAM-1) on the cell surface[12]. This leads to recruitment and transmigration of monocytes through the endothelial monolayer. In the intima, monocytes then proliferate and differentiate into macrophages in response to the growth factor macrophage colony-stimulating

factor (M-CSF)[13]. OxLDL is taken up by the macrophages via scavenger receptors, accumulating as cytosolic droplets and these cells are ultimately transformed into foam-cells[14]. With time, the foam cells die and their lipid-filled contents leak out and form the necrotic core of the lesion. T-cells also infiltrate the atherosclerotic lesion, initiating production of many inflammatory and cytotoxic molecules and further accelerating the inflammatory process[15]. The last step in the development of an atherosclerotic lesion is the formation of a fibrous plaque. Cytokines and growth factors secreted from T-cells and macrophages initiate proliferation and migration of VSMCs into the intima[10]. A fibrous cap is formed in the intima by the accumulation of VSMC and VSMC-derived extracellular matrix over the lipid core of the lesion.

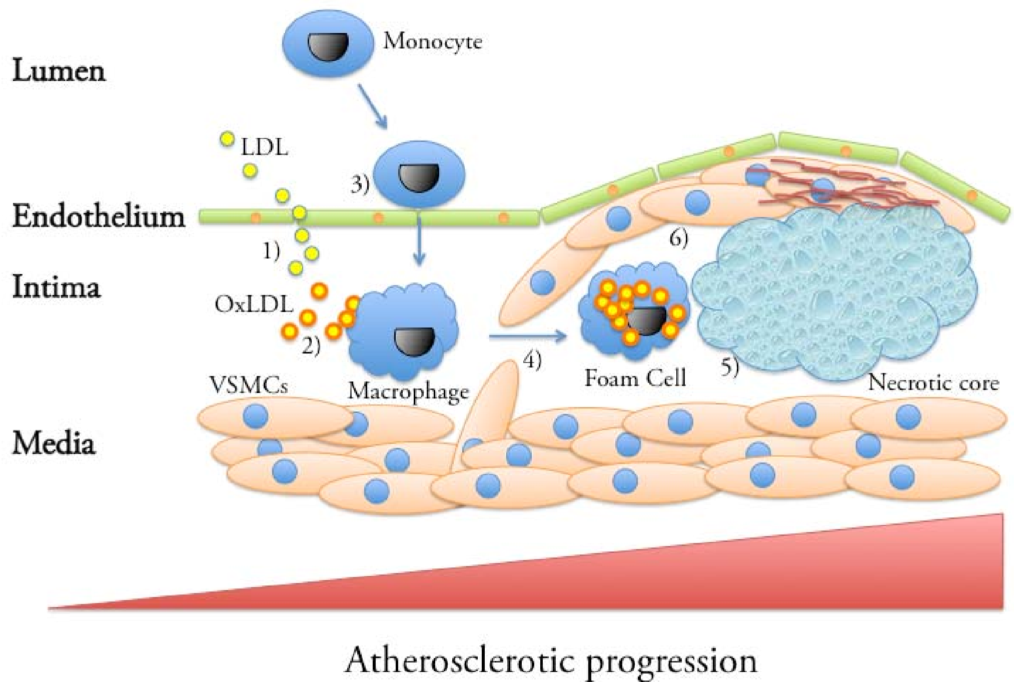

Figure 1. Depiction of atherosclerotic progression. 1) Accumulation of lipids (LDL) in the vessel wall 2) Oxidation of LDL by reactive oxygen species initiates an inflammatory process where endothelial cells upregulate expression of adhesion molecules 3) Monocytes attach to the endothelial cells and transmigrate into the intima where they proliferate and differentiate into macrophages. 4) OxLDL binds to macrophage scavenger receptors and promote uptake of OxLDL 5) Macrophages turn into foam-cells which become apoptotic and leave behind a growing mass of extracellular lipids and cell debris which make up the necrotic core of the lesion 6) Cytokines and growth factors secreted by inflammatory cells in the lesion induce proliferation and migration of VSMC towards the plaque surface and accumulation of extracellular matrix components over the necrotic core

Coronary artery disease and myocardial infarction

Atherosclerosis in the coronary arteries can cause narrowing of the vessel lumen (i.e. stenosis), causing ischemia and thereby giving rise to clinical manifestations defined as coronary artery disease (CAD).

The extent and character of CAD symptoms and manifestations depend largely on the composition of the atherosclerotic plaque[16]. A plaque rich in VSMCs and extracellular matrix is relatively stable but can cause stenosis of the vessel, giving rise to angina pectoris. A lipid-rich plaque covered by a thin fibrous cap is unstable and might rupture, exposing sub-endothelial molecules which causes platelet activation, thrombus formation and partial or complete occlusion of a coronary vessel, ischemia and/or cardiac cell death. In this case, the CAD is defined as an acute coronary syndrome (ACS), which includes unstable angina and myocardial infarction.

Ischemic heart disease is the leading cause of death in the world causing 12.2% of all worldwide deaths, a total of 7.2 million deaths each year[17]. In high income countries 16.3% of all deaths are caused by ischemic heart disease. Worldwide there are more than 7 million patients diagnosed with acute coronary syndromes (ACS) each year[18].

Clinical aspects of myocardial infarction

Symptoms of a myocardial infarction can vary considerably between patients but include chest pain, acute dyspnea, nausea and palpitations. In addition to clinical symptoms the analysis of electrocardiogram (ECG) patterns and markers of myocardial damage in the circulation are required to diagnose a patient with myocardial infarction.

ECG

On the ECG, the extent of myocardial damage is mainly reflected by the ST-segment (Figure 2). An elevated ST-segment in the acute phase is indicative of complete occlusion of the coronary vessel and patients are commonly diagnosed as ST-elevation myocardial infarction (STEMI) or non-ST elevation myocardial infarction (NSTEMI).

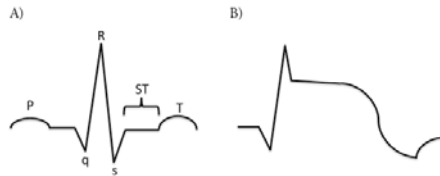

Figure 2. Schematic drawings of ECG patterns. A) A normal ECG with the ST-segment indicated. B) ECG with ST-elevation, indicative of myocardial infarction.

Biomarkers

There is a release of intracellular proteins from the necrotic myocardium into the circulation, which can serve as biomarkers for myocardial infarction[19]. Creatine Kinase, isoform MB, or CKMB, is an enzyme that is relatively cardiac-specific and has been used extensively to monitor myocardial damage. It increases in the blood within 4-6 hours of an infarction and reaches a peak at approximately 10-24 hours. It has a high sensitivity and specificity but is inferior to the current gold standard biomarkers Troponin-T and –I (TnT, -I). An initial rise in TnT/I can be seen within 3-9 hours and a peak is reached within 10-24 hours. TnT/I is extremely sensitive in most cases, but due to the delay in release, these biomarkers are not suitable for determining a diagnosis in the acute phase. Also, elevated levels of troponins, unrelated to MI, can be seen in elderly patients as well as in patients with kidney dysfunction. These issues indicate that in some cases, there is a need for additional biomarkers.

Therapies

The anti-platelet drugs aspirin and clopidogrel are usually given upon suspicion of MI and after diagnosis of MI, respectively. The main objective is however to restore blood flow to the ischemic myocardium. This is achieved primarily by percutaneous coronary intervention (PCI), where an angioplasty balloon or metal stent are introduced into the occluded coronary artery via a catheter from the inguinal femoral or radial artery. In cases where PCI is not immediately available, thrombolysis, i.e. breakdown of the thrombus by pharmacological means, is an alternative way of restoring coronary blood flow.

Prognosis

Long-term prognosis after MI varies greatly and is affected mainly by infarction size. Risk-stratifying factors include age, hemodynamic parameters, ST-segment deviation, diabetes and elevation of cardiac markers[20, 21]. Assessment of left ventricular ejection fraction (LVEF) may also improve prognostic ability[22].

The role of platelets in vascular biology and CAD

Platelets are small anucleate subcellular fragments originating from megakaryocytes in the bone marrow. They are essential for maintaining hemostasis[23] but more recent research suggest they may also contribute significantly to vascular inflammation[24-26]. Although void of a nucleus and genomic DNA, platelets contain mRNA and ribosomes and have the capability of de novo protein synthesis[27-29]. Platelets also contain a multitude of vasoactive substances, stored in either of two types of granules: α- and dense granules. The contents of these granules, such as adhesion proteins (e.g. fibrinogen, fibronectin, von Willebrand factor, thrombospondin), growth factors (e.g. PDGF, TGF-β, EGF), chemokines (e.g. RANTES, platelet factor 4), cytokines (e.g. IL-1β, CD40 ligand), coagulation factors (e.g. factor V and IX, PAI-1) and extracellular nucleotides (ATP and ADP) are released or exposed upon platelet activation, affecting endothelial cells, leukocytes as well as other platelets[25]. Platelet-derived IL-1β is for example a potent activator of endothelial cells, inducing cytokine secretion and monocyte recruitment[24, 26]. Release of CD40 ligand from platelets further promotes endothelial inflammation through IL-8 and MCP-1, resulting in adhesion of neutrophils and monocytes[30]. ADP plays a major role in initiating the activation of other platelets and amplifying the platelet response[31, 32]. The exposure of p-selectin on activated platelets interacts with monocyte PSGL-1, inducing secretion of pro-inflammatory and pro-coagulant factors from monocytes[33, 34].

Release of extracellular vesicles from platelets

Platelets are also capable of releasing extracellular vesicles in response to agonists and mechanical forces (e.g. high shear stress)[35-37]. Thrombin receptor agonist peptide (TRAP) has been shown to cause release of both microparticles and exosomes from platelets *ex vivo*[38]. Platelet microparticles (PMP) are 0.1-1 μm in size, released by membrane shedding and contain membrane proteins from the cell of origin, e.g. GPIIbIIIa[35] whereas exosomes are 40-100 nm in size and derived from exocytosis of multivesicular bodies and α-granules[38]. PMPs are pro-coagulant, mainly due to the presence of anionic phospholipid phophatidyl serine (PS), providing a membrane surface for assembling the components of the coagulation cascade[39]. The pro-coagulant activity is further enhanced by the presence of GPIIbIIIa[40] and tissue factor[28, 41, 42]. PMPs can also act as promoters of vascular inflammation by inducing cytokine release and expression of adhesion molecules in endothelial cells[43]. Moreover, microparticles and exosomes can fuse with the membrane of other cells, potentially acting as delivery vessels for proteins and genetic material[44, 45].

Platelets in ACS

Upon rupture of an unstable atherosclerotic plaque, molecules in the sub-endothelial intima such as collagen and von Willebrand factor are exposed. These molecules are recognized by glycoproteins on the surface of platelets, mainly GPIIb/IIIa, GPIb-IX-

V and GPIb-IIa. This interaction causes platelet adhesion to the vessel wall and release of the granular contents. Platelets spread over the endothelial surface and recruit additional platelets via the interaction of GPIIb/IIIa and fibrinogen. Within minutes, a thrombus is formed which occludes blood flow, causing ischemia and cell death in the tissue downstream of the ruptured plaque.

microRNA

MicroRNA (miRNA) is a class of short, non-coding RNA with pervasive roles in animal biology. Approximately 22 nucleotides (nt) in length, miRNAs mediate repression of gene expression through base pairing with complimentary sequences of mRNA. There are now almost 2000 miRNA species reported in man (miRBase v. 20) and miRNAs have been shown to play a part in as varying aspects of biology as organ development[46-49], energy homeostasis[50] and inflammation[51-54].

Dysregulation or aberrant expression of miRNA has been associated with a vast array of pathological processes, including cancer, neurological disorders and cardiovascular disease[55-61]. Although the regulatory potential of miRNA is huge, one estimation suggests that ~50% of the genome is regulated by miRNAs[62], the effect of a single miRNA:mRNA-interaction is usually modest and genetic ablation of single miRNAs often yield mild phenotypes. It is likely that miRNAs are fine-tuners rather than master regulators, and that their main function is to confer robustness to various biological processes[63, 64].

miRNA biogenesis and function

MiRNAs are transcribed as long hairpin precursor molecules (pri-miRNAs) by RNA polymerase II, either from independent genomic loci or introns of protein-coding genes[65, 66]. These precursor molecules act as substrates for the RNase III family member Drosha, which cleaves the pri-miRNA to produce a ~70 nt pre-miRNA. This immature miRNA is subsequently exported into cytoplasm via an Exportin 5-dependent mechanism[67], where it is further processed by the RNase Dicer, yielding a ~20 nt RNA duplex. One of the strands, the mature miRNA, is incorporated into the RNA-induced silencing complex (RISC), where it acts as a guide for target mRNA degradation or translational repression. A ”seed” region of 2-6 nucleotides near the 5’ end of the miRNA binds with full or partial complimentarity to sites within the mRNA[68]. The mRNA usually contains several target sites, which are often located in the 3’-untranslated region. The mode of action is not clearly defined, but is thought to be dependent on the extent of complimentarity in the miRNA:mRNA duplex. Mismatches within the duplex create bulges, which are believed to block the

ribosomal machinery and repress translation, whereas full complimentarity instead leads to degradation of the mRNA[68, 69].

Regulation of miRNA transcription

The promoter regions of miRNA genes are highly similar to those of protein-coding genes, suggesting that miRNA transcription is also regulated by transcription factors, enhancers and chromatin modifications. For example, two miRNAs important for cardiac development, miR-1 and -133 are transcriptionally regulated in the embryonic heart by the transcription factors SRF and MEF2[70, 71]. p53 induces transcription of miR-34 and -107, enhancing cell cycle arrest and apoptosis[72] and expression of miR-148 is dependent on promoter methylation status [73].

What extracellular stimuli that drive miRNA expression and which signalling pathways are involved is an area that is still relatively unexplored. However, β-adrenergic stimuli have been shown to drive transcription of several miRNAs in rat heart[74, 75]. In adipocytes, prostacyclin induces transcription of miR-711, -148b and -744[76] whereas activation of the G-protein coupled receptor GPR30 upregulates transcription of miR-338-3p in pancreatic β-cells[77].

microRNA in the cardiovascular system

It has become increasingly obvious during the last decade that microRNAs play an important role in cardiovascular development and physiology. Certain microRNA species enriched in cardiovascular tissues help orchestrate processes such as heart development, angiogenesis and cardiac contractility. It is also clear that dysregulation of microRNAs correlate with different cardiovascular disorders, including heart failure, vascular remodelling and atherosclerosis.

Myocardium

miR-1 and -133 stem from the same pri-miRNA and are two of the most abundant microRNAs in cardiomyocytes[78]. These miRNAs are involved in cardiac development and have opposing effects on cardiomyocyte differentiation[79, 80]. miR-208a is encoded by an intron of the α-MHC gene and is expressed exclusively in the heart[60]. Genetic ablation of miR-208a in mice revealed that it is required for cardiomyocyte hypertrophy and fibrosis in response to stress. miR-499 is encoded in an intron of the myosin gene Myh7b and is enriched in cardiac and skeletal muscle. It is strongly associated with cardiac differentiation[81, 82] and prevents cardiomyocyte apoptosis by targeting calcineurin-mediated Drp1 activation and mitochondrial fission[83].

miR-22 is another microRNA which has been extensively studied in the context of cardiac disease. Attenuation of miR-22 protected rat cardiomyocytes from hypertrophy *in vitro* through de-repression of the cell cycle regulator PTEN[84]. On the other hand, targeted deletion of miR-22 in mice promoted cardiac dilation and decreased contractile function in response to stress[85]. Moreover, Huang et al reported that miR-22 is a critical regulator of cardiac hypertrophy and cardiac remodeling in response to stress[86].

The therapeutic potential of cardiovascular miRNAs has been explored in a few studies lately. miR-24, which is enriched in cardiac endothelial cells and shown to be an inhibitor of angiogenesis, was induced after cardiac ischemia in mice[87]. Inhibiting miR-24 reduced infarct size via reduced endothelial apoptosis and increased vascularity. Injecting synthetic miR-210 intramyocardially in mice was shown to improve cardiac function after MI, reducing infarct size and cardiomyocyte apoptosis[88].

Platelets

Landry *et al* were the first to report the presence of miRNA in platelets and RNA profiling revealed that platelets actually seemed to be enriched in small RNA species[89]. Apart from approximately 200 mature miRNAs, platelets were reported to contain pre-miRNAs as well as functional Dicer and miRNA effector complexes. The presence of miR-223 in complex with RISC suggested that the miRNA was functional and might have a regulatory role in platelets. This was confirmed by a report showing that miR-96 could regulate VAMP8 mRNA in platelets, suggesting a role for miRNA in regulating platelet reactivity[90]. Nagalla *et al* then reported that, based on the expression profiles of the seven platelet miRNAs miR-19b, -34b, -190, -320a, -320b, -320c and -320d, the authors could accurately predict platelet reactivity[91].

Endothelial and vascular smooth muscle cells

Genetic ablation of the Dicer gene causes mice to die early in embryogenesis due to impaired angiogenesis[46], suggesting that miRNAs play an important part in the development of the vasculature. Similarly, vascular smooth muscle-specific Dicer knock down caused late embryonic lethality due to extensive internal hemorrhage[92]. The blood vessels of these mice were dilated, thin-walled and had reduced contractility.

miR-126 is one of the most enriched miRNA species in endothelium[93, 94] and its effects are quite well characterized. It is encoded within and coexpressed with the endothelial-restricted Egfl7 gene[95], promotes pro-angiogenic signaling and vascular integrity[96, 97] and acts as an anti-inflammatory mediator by suppressing vascular cell adhesion molecule-1 (VCAM-1)[53].

miRNAs miR-221 and -222 are clustered together on chromosome Xp11.3[98, 99] and are abundant in both endothelial cells and VSMCs[100]. In endothelial cells, miR-221/222 reversed the pro-inflammatory effects of Angiotensin II and reduced leukocyte adhesion *in vitro*[101], possibly suggesting a role for this miRNA cluster in atherosclerosis susceptibility.

The endothelial-enriched miR-92a has been proposed as a valuable therapeutic target in the setting of cardiovascular disease after a report showed that systemic administration of an anti-miR-92a oligonucleotide enhanced blood vessel formation and functional recovery after acute myocardial infarction in mice[102]. Moreover, atheroprotective flow decreases the levels of miR-92a, causing derepression of the transcription factor KLF2 and maintaining endothelial homeostasis[103].

Two of the most abundant and well-characterized miRNAs in VSMC are part of the same bi-cistronic cluster, miR-143/145. These miRNAs cooperatively target a multitude of transcription factors to promote VSMC differentiation and simultaneously inhibit proliferation[104]. Lentiviral delivery of miR-145 decreased macrophage infiltration and thus limited plaque inflammation, representing a novel therapeutic target to limit atherosclerotic progression and increase plaque stability[105].

microRNA in intercellular communication

Not only are miRNAs important intracellular signalling molecules, it has become increasingly clear during the last few years that miRNAs might also represent a new class of paracrine mediators, being transferred in extracellular vesicles between different cells and tissues and exerting their functions in an intercellular manner.

The first evidence of intercellular miRNA transfer was reported by Valadi *et al* in 2007[106]. The study showed that exosomes originating from a mast-cell line not only contained specific mRNA and miRNAs, but also that this genetic material was functional and could be delivered to other mast cells. Mittelbrunn *et al* then showed that there is a transfer of functional miR-335 from T-cells to antigen presenting cells via exosomes during immune synapsis[107]. Another example is miR-150, which was shown to be selectively packed into microvesicles upon monocyte activation and delivered to endothelial cells, enhancing cell migration[108] and angiogenesis[109].

Intercellular transfer of miRNA has also been implicated in atheroprotection. Stressed endothelial cells were shown to release apoptotic bodies containing miR-126, which could be delivered to adjacent cells in a paracrine manner, induce progenitor cell recruitment via CXCL12 and protect mice from atherosclerosis[110]. In another paper, Hergenreider *et al* showed transfer of miR-143/145 from endothelial cells subjected to shear stress to VSMCs in extracellular vesicles[111]. Uptake of endothelial miR-

143/145 caused an induction of an atheroprotective VSMC phenotype and injection of miR-143/145 containing vesicles reduced atherosclerotic lesions in a mouse model.

Apart from exosomes and extracellular vesicles, Vickers *et al* showed that miRNA could also be transferred by high-density lipoproteins[112]. Human HDL was shown to contain small RNAs and that the miRNA profile differed distinctly between normal and hypercholesterolemic patients. Delivery of functional HDL-bound miR-223 to cultured hepatocytes could affect target gene expression in the recipient cells.

The mechanism for sorting and packaging of miRNA, as well as release and uptake of the extracellular vesicles remain elusive, but one study suggests that secretion of miRNAs is controlled by neutral sphingomyelinase 2 (nSMase2), an enzyme involved in ceramide biosynthesis[113]. This is supported by the fact that Mittelbrunn *et al* could inhibit miRNA transfer with the nSMase2-inhibitor manumycin-A as well as siRNA-mediated knock down of nSMase2 [107]. Brefeldin A, an inhibitor of guanine nucleotide-exchange protein BIG2 and regulator of exosome release, could also be used to effectively block miRNA transfer in the same study.

microRNA as biomarkers

The surprising discovery that miRNAs are stable in plasma despite high RNase activity[114], the fact that the expression of many miRNAs are more or less tissue-specific[94, 115, 116] and a realization that many organs and tissues release or secrete miRNAs in response to stress or injury[117, 118] have made many researchers realize the great potential of miRNAs as biomarkers for various diseases.

The notion of using miRNA as blood-borne biomarkers for disease first sprang from a report by Mitchell *et al*, showing that miRNA is remarkably stable in plasma, even after prolonged storage at room temperature or repeated freeze/thaw-cycles and that tumor-derived miRNAs could easily be detected in blood samples from cancer patients with standard laboratory techniques[114]. Since then, the usefulness of circulating miRNA has been assessed not only for diagnostics of various cancer forms[119-121] but also for other clinical manifestations, including diabetes[122], heart failure[123] and hepatic diseases[124, 125]. Moreover, miRNAs have been investigated e.g. as markers of platelet activation[126] and organ rejection[127].

Purinergic signaling

In addition to their function as an intracellular energy source and as the building blocks of DNA and RNA, the purines and pyrimidines ATP, ADP, UTP and UDP can also act as extracellular signaling molecules, affecting many fundamental biological processes. Extracellular purines and pyrimidines exert their effects through binding to the family of purinergic receptors, or P2 receptors. There are fifteen P2 receptors subdivided into eight P2Y- (G-protein coupled receptors) and seven P2X-receptors (ligand-gated ion channels). The P2Y-receptors can be further subdivided based on agonist specificity, into adenine-preferring receptors ($P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{12}$), uridine-preferring receptors ($P2Y_2$, $P2Y_4$ and $P2Y_6$) and uridine sugar-prefering recptors ($P2Y_{14}$), while the P2X-receptors ($P2X_{1-7}$) all bind ATP[128, 129]. In terms of G-coupling, the P2Y receptors can be divided into G_q-coupled receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) which stimulates phospholipase C (PLC) to generate inositol phosphate and diacylglycerol and e.g. induce mobilization of Ca^{2+} from the endoplasmatic reticulum and G_i-coupled receptors ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) which inhibits the generation of the second messenger cAMP. $P2Y_{11}$ is unique in that it can couple to both G_q and G_s[128, 130, 131], which stimulates adenylyl cyclase to generate cAMP.

Purinergic signaling in vascular inflammation and atherosclerosis

In the cardiovascular system, purinergic signaling has been implicated in processes such as platelet activation[132-135], vascular tone [136, 137], cardiac contractility[138, 139] and inflammation[140, 141]. The nucleotides ATP, ADP and UTP are released into the extracellular space from vascular cells subjected to shear stress and mechanical forces[142], activated platelets[128] and hypoxic erythrocytes[143], acting on the many subtypes of P2-receptors expressed in the cardiovascular system.

The principal P2Y-receptors in endothelial cells are $P2Y_1$ and $P2Y_2$, although functional roles for $P2Y_4$[144] and $P2Y_6$ [140, 145, 146] have also been described. Binding of ADP to $P2Y_1$ has been shown to regulate vasodilation through activation of NO-synthase and endothelial-dependent relaxing factor (EDRF)[147]. Vasorelaxing effects have also been linked to the release of NO and PGI_2 following activation of $P2Y_1$ and $P2Y_2$ receptors in endothelial cells[148]. Activation of $P2Y_2$ simultaneously induces monocyte/macrophage recruitment and infiltration by vascular endothelium[149], possibly through up-regulation of VCAM-1 expression[150]. Furthermore, stimulation of P2-receptors on monocytes and other leukocytes give rise to release of several

proinflammatory cytokines from leukocytes, including IL-1β, IL-1α, IL-8 and TNF-α, which can affect vascular cells and drive the atherosclerotic process[147]. For example, IL-1β has been shown to induce $P2Y_2$ expression on vascular cells[151], inducing mitogenic responses and should hypothetically make the vasculature more susceptible to inflammation. Migration of VSMC is a well-documented phenomenon in the later stages of atherosclerosis and this process could be facilitated by ATP or UTP acting on the $P2Y_2$ receptor[152]. Moreover, Chaulet *et al* showed that UTP in perticular exuded chemotactic effects on cultured rat aortic SMC via induction of osteopontin expression[153].

Aims and Hypotheses

The general aim of this thesis was to study the biological roles and potential clinical utililty of miRNAs in the cardiovascular system in the context of coronary artery disease.

- The aim of Study I and II was to investigate the dynamics of cardiac-enriched miRNA in the circulation in response to myocardial ischemia and to evaluate the diagnostic and prognostic usefulness of these miRNAs in patients with ACS.
- The aim of Study III was to compare the miRNA profiles in platelets from healthy individuals and MI patients and test the hypothesis that platelet miRNAs are released upon activation and exert paracrine effects on endothelial cells.
- In Study IV we hypothesized that miRNA expression and function in endothelial cells is driven partly by stimulation with extracellular nucleotides and can have effects that are of importance to vascular inflammation and atherosclerosis

Methods

The aim of this section is to give an overview of the methods and experimental procedures used in this thesis. For detailed information please refer to the individual papers.

Samples

Human patients and controls

All patients and healthy volunteers gave their informed consent and the ethics committee of the Faculty of Medicine at Lund University approved all studies. All studies were conducted in accordance with the declaration of Helsinki.

In studies I-III, patients diagnosed with ACS, STEMI or NSTEMI were identified at the coronary care unit at Lund University hospital and recruited after giving their informed consent.

Plasma samples

Plasma from patients and controls was prepared by centrifugation 1600*g for 15 minutes, aliquoted and stored at -80 °C until analysis.

Platelet preparation

In order to avoid platelet activation and contamination with leukocytes, platelets need to be prepared very carefully and stringently[154]. Blood was drawn with self-propagated flow into a citrate-dextrose solution containing the platelet inhibitors EDTA, prostaglandin E1 and aspirin to minimize platelet activation. Leukocytes and erythrocytes were removed with combined centrifugation, filtration and magnetic depletion steps. Purity of the platelet preparation was determined with FACS and qRT-PCR.

Thrombi

In Study III, trombectomy was performed on two STEMI patients. The thrombi causing the myocardial infarctions were removed using a catheter with a vacuum function, collected and stored at -80 °C until analysis.

Pig model of myocardial infarction

In Study I, plasma samples from a pig model of myocardial infarction were analyzed. Healthy, domestic pigs weighing 40-50 kg were fasted over night and anesthetized. Cardiac ischemia was induced by inflation of an angioplasty balloon in the proximal left anterior descending coronary artery for 40 minutes. Total occlusion of the coronary vessel and restoration of blood flow after deflation of the balloon was confirmed with coronary angiograms.

Cell lines

In Studies III and IV, the endothelial cell line human microvascular endothelial cell-1 (HMEC-1) was used. It was cultured in MCDB 131 medium (Life Technologies) containing 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 1% L-Glutamine, 0.1% Amphotericin B and 0.1% epidermal growth factor (EGF). In Study III, human embryonic kidney 293 (HEK293) cells were cultured in DMEM + GlutaMAX (Life Technologies) supplemented with 10 % FBS and 1 % Penicillin/Streptomycin. Cell culture was carried out in a standard cell incubator at 37 °C with 5% CO_2.

Preparation of RNA

In case of cells or tissues (Studies III and IV), a column-based RNA-preparation kit was used according to the manufacturers recommendations. However, due to the low concentrations of RNA in plasma and the high abundance of proteins and lipids which can interfere with RNA isolation, special considerations were required when preparing RNA from plasma in Studies I and II. The highly concentrated TRIzol LS was mixed with plasma in a 3:1 ratio in order to denature proteins and lipids, before preparing total RNA (including small RNA) using miRNeasy kit (Qiagen, Hilden, Germany). Because the yields are insufficient for quantification of plasma RNA with standard methods such as NanoDrop, we used a fixed volume of plasma when preparing RNA from plasma samples.

Quantitative Real-Time PCR

For analysis of mRNA, cDNA was prepared from RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and expression levels were assessed with TaqMan Master Mix and TaqMan assays specific for the genes of interest.

For assessment of miRNA levels, cDNA was prepared from either a fixed volume of RNA preparation for plasma samples, or a fixed amount of RNA for cell/tissue samples, using the miRCURY LNA Universal RT microRNA cDNA kit (Exiqon, Vedbaek, Denmark) and expression levels were analyzed using SYBR Green (Life Technologies, Carlsbad, USA) and primers specific for the miRNAs of interest.

All samples were run in duplicate on a StepOne Plus qRT-PCR instrument (Life Technologies) and the threshold was set manually where exponential amplification had been reached in all samples. A no-template control was added for each primer on each plate used to assess contamination/non-specific amplification and no-reverse transcriptase controls were run for each set of samples to rule out genomic DNA contamination in the RNA preparations. Melt-curves were included in each run in order to ensure no unspecific PCR-products were formed.

In case of mRNA analysis, expression levels were expressed relative to a housekeeping gene (cyclophilin in most cases) and normalized to the mean of the controls according to the formula 2^{-ddCt} [155]. For miRNA analysis in cells and tissues, miR-16 or U6 small nucleolar RNA, which were highly abundant and determined to be stable under the conditions examined in this thesis, were used as housekeeping genes. For assessing miRNA levels in plasma, miR-17 has been validated as a stable housekeeping gene in the context of myocardial infarction[156] and has been used for this purpose in studies I and II.

RNA-sequencing

RNA-seq is a relatively new technique for transcriptome profiling, based on the “next generation sequencing” or “deep sequencing” approach, where millions of cDNA fragments are sequenced in parallel. Total RNA is converted to a library of cDNA fragments with adaptor sequences attached to both ends (Figure 3). On the Illumina platform, which has been used to profile the microRNA transcriptome in Study III and IV, each fragment is then reverse transcribed and PCR-amplified with primers specific for the adaptor sequences to enrich for fragments that have incorporated the adaptors. Because microRNAs have a length close to the optimal read length of the sequencer, no fragmentation of the RNA is necessary before attaching the adaptors, as

is the case for mRNA. The cDNA is then gel-purified and run on a next generation sequencing instrument, in this case the Illumina Genome Analyzer IIx. The cDNA library is attached to a flow cell and up to a 1000 copies of each cDNA fragment is created by solid-phase amplification ("cluster generation"). The cDNA fragments are then sequenced in parallel by the "sequencing by synthesis"-technique, whereby fluorescently labeled nucleotides are added to the flow cell one by one in a cyclic manner. After each cycle, the incorporation of a specific nucleotide into the nucleic acid chain is called by measuring the fluorescent signal. The sequencing reads are aligned to a genomic reference sequence using various bioinformatics tools (e.g. TopHat) to provide information about transcript abundance, differentially expressed transcripts, splice variants and non-coding RNAs.

One of the strengths of RNA-seq compared to other transcriptomics methods (e.g. microarray) is that it is totally unbiased. All RNA, both known and unknown, in your sample is sequenced which means there is great potential for discovery of e.g. new RNA-species and splice variants. Moreover, no hybridization is necessary, which means that e.g. GC-content does not influence or bias the results, which might be the case in microarray analysis.

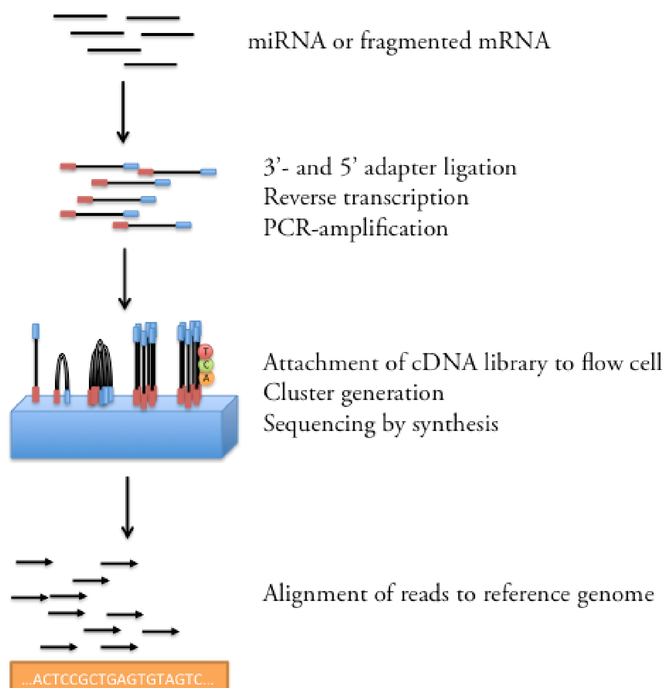

Figure 3. RNA-seq work flow.

Flow cytometry

Assessment of platelet microparticles

It is only recently that flow cytometers have achieved sufficient resolution to distinguish sub-micron extracellular vesicles from noise. To set the gates on size, we have used 800 nm latex beads for reference. Even though it is possible to distinguish extracellular vesicles based on size alone (Figure 4), staining with specific cell surface markers increases the specificity and gives additional information about the cell of origin. In the case of platelets, we have used a monoclonal antibody specific for the pan-platelet marker CD42a (GPIX), conjugated with FITC.

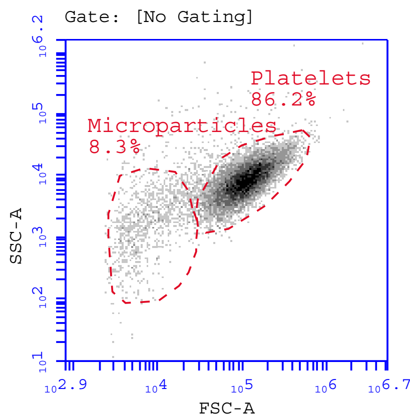

Figure 4. FACS plot showing gating of platelets and platelet microparticles based on size and granularity.

ICAM-1 surface expression

To assess the cell surface expression of ICAM-1 in studies III and IV, we used flow cytometry. Experimentally treated HMEC-1 cells were detached from the culture plates using trypsin, washed and stained with a monoclonal ICAM-1 antibody conjugated with the fluorophore APC. HMEC-1 cells were identified based on size as well as the expression of PECAM-1 (CD31) and ICAM-1 expression was measured as the mean fluorescence in the FL-4 channel. Using mean fluorescence instead of proportion of positive cells was due to the fact that there was not two distinctly negative and positive populations but rather a sliding scale from negative to positive cells.

Transfection of platelets

In order to track the transfer of miRNA from platelets to endothelial cells in Study III, we transfected purified platelets with fluorescently labeled, synthetic miRNA. Platelets have long been considered unsusceptible to transfection, but a recent report showed that, although transfection by electroporation caused aggregation of platelets, lipofectamine-based transfection could effectively introduce siRNA into washed platelets (Ho et al, 2011). We used this protocol for transfecting platelets with synthetic miRNA and with minor adjustments were able to get transfection efficiencies of approximately 15%. To ensure that the miRNA was not just attached to the surface of the platelets, we performed control experiments where transfected platelets were treated with RNase. RNase-treatment did not affect the transfection efficiency and we therefore concluded that the miRNA resided in the cytoplasm of the platelets.

Knock down/over expression of miRNA

To be able to study the function of single miRNAs *in vitro* in studies III and IV, we wanted to overexpress and knock down miRNAs of interest and study effects on target mRNA expression. In order to overexpress miRNAs, we transfected cells with synthetic pre-miR miRNA precursors (Life Technologies), which are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules. For knock down of endogenous miRNAs, we transfected cells with anti-miR miRNA inhibitors (Life Technologies), which are chemically modified synthetic single stranded nucleic acids designed to specifically bind to and inhibit the endogenous miRNA of interest. miRNA over expression and knock down as well as target mRNA expression was confirmed with qRT-PCR.

microRNA target prediction and validation

Most miRNAs pair imperfectly with their target mRNAs making it difficult to identify valid and relevant targets. Bioinformatic analysis based on pairing of the miRNA seed region with evolutionary conserved, complimentary sites in the 3'-UTR can successfully predict *bona fide* miRNA targets, but typically yield hundreds of potential target genes, including a high ratio of false positives. In Study III, the bioinformatics tools TargetScan (www.targetscan.com) was used. The TargetScan algorithm requires an exact match of ≥7 nucleotides of the seed sequence and

improves the prediction by taking into account evolutionary conservation[157]. Other algorithms, such as miRanda, is less strict in aligning miRNA and mRNA, and allows wobbles and mismatches in the seed pairing[158]. However, only conserved pairings are considered. To reduce the number of target hits and to increase the likelihood of finding valid targets, chosing targets that are consistently found with different algorithms can be effective.

Validation of predicted mRNA:miRNA-interactions were performed using a luciferase reporter assay. We used reporter plasmids where the 3'-UTR of the predicted mRNA targets had been cloned downstream of the firefly luciferase gene and controlled by the SV40 enhancer (GeneCopoeia, Rockville, USA). The plasmids were co-transfected with the corresponding pre-miR to over express the miRNA and the luminescent signal was measured. In case of miRNA:mRNA-interaction, the luminescent signal was quenched in a dose-dependent manner when cells were transfected with increasing amounts of pre-miR.

Leukocyte adhesion assay

In Study IV, we wanted to assess the functional consequences of miRNA-mediated ICAM-1 repression with a leukocyte adhesion assay. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy individuals, stained with a cell-penetrant fluorescent dye and briefly co-cultured with HMEC-1. The PBMCs that had not attached were removed, the endothelial cells were thoroughly washed and the fluorescence was assessed on a plate reader. The fluorescent signal was then assumed to be proportional to the amount of attached PBMC.

Statistics

In Study I and II, receiver operating characteristic (ROC) analysis was carried out to estimate the diagnostic and prognostic properties of circulating cardiac-enriched miRNA in MI and ACS. Area under the curve (AUC) in the ROC plot was used as a measure to evaluate and compare miRNA to current biomarkers. Correlation between miRNA levels and established biomarkers was tested with Spearman's rank correlation coefficient.

In Study II, logistic regression was used to test for association between the levels of circulating miRNAs and a primary endpoint including death and development of heart failure. Odds ratios were adjusted for age, sex and time between admission and sampling.

In all studies, Student's T-test or one- or two-way ANOVA was used as appropriate to compare differences in distributions between groups. Statistical significance was usually considered where $p<0.05$.

All statistical analyses were performed either in GraphPad Prism v. 5(GraphPad Software Inc., La Jolla, USA) or SPSS v. 19 (IBM, New York, USA).

Results

Cardiospecific microRNA Plasma Levels Correlate with Troponin and Cardiac Function in Patients with ST Elevation Myocardial Infarction, Are Selectively Dependent on Renal Elimination, and Can Be Detected in Urine Samples (Study I)

The aim of this study was to assess the abundance of cardiac-enriched miRNA in the circulation and urine of patients after STEMI and to evaluate their usefulness as diagnostic biomarkers. Based on reported miRNA expression patterns in myocardium and skeletal muscle, we chose to focus this study on the five miRNAs miR-1, -133, -208a, -208b and -499-5p.

Cardiac miRNAs can be detected rapidly in circulation after MI in a porcine model

To test our hypothesis that miRNAs are rapidly released into the circulation after myocardial ischemia and necrosis, we used a porcine model of MI. Six pigs were included and subjected to 40 minutes of myocardial ischemia via inflation of an angioplasty balloon in the proximal LAD. All miRNAs except miR-208a rose rapidly and could be detected within 20 minutes of reperfusion. miR-1, -133 and -208b peaked at 120 minutes after induction of ischemia, while miR-499-5p continued to rise even after 150 minutes. miR-208a could not be detected in any of the samples.

The levels of cardiac miRNAs is elevated in STEMI patients

To confirm our findings from the pig model in patients and to evaluate the clinical usefulness of miRNAs as biomarkers, we measured the levels of the five cardiac-enriched miRNAs in plasma from 25 STEMI patients and 11 healthy controls. Patient blood was collected in a time series with the first blood sample taken within 24 hours, the second within 48 hours, the third within 72 hours and a fourth sample was taken at follow up >1 month after the MI. As seen in Figure 5, miR-1, -133, -208b and -499-5p were all significantly elevated within 12 hours of onset of symptoms compared to healthy controls and had returned to baseline at follow-up.

The most pronounced dynamics was seen for miR-208b, which increased approximately 3000-fold over baseline within 12 hours.

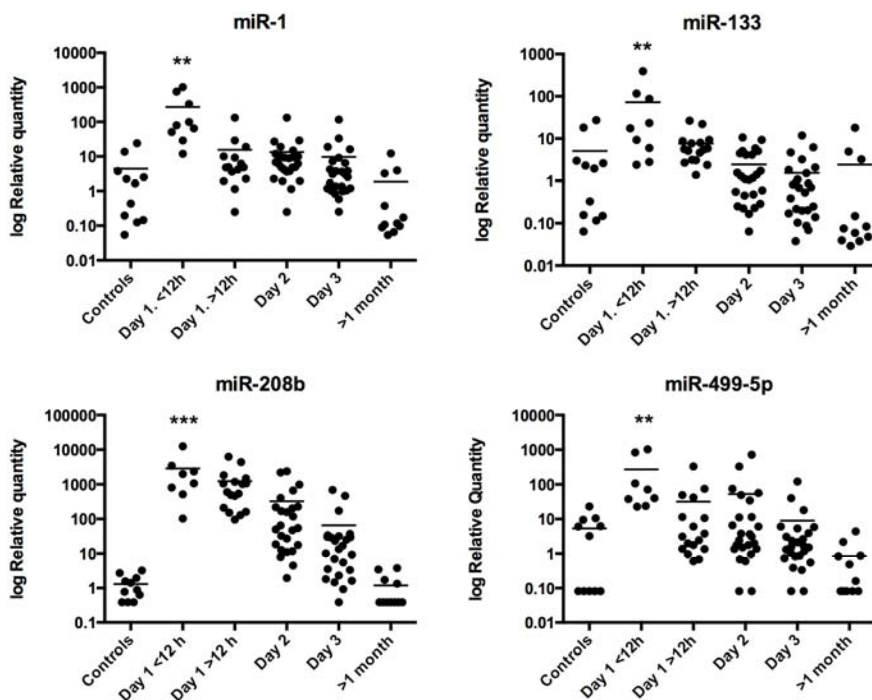

Figure 5. Plasma levels of cardiac-enriched miRNA in STEMI patients and healthy controls.

Cardiac-enriched miRNAs can be detected in urine samples from STEMI-patients

Given the rapid clearance of especially miR-1 and miR-133 from the circulation, we hypothesized that miRNA might be excreted in urine. We were able to detect miR-1 and -133, but not -208b and -499-5p in urine samples from STEMI patients taken within 24 hours of onset of symptoms.

The levels of circulating cardiac-enriched miRNAs correlate with Troponin and myocardial function

We found a significant correlation between the peak level of the current gold standard biomarker Troponin T and the peak level of miR-208b. Moreover, we found a significant negative correlation between the peak level of miR-208b and left ventricular ejection fraction LVEF, a measure of cardiac function and an important prognostic predictor after MI.

Circulating cardiac-enriched miRNAs accurately discriminate STEMI patients from healthy controls

To assess the diagnostic potential of circulating miRNAs, we performed ROC analysis of a subset of patients (n=12) where samples had been taken within 12 hours of onset of symptoms and healthy controls (n=11). All miRNAs accurately discriminated between patients and controls but miR-208b stood out with 100% specificity and sensitivity.

Circulating cardio-enriched microRNAs are associated with long-term prognosis following myocardial infarction (Study II)

The aim of this study was to confirm the results of Study I and to evaluate both the diagnostic and prognostic potential of circulating cardio-enriched miRNAs as biomarkers in a larger material of patients with ACS. We utilized the LUNDHEARTGENE biobank to assess the levels of miR-1, -208b and -499-5p in plasma from 407 patients with acute coronary syndrome, including 173 STEMI, 146 NSTEMI and 88 non-MI (angina or general chest pain). Patient characteristics can be found in Table 1.

Elevated levels of miR-208b and -499-5p in STEMI compared to NSTEMI and non-MI patients

The levels of circulating miR-208b and -499-5p correlated with the severity of coronary disease, with higher levels in the NSTEMI group than in the non-MI group and higher levels in the STEMI group than in the NSTEMI group (Table 1).

miR-208b and -499-5p accurately discriminate betweeen MI and non-MI

ROC analysis revealed that the levels of both miR-208b and miR-499-5p accurately discriminated MI patients (STEMI+NSTEMI) from non-MI patients, with AUC of 0.82 and 0.79, respectively (Figure 6). However, both were inferior to the current cardiac marker Troponin T, which had an AUC of 0.95.

Elevated levels of circulating miR-208b and -499-5p are associated with an increased risk of death and development of heart failure after MI

To evaluate the prognostic potential of circulating cardio-enriched miRNA, we had defined a primary endpoint including death within 30 days of hospitalization, development of heart failure or an LVEF<40% during follow-up. miRNA levels were divided into quartiles and association with the primary endpoint was tested with logistic regression, adjusting for sex, age and sampling time. 18% of the patients experienced the primary endpoint. Plasma levels of miR-208b and -499-5p were

associated with the primary endpoint, with odds ratios of 1.79 (95% CI = 1.38-2.33, $p=1\times10^{-5}$) and 1.70 (95% CI=1.31-2.20, $p = 5 \times 10^{-5}$), respectively. Statistical significance was lost when also adjusting for Troponin T. There were weak negative correlations between all three miRNAs and LVEF in MI, but not in non-MI patients.

Table 1. Patient characteristics and miRNA levels in Study II

Characteristic	Total cohort	Experienced endpoint	No end-point	p
Sample size	407	74	333	
Age	65 (11.1)	67.8 (11.8)	64.5 (10.9)	**0.02**
Men	76.9 %	78.1 %	76.6 %	0.79
Diabetes Mellitus	17.2 %	17.8 %	17.1 %	0.88
Hypertonia	43.5 %	52.1 %	41.6 %	0.10
Current Smoking	24.8 %	23.3 %	25.1 %	0.74
Mean time to sample (hours)	38.4 (32.2)	42.7 (13.7)	38.2 (6.9)	0.28
Diagnosis				**0.04**
STEMI	42.5 %	53.4 %	40.1 %	0.79
NSTEMI	35.9 %	35.6 %	35.9 %	
Non-MI	21.6 %	11.0 %	24.0 %	
Unstable angina	63,6 %	85.7 %	62,5 %	
Stable angina	21,6 %	0 %	26.2 %	
Chest pain	14,8 %	14.3 %	11.2 %	
Troponin T (ng/l)	2.55 (3.76)	5.14 (5.25)	1.98 (3.08)	**<0.0000001**
Death within 3 months (%)	1.2 %	6.8 %	0 %	**0.000001**
LVEF				**<0.0000001**
>50%	62.8 %	5.6 %	78.7 %	
40-49%	17.4 %	5.6 %	21.3 %	
30-39%	14.5 %	67.6 %	0 %	
<30%	4.4 %	21.1 %	0 %	
miRNA (ln $2^{-\Delta Ct}$)				
miR-1	-2.69 (0.99)	-2.58 (1.08)	-2.71 (0.97)	0.32
miR-208b	-4.59 (2.22)	-3.70 (2.38)	-4.78 (2.14)	**0.0001**
miR-499-5p	-4.77 (1.80)	-4.03 (2.04)	-4.93 (1.71)	**0.00009**

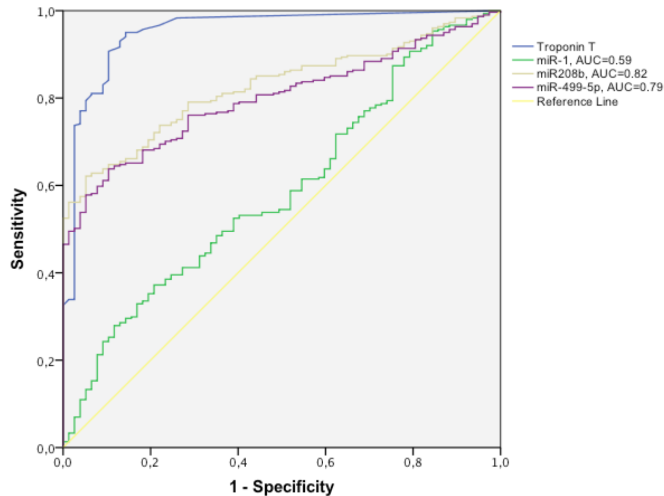

Figure 6. ROC curves for cardiac-enriched miRNAs and Troponin T describing the discrimination of patients with MI from non-MI patients.

Cardiac-enriched miRNAs in the circulation emanate from the myocardium

To confirm that circulating miR-208b and -499-5p in fact come directly from the myocardium, we measured the levels of these miRNAs in the coronary sinus before and after cardioplegia in four patients undergoing coronary artery bypass graft (CABG) surgery. The levels of both miRNAs were undetectable in the coronary sinus before cardioplegia but were detectable at levels substantially higher than in the periphery immediately after restoring cardiac contraction, strongly suggesting that the presence of this miRNAs in the circulation is due to stressed or necrotic cardiomyocytes.

Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression (Study III)

Activated platelets play a key role in the pathophysiology of MI. The primary aim of this paper was to study differences in the platelet miRNA transcriptome between

patients with MI and healthy controls. The results led us to investigate a possible role for platelet miRNAs as paracrine signaling molecules.

MI patients have an altered platelet miRNA profile compared to healthy individuals

We performed an RNA-seq screening of platelet small RNA from two STEMI patients and two age and sex matched controls. The results showed nine differentially expressed miRNAs in the patients, eight (miR-22, -127-3p, 139-3p, -185, -320b, -423-5p, -1250 and -1307) were down-regulated and one (miR-320a) was up-regulated. Four candidate miRNAs (miR-22, -185, -320b and -423-5p) were selected for further analysis based on their putative mRNA targets, abundance in platelets and magnitude of difference between patients and controls. Differential expression of the four candidate miRNAs was confirmed in a separate set of patients and controls (n=10 and 15, respectively) with qRT-PCR (Figure 7A).

Platelets release miRNA upon activation which can be taken up by endothelial cells

The fact that eight of the nine differentially expressed miRNAs in platelets from MI patients were downregulated led us to hypothesize that platelets shed miRNA upon activation. We tested this by examining the levels of the four candidate miRNAs in the supernatant of platelets *ex vivo,* before and after aggregation with thrombin. The levels of all four miRNAs were significantly elevated in the supernatant after aggregation, suggesting activation-dependent secretion of platelet miRNA (Figure 7B).

The question was then whether platelet-derived miRNA could be taken up by other cells in the cardiovascular system. We tested this hypothesis by co-culturing platelets transfected either with a fluorescently labeled synthetic miRNA (miR-FITC) or an exogenous *C. Elegans* (cel-miR-39) miRNA together with the endothelial cell line HMEC-1. The experiment was carried out both in the presence and absence of the platelet agonist thrombin. After 3 hours of co-culture, there was a clear thrombin-induced uptake of miR-FITC and cel-miR-39, as analyzed by confocal microscopy and qRT-PCR, respectively (Figure 8).

Measuring the levels of the candidate miRNAs in HMEC-1 after co-culture with platelets revealed that there was a transient, thrombin-dependent increase in all four miRNAs between 3-8 hours after washing away the platelets, further supporting the idea of transfer of platelet-derived miRNA.

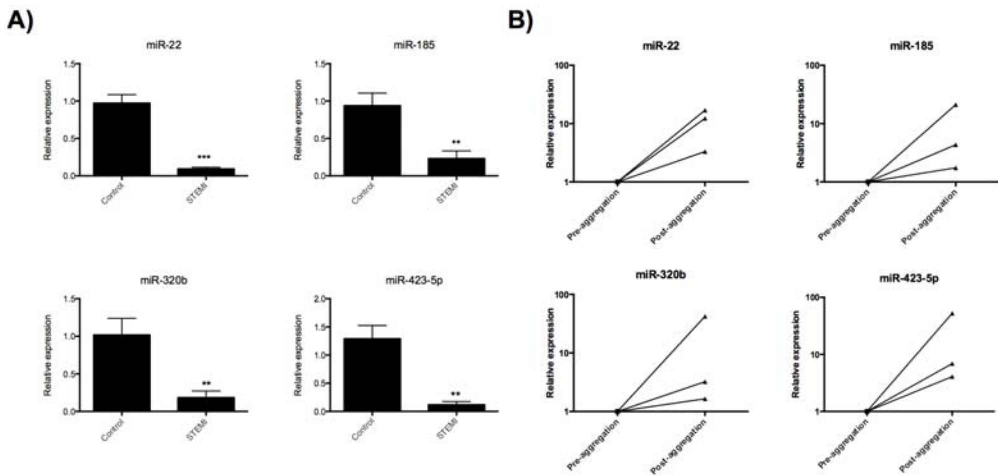

Figure 7. A) miRNA levels in platelets of healthy individuals and STEMI patients. B) Levels of miRNA in the supernatant of platelets before and after aggregation with thrombin.

Transfer of platelet miRNA is microvesicle-dependent

Considering recent reports on cell-to-cell transfer of miRNA via extracellular vesicles, we hypothesized that platelet miRNA is transferred via microparticles to endothelial cells. In order to elucidate the mechanism of miRNA transfer we performed a screening of several compounds reported to inhibit platelet microparticle and exosome formation and found that Brefeldin A, an inhibitor of guanine nucleotide-exchange protein BIG2, was the most efficient inhibitor. Brefeldin A decreased the amount of miR-FITC in the platelet microparticles and inhibited miRNA transfer in the co-culture experiments, supporting the idea that the transfer is microparticle-dependent.

Platelet-derived miR-320b attenuates ICAM-1 in endothelial cells

To show that the platelet-derived miRNA was actually functional in the recipient cells, we first attempted to validate some of the most interesting putative mRNA targets of the candidate miRNAs. Using reporter plasmids containing the target gene 3'-UTR we could confirm the interaction of miR-22 and miR-320b with ICAM-1 mRNA and chose to focus the rest of the study on these two miRNAs. We could also confirm that HMEC-1 downregulated ICAM-1 expression in response to platelet releasate, consistent with the hypothesis that functional miR-22 and -320b are delivered to the endothelial cells. To test if the effect on ICAM-1 was miRNA-dependent, we knocked down endogenous miR-22 and -320b in HMEC-1 and then treated the cells with platelet releasate. In cells where miR-320b, but not miR-22, had been knocked down, there was a rescue of ICAM-1 de-repression, suggesting that the effect on ICAM-1 is dependent on the effect of specific miRNA species.

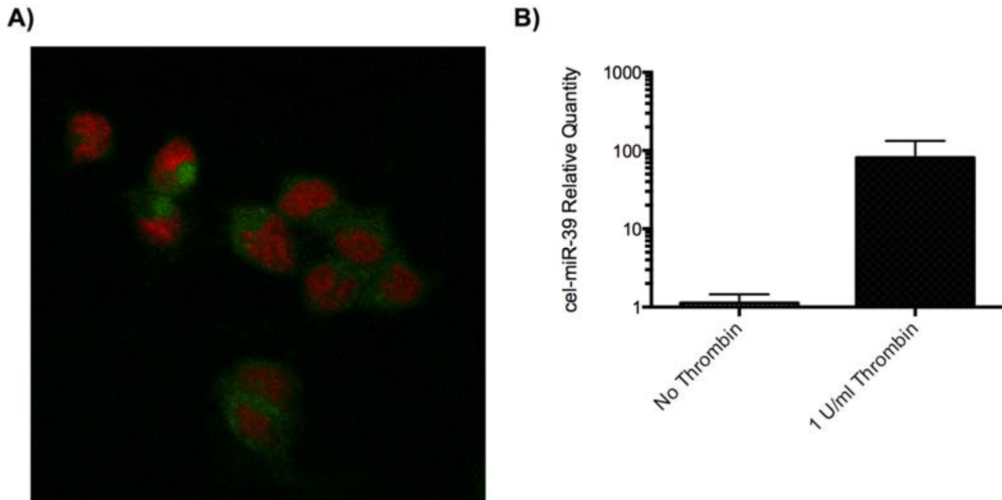

Figure 8. A) Confocal image of HMEC-1 containing fluorescent miRNA (green) after co-culture with activated platelets. Nuclei are stained red. B) Levels of cel-miR-39 in HMEC-1 after co-culture with transfected platelets in the presence or absence of thrombin.

Extracellular UTP and ATP attenuate endothelial inflammation through miR-22 mediated ICAM-1 inhibition (Study IV)

Extracellular nucleotides influence important aspects of vascular physiology and pathophysiology, including inflammation and progression of atherosclerosis. miRNAs are key intercellular regulators but few studies have tried to elucidate what extracellular stimuli enhance or attenuate miRNA transcription. In this study, the aim was to investigate the effects of extracellular UTP and ATP on endothelial miRNA transcription and if the downstream effects have any relevance to vascular physiology.

Differential expression of miRNA in endothelial cells upon ATP/UTP-stimulation

We performed RNA-seq on cDNA from HMEC-1 cells stimulated with UTP to screen for differentially expressed miRNAs. 10 μM of UTP for 3 hours resulted in the differential expression of 15 miRNAs (Table 2). miR-22 was chosen for further analysis since we had established miRNA:mRNA interaction for this miRNA and ICAM-1 mRNA in Study III. The effect of UTP on miR-22 expression was confirmed with qRT-PCR in a time course experiment. Interestingly, 10 μM of the stable ATP-analogue ATPγS had the same effect on miR-22 expression.

Table 2. Differentially expressed miRNAs in HMEC-1 treated with UTP

Gene symbol	No. Of Transcripts - Control	No. Of Transcripts - UTP	Ratio UTP:Control	p-value
MIR1262	3106,06	28241,1	9.09	0.16
MIR1294	2584,12	9390,44	3.63	0.15
MIR1296	9520,59	14973,9	1.57	0.18
MIR137	8851,82	13606,7	1.54	0.48
MIR142	10187,4	34624,2	3.40	0.0057
MIR144	2451,51	5065,45	2.07	0.43
MIR185	22924,8	8797,39	0.38	0.055
MIR216A	2729,64	1328,76	0.49	0.40
MIR219-1	9848,55	3702,22	0.38	0.19
MIR22	1,28E+07	1,83E+07	1.42	0.0017
MIR34C	4181,2	1647,81	0.39	0.31
MIR451	177373	2,22E+06	1.52	1.79e-10
MIR485	1464,78	697,607	0.48	0.0078
MIR486	1,25E+06	2,82E+06	2.26	0.0012
MIR7-2	160625	48238,8	0.30	0.062

miR-22 attenuates ICAM-1 expression and leukocyte adhesion to endothelial cells in vitro

After confirming that miR-22 overexpression resulted in decreased levels of ICAM-1 protein on the cell surface of HMEC-1 cells, we wanted to assess the functional consequences of this miRNA:mRNA-interaction. As ICAM-1 has an important role in recruiting leukocytes to the endothelium and thereby promoting vascular inflammation, we evaluated leukocyte adherence to HMEC-1 overexpressing miR-22. Leukocyte adhesion was decreased 20% in cells overexpressing miR-22, an effect comparable to that of treating cells with an ICAM-1 blocking antibody (Figure 9).

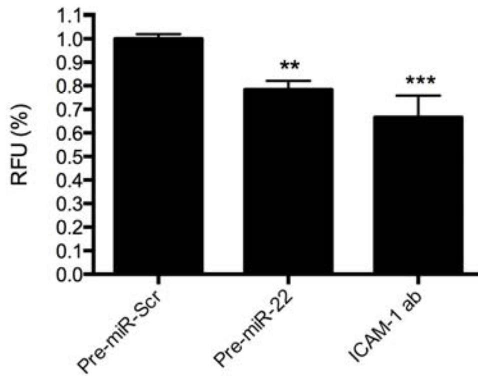

Figure 9. Leukocyte adhesion to HMEC-1 transfected with pre-miR-22, scrambled control pre-miRNA or incubated with ICAM-1 blocking antibody.

UTP and ATP attenuate ICAM-1 expression and leukocyte adhesion in HMEC-1

In order to connect the effect of miR-22 on ICAM-1 expression to UTP and ATP, we measured cell surface ICAM-1 and leukocyte adhesion in HMEC-1 treated with increasing doses of UTP and ATP. After 24 hours there was a dose-dependent negative effect of both UTP and ATPγS on ICAM-1 protein levels and a reduction in leukocyte adherence.

Discussion

The studies included in this thesis deal with two distinct aspects of cardiovascular miRNA; in Study I and II we assessed the clinical utility of miRNAs in the circulation as potential biomarkers in the context of coronary artery disease, whereas in Study III and IV we investigated more basic biological questions about intracellular and paracrine functions of miRNAs in vascular inflammation.

Circulating miRNA as biomarkers in CAD

The field of circulating miRNA as biomarkers for various diseases has been expanding extremely rapidly since the first few studies in 2008[114, 159] and now spans from cancer[160-163] to autoimmune disease[164, 165] via diabetes[166], neurological disorders[167] and organ failure[168, 169]. In the case of coronary artery disease, the presence of cardiac-enriched miRNAs in the circulation of animals subjected to myocardial ischemia[170] prompted us and others to evaluate the clinical utility of these molecules in human patients. In Study I, we examined the early dynamics of cardiac miRNA release in a large animal model of MI and could detect a massive increase of several cardiac-enriched miRNAs in the circulation of MI patients within 12 hours of symptom onset. In Study II, we extended our aim to include general ACS and performed a larger study on over 400 patients. Both miR-208b and miR-499-5p could accurately discriminate between patient groups and had prognostic value in estimation of death and development of heart failure upon follow up, but was slightly inferior to the current cardiac marker Troponin T.

During the work on this thesis, several other studies reported on the validity of circulating miRNAs as biomarkers in the context of ACS and myocardial infarction. The first was a smaller study by D'Alessandra *et al* on approximately 30 STEMI patients, which found a significant increase in the four cardiac-enriched miRNAs miR-1, -133a, -133b and -499-5p[156]. Interestingly, peak levels of miR-1 and -133a/b were found within 160 minutes after onset of symptoms whereas miR-499-5p had a slower release pattern, confirming the very rapid dynamics of miRNA release we could see in the pig model. A similar study was published shortly after, confirming a massive increase of miR-208b and -499-5p in 30 myocardial infarction patients, but

could also see a less pronounced release in patients with viral myocarditits and acute heart failure[171]. Another study performed miRNA profiling of plasma from patients with stable CAD and healthy controls[172] and found several endothelial-enriched miRNAs (miR-126, -17, -92a) to be downregulated in patients, which might be part of the same endothelial uptake mechanism as we report in Study III. Interestingly, miR-126 was shown to be consumed during trans-coronary passage in patients with evidence of myocardial injury[173] in a later study.

The first larger study was reported by Widera *et al* and included 444 patients with ACS[174]. miR-1, -133a/b, -208a/b and -499-5p were all measured in plasma samples obtained on admission. The miRNA levels differed across patient groups and correlated with Troponin T, reflecting the results of Study II. Moreover, miR-133a and miR-208b were significantly associated with the risk of death, defined as all-cause mortality within six months. Similar to Study II, however, statistical significance was lost upon adjusting for Troponin T. Similarly, Devaux *et al* reported that adding circulating miR-499 to Troponin T did not result in an improvement of diagnosis[175]. Taken together, these results confirm our conclusion that miRNA does not offer any additional prognostic or diagnostic information in this context. However, the rapid dynamics and substantial increase in plasma upon myocardial injury make them excellent candidates for rapid diagnosis of MI in the emergency department or e.g. in cases of early reinfarction or kidney failure, where traditional biomarkers perform poorly. One such study was recently published, were Olivieri and colleagues evaluated the diagnostic potential of circulating miR-499-5p in elderly patients with NSTEMI[176]. Geriatric NSTEMI patients frequently present with atypical symptoms, ambiguous ECGs and can have modest Troponin-elevation due to heart failure. miR-499-5p was reported to exhibit diagnostic accuracy superior to that of TnT in patients with modest elevation at presentation.

One prospective study on circulating miRNAs and risk of myocardial infarction has been published to date. Zampetaki *et al* showed in a cohort of >800 participants that miR-126 was associated with an >2-fold increased risk of incident MI whereas miR-223 and miR-197 were both associated with a ~50 % reduced risk[177]. Interestingly, miR-223 was shown to be highly abundant in platelets and platelet microparticles, and might be released by the same mechanism as we describe in Study III

miRNA in the circulation – origin and mechanism of release

Considering the tissue specific expression of most miRNAs it is tempting to make assumptions about the origin of specific miRNA species in the circulation. In Study II we made an effort to confirm the cardiac origin of miR-208b and miR-499. We sampled blood from the coronary sinus of CABG patients during and immediately after cardioplegia (i.e. intentional and temporary cessation of cardiac activity). The levels of miR-208b and -499-5p were below the detection limit during cardioplegia

and present at levels far above those in the periphery immediately after, strongly indicating that the release of these miRNAs originate from cardiomyocytes specifically. This was supported by another study where concentration gradients of cardiac-enriched miRNAs were measured across the coronary circulation after myocardial injury[173]. Interestingly, during cessation of cardiac activity, cardiomyocytes are protected from cell death by reducing cardiac metabolism. This indicates that miRNA-release from cardiomyocytes is not only a byproduct of leakage from necrotic cells but an active process initiated by cell stress. We have demonstrated that the liver miRNA miR-122 is massively increased in the circulation of pigs subjected to cardiogenic shock without any signs of liver necrosis based on clinical liver markers (AST/ALT)[178], further indicating that in some cases tissue stress is sufficient for miRNA release. Sensitive biomarkers for tissue stress might be of high clinical relevance and merits further research.

Considerable effort has gone into characterizing in which form miRNAs are transported in the circulation. Arroyo *et al* described two types of miRNA-complexes in plasma of healthy individuals: a microvesicle-bound fraction and a fraction bound to the key effector protein of miRNA-mediated silencing, Ago2, which carried the majority of circulating miRNA[179]. These complexes protect circulating miRNA from degradation by RNase and may serve as extracellular delivery vehicles for cell-to-cell transfer of miRNA, as shown in Study III.

Technical considerations

There are many considerations to bear in mind when discussing circulating miRNAs as potential biomarkers for disease. The rapid dynamics of miRNA release following myocardial injury is good for swift diagnosis but also means that timing of the sampling is crucial. The diagnostic accuracy of miR-499-5p increased considerably when only analyzing samples taken within 24 hours in Study I, and it is likely that the accuracy is improved the closer to admission the sampling occurs.

All studies to date have used qRT-PCR to detect miRNA in plasma, which raises several questions. One technical consideration concerns normalization of data from qRT-PCR experiments. No established housekeeping miRNA for normalization of biological variation exists and many groups rely solely on synthetic RNA spike-ins for normalization, which will only adjust for the technical variation caused by RNA isolation and cDNA synthesis. Insufficient normalization for biological variation will most likely affect the diagnostic and prognostic accuracy negatively, and more effort must be put into finding stable, reliable blood miRNAs in the context of CAD. Another consideration when analyzing blood samples is the use of heparin as an anti-coagulant. Heparin is an inhibitor of Taq Polymerase[180] and it had pronounced effects on the ability to detect miRNA in the circulation of patients with CAD in a

recent study[181]. Considering the low abundance of RNA in plasma, meticulous handling of the samples during isolation is required in order to get consistent results. For accurate quantification of RNA yields, standard methods such as NanoDrop are insufficient and the use of more sensitive techniques, e.g. Bioanalyzer, is required.

From a clinical perspective, qRT-PCR is time-consuming and expensive and other means of detecting miRNA in plasma samples would be useful if miRNA were to be used clinically. A recently developed method based on an electrochemical genosensor that can directly detect miRNAs without labeling or PCR has been proposed as a feasible alternative for routine clinical use[182]. Another technique, called Nanopore, which utilizes a programmable oligonucleotide probe, has been used to detect sub-picomolar levels of cancer-associated miRNAs in lung cancer patients[183].

The function of platelet miRNA

The existence of mature miRNA and pre-miRNA in platelets, as well as the enzymatic infrastructure for miRNA processing and function, was shown in 2009 [89]. Transcriptome profiling seems to suggest that platelets are even enriched in miRNA[89, 184]. However, only one study have been able to confirm a functional miRNA:mRNA-interaction in platelets[90]. That, together with the limited *de novo* synthesis of proteins in platelets, suggests that there is additional roles for miRNA in platelets. When we compared the small RNA profiles of platelets from healthy individuals and MI patients in Study III, it was evident that the activated platelets of patients had lost certain miRNAs. This was confirmed by the depletion of the same miRNA species in thrombi aspirated from MI patients as well as the evidence of miRNA release from activated platelets *ex vivo*. The question was, where does the miRNA end up? Based on the few studies available on miRNA transfer, the endothelium seemed to be an active site of miRNA uptake[109, 110]. Our co-culture experiments confirmed that functional miRNA could be transferred between platelets and endothelial cells to regulate ICAM-1 expression. Soon after Study III was published, another report showed the delivery of functional Ago2-miR-223 complexes from platelets to endothelial cells via microparticles[185], confirming our data. The authors showed the internalization of miR-223 containing platelet microparticles by human umbilical vein endothelial cells and the subsequent downregulation of mRNA targets *in vitro*. These two studies together make a very strong case for the notion of miRNA-mediated platelet/endothelial crosstalk.

miRNA in vascular inflammation and atherosclerosis – modes of action and therapeutic potential

In both Study III and IV, we have investigated the role of miRNA in vascular inflammation. We have established ICAM-1 as a target for both miR-22 and miR-320b in endothelial cells, and in Study IV we showed the functional consequences of miR-22 mediated ICAM-1 regulation on leukocyte adherence. Thus, in Study III, activated platelets were shown to release an anti-inflammatory mediator, which inhibits leukocyte adherence to the endothelium. This might seem counterintuitive, since activated platelets have been shown to release a multitude of pro-inflammatory mediators[25] and even induce ICAM-1 expression on endothelial cells[186]. However, considering the relatively long time frame (~24 h) for miRNA action in study III, we propose that the paracrine effects of platelet miRNA in this case might be to attenuate or fine-tune the inflammatory response in a sort of feedback mechanism. In concordance with this notion, miRNAs have been implied in the resolution of inflammation[187] and paracrine miRNA-signaling has previously been described as anti-atherosclerotic[110, 111].

In Study IV, we connected anti-inflammatory effects of extracellular UTP and ATP on endothelial cells to miR-22 and ICAM-1. This is the first time purinergic signaling has been linked to miRNA expression and function. Our results were quite surprising since ATP had previously been shown to upregulate ICAM-1 expression in HMEC-1 cells[141]. However, considering that we could show both downregulation of ICAM-1 surface expression as well as a functional consequence of this downregulation (reduced leukocyte adhesion), we feel that we have strong support for our hypotheses.

The results of Study IV are intriguing and raise several interesting questions that we aim to answer in the future. For example, what are the receptor(s) responsible for the effects of UTP and ATP on miRNA transcription? The $P2Y_2$-receptor seems to be the most likely candidate, considering the data we have at present. Sensitivity in the low micromolar range suggests that it is a P2Y- rather than a P2X-receptor. Moreover, the magnitude of the effect of UTP and ATP on miR-22 transcription is comparable, indicative of the $P2Y_2$-receptor. Lastly, it is the most abundant P2Y-receptor in HMEC-1 cells. Another interesting question that we will attempt to answer is what intercellular signaling pathways are involved in ATP/UTP-induced miR-22 expression. It has been observed in several kinds of cells that extracellular nucleotides through P2 receptors modulate multiple signaling pathways including phosphorylation of the mitogen-activated protein kinases (MAPKs) and activation of transcription factors such as c-fos and ATF-1[188-192] but the mechanisms in endothelial cells are not yet elucidated. Studies of the miR-22 promoter region to identify the specific elements that drive transcription are also under way. Interestingly, one study

suggested that a region between base pairs 700-1500 upstream of the transcription start site contain enhancer elements whereas the first 700 base pairs upstream contain a strong inhibitory element in the human pre-B cell line NALM-6[193].

The mechanisms of miRNA-mediated attenuation of vascular inflammation shown in Study III and IV might be exploited for therapeutic purposes in atherosclerosis. A few examples of miRNAs as potential therapeutic targets have been reported to date. The *in vivo* administration of miR-181b mimics in mice reduced pro-inflammatory NF-κB signaling and leukocyte infiltration in the endothelium[194]. Moreover, miR-155 and -221/222 have been shown to attenuate angiotensin II-induced endothelial inflammation[101]. The most promising miRNA for anti-atherosclerotic therapy is however miR-33, which is a key regulator of cholesterol transport and fatty acid metabolism[195]. There are several studies were antagonism of miR-33 has beneficial effects on atherosclerotic progression. In one of the few studies on non-human primates, inhibition of miR-33 was shown to raise plasma HDL and lower VLDL triglycerides in African green monkeys[196]. In other studies, genetic ablation or therapeutic silencing of miR-33 in atherosclerosis-prone mice reduced the size and lipid content of atherosclerotic lesions[197-199]. Another interesting target is miR-30c, which upon hepatic overexpression resulted in decreased lipid synthesis and atherosclerosis in mice in a recent study[200]. Macrophage-derived miR-342-5p drives inflammatory pathways in atherosclerotic lesions and systemic treatment with a miR-342-5p inhibitor reduced atherosclerotic progress in the aorta of mice[201]. Lastly, smooth muscle cell-specific expression of miR-145 by injection of lentiviral vectors in mice markedly reduced plaque size, increased fibrous cap area, reduced the necrotic core area and increased plaque collagen content[105]. Even though considerable scientific effort needs to be made before pharmacological miRNA modulation reaches clinical use, these examples show that there is great potential in targeting miRNAs for treatment of cardiovascular disease.

Conclusions

The following conclusions can be drawn based on the results presented in this thesis:

- Cardiac-enriched miRNAs are released into the circulation upon myocardial ischemia and in patients with ACS
- The plasma levels of miR-208b and -499-5p can be used to accurately distinguish MI-patients from non-MI patients and are associated with an increased risk of death and development of heart failure
- The miRNA transcriptome in platelets from patients with MI differ from those of healthy individuals
- Activated platelets release miRNA which can be taken up by endothelial cells and regulate endothelial cell gene expression
- ICAM-1 is a target of miR-22 and miR-320b in endothelial cells
- Extracellular UTP and ATP causes differential expression of endothelial cell miRNAs
- The effects of UTP and ATP on endothelial cell ICAM-1 expression and leukocyte adhesion is in part mediated by miR-22

Populärvetenskaplig sammanfattning på svenska

Hjärtinfarkt är den enskilt vanligaste dödsorsaken i världen såväl som i Sverige. En hjärtinfarkt uppkommer då ett av de kärl som försörjer hjärtat med blod (s.k. kranskärl) täpps igen av en blodpropp och hjärtmuskelceller därmed dör av syrebrist. Orsaken till att en blodpropp bildas är vanligen att ett s.k. åderförkalkningsplack i ett kranskärl brister och bildar ett sår i kärlväggen. Detta sätter igång olika koagulationsmekanismer, där bl.a. blodplättar spelar en viktig roll. Blodplättar, som normalt cirkulerar i ett vilande tillstånd i blodbanan, reagerar på olika strukturer i såret, klumpar ihop sig och bildar en propp för att täcka sårytan. Denna propp riskerar därmed också att täppa till kranskärlet och strypa blodtillförseln till delar av hjärtmuskeln. Åderförkalkning i hjärtats kranskärl är alltså den bakomliggande orsaken till en hjärtinfarkt och åderfökalkningsprocessen är en mycket komplex biologisk mekanism som kan pågå under många år innan några symtom uppstår. Det första steget i processen är att fettmolekyler ackumuleras under kärlväggens ytskikt, endotelet. Detta sker ofta på speciella platser i kärlträdet där blodflödet störs, t.ex. vid förgreningar. Fettmolekylerna i kärlväggen oxideras av fria syreradikaler, vilket sätter igång en inflammatorisk process i endotelet. Endotelcellerna börjar uttrycka speciella molekyler på ytan som attraherar inflammatoriska celler i blodbanan. Dessa celler fastnar och tar sig in genom kärlväggen där de börjar ta upp de oxiderade fettmolekylerna. När de inflammatoriska cellerna sedan dör läcker fett och avfallsprodukter ut och bildar en s.k. nekrotisk kärna inuti kärlväggen. Detta triggar glattmuskelceller i utkanten av kärlväggen att börja röra sig mot blodbanan och omsluta den nekrotiska kärnan. Glattmuskelcellerna bildar också fiberstrukturer och tillsammans bildar detta en slags kapsel över placket som tränger ut i blodbanan och kan orsaka förträngningar av kärlet. Plackets sammansättning är avgörande för hur stor risken är för att det ska brista. Ett fettrikt plack med en tunn kapsel är instabilt medan ett plack med en tjock, fiberrik kapsel ofta är stabilt.

Symptom på hjärtinfarkt kan vara vaga och skilja sig mycket från patient till patient, vilket kan göra det svårt att ställa en snabb diagnos. För att säkerställa diagnos vid misstanke om infarkt krävs antingen att man kan påvisa typiska EKG-mönster eller att man kan uppmäta signifikanta halter av speciella hjärtenzymer i blodet. Dessa enzymer, idag används framförallt s.k. troponiner, finns normalt endast i hjärtat men

läcker ut i blodbanan när hjärtmuskelcellerna dör efter en infarkt. Troponin är ett exempel på en s.k. biomarkör, d.v.s. någon typ av molekyl som associeras med ett specifikt sjukdomstillstånd och kan användas på klinik för att säga något om diagnos eller prognos. Troponin är mycket känsligt men kan bara detekteras i blodet med en viss fördröjning, vilket gör det olämpligt för diagnostik i det akuta skedet. Det finns också patientgrupper som har kroniskt förhöjda halter av Troponin, vilket försvårar diagnostik vid en misstänkt infarkt.

Syftet med delarbete I och II var att utvärdera nyttan med en ny typ av biomarkör, s.k. mikroRNA, i samband med hjärtinfarkt. MikroRNA är ett slags korta RNA-molekyler som normalt finns i alla celler i kroppen men till skillnad från vanligt RNA inte utgör en ritning för proteintillverkning utan istället hämmar uttrycket av specifika gener. En del mikroRNA finns uteslutande i vissa organ och man har sett att stressade eller döende celler frisätter mikroRNA till blodbanan. Detta faktum har vi försökt bekräfta och använda för diagnos och prognos i samband med hjärtinfarkt i delarbete I och II. I delarbete I kunde vi se en snabb frisättning av hjärt-anrikat mikroRNA i blodet hos grisar där vi framtvingat en hjärtinfarkt på konstgjord väg. Det fick oss att också vilja undersöka förloppet hos patienter med stora infarkter (s.k. ST-höjningsinfarkter) och vi kunde se en kraftig ökning av dessa mikroRNA i blodet hos patienterna inom 12 timmar efter symptomdebut. I delarbete II utökade vi arbetet och undersökte om mängden hjärt-anrikat mikroRNA kunde säga något om diagnos och prognos hos ca. 400 patienter med olika former av kranskärlssjukdom. Vi kunde se att förekomsten av två specifika mikroRNA-molekyler särskilt väl kunde särskilja patienter med hjärtinfarkt från andra patienter med kranskärlssjukdom samt att mängden av dessa mikroRNA i blodet avspeglade hur väl patienterna kom att må efter infarkten. Ju högre nivåer av dessa två mikroRNA patienterna hade i blodet desto större var också risken att drabbas av död eller hjärtsvikt inom en månad efter infarkten.

I den andra delen av avhandlingen har vi undersökt biologiska aspekter av mikroRNA i kärl och kärlvägg. I delarbete III studerade vi mikroRNA i blodplättar och kunde se att sammansättningen skiljde sig mellan friska individer och patienter med hjärtinfarkt. Detta fick oss att gå vidare med hypotesen att de ”retade” blodplättarna hos infarktpatienter frisätter mikroRNA och kunde bekräfta det experimentellt med framrenade blodplättar i provrör. Vi ställde oss sedan frågan vart det frisatta mikroRNA:t kunde ta vägen och undersökte om det kunde överföras mellan blodplättar och endotelceller. I ett experimentellt system där vi sammanförde blodplättar och odlade endotelceller i cellkultur kunde vi se att funktionellt mikroRNA överfördes och hämmade uttrycket av en inflammatorisk molekyl i endotelcellerna. Med utgångspunkt i våra resultat verkar det alltså som om mikroRNA i blodplättar kan fungera som ett slags hormon, som frisätts och påverkar andra celler i kroppen.

I det sista delarbetet har vi undersökt hur mikroRNA reglerar inflammatoriska processer i endotelceller som svar på stimulering med nukleotiderna ATP och UTP. ATP och UTP fungerar inte bara som byggstenar för DNA och som energikälla i alla celler utan kan också frisättas och påverka viktiga processer i andra celler, bl.a. har det visat sig påverka rekrytering av inflammatoriska celler till kärlväggen. Vi tittade på hur samtliga mikroRNA i endotelceller påverkades av stimulering med ATP och UTP och kunde se många förändringar i nivåerna före och efter stimuleringen. Bl.a. ökade mikroRNA:t miR-22 kraftigt vid stimulering, ett mikroRNA som vi i delarbete III kunnat visa reglerade uttrycket av ett specifikt inflammatoriskt protein. Vi kunde bekräfta detta samband samt visa att ökade nivåer av miR-22 i odlade endotelceller ledde till minskad rekrytering av inflammatoriska celler. Slutsatsen från studien är att UTP och ATP har anti-inflammatoriska egenskaper på endotel genom en mikroRNA-beroende mekanism.

Slutsatserna man kan dra av resultaten i denna avhandling är att mikroRNA dels spelar roll för inflammatoriska processer i kärlväggen, något som är viktigt för vår förståelse av hur åderförkalkning kan hämmas och behandlas, samt att nivåerna av hjärt-anrikade mikroRNA i blodet bär på diagnostisk och prognostisk information i samband med hjärtinfarkt.

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