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# Biomarkers in out-of-hospital cardiac arrest and cardiogenic shock

- with focus on plasma microRNAs



# Biomarkers in out-of-hospital cardiac arrest and cardiogenic shock

- With focus on plasma microRNAs

Patrik Gilje, MD



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DOCTORAL DISSERTATION

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To be defended at Segerfalksalen, BMC on the 9<sup>th</sup> of December at 9 a.m.

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Title and subtitle  <b>Biomarkers in out-of-hospital cardiac arrest and cardiogenic shock - with focus on plasma microRNAs</b>		
Abstract Introduction: Survivors of out-of-hospital cardiac arrest (OHCA) and patients in cardiogenic shock (CS) are difficult to treat and have a high mortality. The aims of this thesis were to evaluate plasma microRNAs and high-sensitivity troponin-T (hs-TnT) to assess prognosis and organ function among these patients. Methods: In 4 of the 5 studies, we used blood samples and clinical data to evaluate the plasma levels of the liver-specific miR-122, the brain-specific and miR-124-3p and hs-TnT after OHCA. In 1 of the studies, a porcine hypothermia ischemic cardiogenic shock model was used. Results: At 48 h after OHCA, plasma miR-124-3p was similar predictive of a poor neurological outcome as the neuron-specific enolase (NSE), but did not provide any prognostic information when added to NSE. In the porcine ischemic cardiogenic shock model, plasma miR-122 increased 460.000-fold and this increase was attenuated by hypothermia treatment. miR-122 correlated to markers of organ hypoperfusion. At admission after OHCA, plasma miR-122 was 404-fold elevated compared to controls. Although not being predictive of outcome, miR-122 correlated to markers of hypoperfusion and bystander CPR. At 48h after OHCA, hs-TnT was an independent marker of all-cause mortality and death due to cardiovascular causes or multi-organ failure. Conclusions: This thesis has potential future implications for prognostication and care after OHCA and CS.		
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- with focus on plasma microRNAs

Patrik Gilje, MD



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*To Michelle*

*-I love you more than hearts*



”Never be the smartest guy in the room”

James D Watson

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# List of Publications

- I            **Gilje P**, Gidlöf O, Rundgren M, Cronberg T, Al-Mashat M, Olde B, Friberg H, Erlinge D. **The brain-enriched microRNA miR-124 in plasma predicts neurological outcome after cardiac arrest.** Crit Care. 2014 Mar 3;18(2)
  
- II           **Devaux Y**, Dankiewicz J, Salgado-Somoza A, Stammet P, Collignon O, **Gilje P**, Gidlöf O, Zhang L, Vausort M, Hassager C, Wise MP, Kuiper M, Friberg H, Cronberg T, Erlinge D, Nielsen N. **Association of Circulating MicroRNA-124-3p Levels With Outcomes After Out-of-Hospital Cardiac Arrest: A Substudy of a Randomized Clinical Trial.** JAMA Cardiol. 2016 Jun 1;1(3):305-13.
  
- III          **(Gilje) Andersson P**, Gidlöf O, Braun O, Götzberg M, van der Pals J, Olde B, Erlinge D. **Plasma levels of liver-specific miR-122 is massively increased in a porcine cardiogenic shock model and attenuated by hypothermia.** Shock. 2012 Feb;37(2):234-8
  
- IV          **Gilje P**, Frydland M, Bro-Jeppesen J, Dankiewicz J, Al-Mashat M, Jögi J, Kjaergaard J, Hassager C, Erlinge D. **The liver-specific miR-122 is a hyper-acute biomarker of liver hypoperfusion and traumatic chest compressions after out-of-hospital cardiac arrest.** Manuscript.
  
- V           **Gilje P**, Koul S, Hartvig Thomsen J, Devaux Y, Friberg H, Kuiper M, Horn J, Nielsen N, Pellis T, Stammet P, Wise MP, Kjaergaard J, Hassager C, Erlinge D. **High-sensitivity Troponin-T as a prognostic marker after out-of-hospital cardiac arrest – a Targeted Temperature Management (TTM) trial substudy.** Resuscitation. 2016 Oct;107:156-61

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In addition to the papers included in this thesis, the author has published 8 other articles in international peer-reviewed journals.

# Populärvetenskaplig sammanfattning

Biomarkörer är ämnen som detekteras i kroppsvätskor (vanligtvis i blodet) och som ger information om tillståndet i kroppen (t.ex. ett ämne som läcker ut i blodet vid hjärtinfarkt) eller ger information om prognos vid ett visst sjukdomstillstånd. I denna avhandling har jag undersökt hur biomarkörer kan användas för att värdera prognosen hos patienter som återupplivats efter hjärtstillestånd. Jag har även i gris kartlagt hur en stor hjärtinfarkt med efterföljande lågt blodtryck (chock) påverkar vävnadsspecifika biomarkörer i ett försök att hitta nya markörer för organskada vid detta tillstånd.

Två olika typer av biomarkörer har studerats i avhandlingen – microRNA och höggänsliga troponiner. MikroRNA bildas direkt från DNA i alla kroppens celler. När den genetiska koden som finns lagrad i form av DNA översätts till proteiner i cellen sker detta med hjälp av RNA. RNA kopierar den kod som finns i DNA och RNA-molekylen kan därefter gå vidare till cellens proteinfabrik. En del RNA har dock andra uppgifter än att fungera som länk mellan DNA och proteintillverkning. Korta RNA-strängar, så kallat mikroRNA används istället av cellen för att binda till specifika RNA och hindrar på detta vis detta RNA från att nå cellens proteinfabrik. Konsekvensen av denna process blir att proteinet inte tillverkas och att genen som skulle ge upphov till proteinet ifråga indirekt stängs av. MikroRNA hittades i blodet hos människa 2008 och eftersom vissa mikroRNA enbart finns i ett organ skulle de potentiellt kunna användas som biomarkör för att detektera organskada och kanske även ge information gällande prognos vid specifika sjukdomstillstånd. Den andra typen av biomarkör som studerats i avhandlingen, Troponin T, finns enbart i hjärtmuskelcellen och är den bästa markören för att detektera hjärtskada.

I artikel I och II studerades om miR-124-3p, som är ett hjärnspecifikt microRNA, kunde förutsäga död eller omfattande hjärnskada hos patienter som återupplivats efter hjärtstillestånd. Dessa patienter har dålig prognos och cirka 50 % avlider, vanligtvis på grund av omfattande hjärnskador. Eftersom patienterna är medvetslösa och ligger i respirator är det under de första dygnet svårt värdera om de kommer överleva med hjärnan i behåll eller om de kommer att avlida. En biomarkör som tidigt i förloppet kan förutsäga prognosen hos dessa patienter liv hade haft stor

betydelse. Det finns idag en etablerad markör för hjärnskada efter hjärtstillestånd som heter NSE. Vi visade i artikel I och II att miR-124-3p är en bra markör för att kunna förutsäga dålig prognos hos återupplivade hjärtstoppspatienter, men den var inte bättre än NSE. Att kombinera miR-124-3p med NSE gav inte heller någon ytterligare information.

I artikel III utforskade vi i sövda grisar hur miR-122-5p (leverspecifikt) och miR-208b-3p (hjärtspecifikt) i blodet påverkades av stor hjärtinfarkt med lågt blodtryck (chock). Nedkylning har tidigare visat sig hämma organskada och hälften av grisarna kylades till 33°C medan andra hälften hade normal kroppstemperatur (38°C). Vi upptäckte att miR-122 steg mycket snabbt och kraftigt i blodet (460000 gånger) i samband med hjärtinfarkt och chock hos gruppen som inte kylades. I den kylda gruppen sågs istället sjunkande nivåer av miR-122. Vi kunde visa att nivåerna av miR-122 korrelerade med nedsatt blodcirkulation i djuren. Även miR-208b-3p steg kraftigt (63000 gånger), men påverkades inte av kylbehandling.

I artikel IV gick vi vidare med resultaten från artikel III till patienter som återupplivats efter hjärtstopp. Även hos dessa patienter sågs en mycket snabb ökning av miR-122 i blodet direkt efter återupplivningen. miR-122 korrelerade inte med patienternas prognos men liksom i artikel III fick vi indikationer på att miR-122 korrelerade med nedsatt blodcirkulation i kroppen. Vi såg även en korrelation till hjärt/lungräddning vilket sannolikt representerar traumatisk leverskada i samband med bröstkompressioner. Hälften av patienterna kylades till 33°C medan hälften kylades till 36°C, men vi såg ingen skillnad med avseende på miR-122 mellan dessa grupper.

Slutligen i artikel V studerade vi om förhöjda nivåer av troponin-T i blodet kan förutsäga prognos hos patienter som initialt överlevt hjärtstopp. Det visade sig att troponin T predikterar död även när andra kända faktorer som påverkar prognosen tas i beaktande.

Sammanfattningsvis har vi hittat intressanta biomarkörer för såväl prognos efter hjärtstillestånd som organpåverkan vid chock utlöst av stor hjärtinfarkt. Fler studier kommer dock behövas innan dessa biomarkörer kan föras in i klinisk vardag.

# Abbreviations

ALT	alanine aminotransferase
AMI	acute myocardial infarction
AUC	area under the curve
CA	cardiac arrest
CO	cardiac output
CPC	Cerebral Performance Category
CPR	cardiopulmonary resuscitation
CS	cardiogenic shock
CV	coefficient of variation
EVs	extracellular vesicles
Hs-TnT	high-sensitivity troponin-T
LAD	left anterior descending (artery)
LVIDD	left ventricular internal diameter end diastole
MAP	mean arterial pressure
miRNA	microRNA
MOF	multi-organ failure
NSE	neuron-specific enolase

NSTEMI	non-ST-elevation myocardial infarction
OHCA	out-of-hospital cardiac arrest
qRT-PCR	Quantitative Real-Time PCR
RISC-complex	RNA-induced silencing complex
RNA	Ribonucleic acid
ROSC	return of spontaneous circulation
STEMI	ST-elevation myocardial infarction
TnT	troponin-T



# Introduction

Prognostication is a fundamental part of everyday life in the clinic. In addition to the clinic examination and patient history, we daily use tools to assess the prognosis of our individual patient and constantly strive for better prediction of outcome. This becomes even more important when dealing with unconscious survivors of cardiac arrest (CA), who is notoriously difficult to assess by clinical examination alone and where extensive intensive care treatment of patients with a poor prognosis leads to high costs, both human and economical. Hence, there is a need after CA for methods that are better able to distinguish patients that would gain from continuous treatment efforts from those where palliation should be initiated.

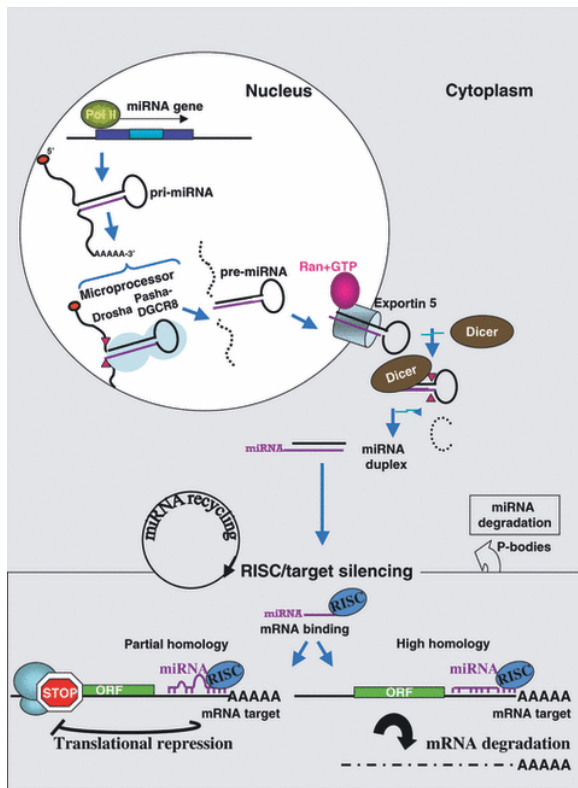
This thesis has plasma biomarkers as unifying theme. Plasma biomarkers have the advantage to be easily obtained and can be used for diagnostic as well as prognostic purposes. In many cases, biomarkers can also be used to monitor the development of disease. In the cardiovascular field, we rely heavily on biomarker such as pro-BNP and high-sensitivity troponins for both diagnostic and prognostic purposes. As we shall see, a new class of interesting plasma-derived biomarkers has emerged during recent years – microRNAs (miRNAs).

## MicroRNAs

The scientific community was for long troubled by an apparent paradox – why do many plants and animals such as lizards have more protein-coding genes than humans? Why do not the size of the genome correlate to developmental complexity? This paradox was solved by the discovery that much of the genome codes for RNAs that do not participate in the classic DNA-RNA-protein pathway. Instead, some of these non-coding RNAs participate in regulatory functions in the cell and the more non-coding RNAs in a species, the higher the biological complexity. Hence, increased complexity do not correlate to more protein-coding genes but rather to more complex regulatory systems (1). There is a rich palette of different types of non-coding RNA:s and this thesis focus on one of them – miRNAs.

After first being discovered in *Caenorhabditis elegans* in 1993, it has been progressively clear that microRNAs are vital for the development of multicellular organisms and plays key regulators in fundamental processes in the cell (2). miRNAs are short (18 to 23 nucleotides) non-coding RNAs that have a central role in regulating translation and degradation of mRNAs. By post-transcriptionally interact with messenger-RNAs (mRNAs) they suppress gene expression by complimentary binding to specific sites on the mRNA, thereby preventing the mRNAs to gain access to the ribosomes. Partial homology results in repression of translation whereas high homology leads to degradation of the mRNA-transcript (3).

Mature miRNAs are not simply transcribed directly from the DNA, but are the result of a complex process involving several steps (figure 1). First, a primary transcript is created through transcription of DNA. These primary transcripts are relatively long and contains a stem-loop structure, which harbor the mature miRNAs sequence. The miRNA primary transcript is cleaved by the by a nuclear protein complex including the RNase Drosha, resulting in a hairpin-shaped pre-miRNA that is transported out of the nucleus to the cytoplasm. Here, the pre-miRNA is further processed by the RNase Dicer into a miRNA-duplex. This duplex interacts with proteins (Ago-proteins) to form the RISC-complex (RNA-induced silencing complex). One of the miRNA-strands (the passenger strand) is degraded while the guide strand remains in the RISC-complex and represses translation by binding to mRNAs with complimentary RNA-sequences (3, 4).



**Figure 1.** Overview of the miRNA-synthesis pathway. The primary miRNA-transcript is processed in several steps while being transported from the nucleus to the cytoplasm. By binding to the RISC-complex, the miRNA binds to mRNAs which lead to repression of translation or degradation of mRNA. Reprinted with permission from John Wiley and Sons: Function of microRNA-375 and microRNA-124a in pancreas and brain. *FEBS J.* 2009 Nov;276(22):6509-21.

Today, over 3000 different miRNAs have been sequenced but it remains controversial how many of these miRNAs that are actually functional *in vivo* (5). It has been estimated that half of the protein coding genes are being regulated by miRNAs (3, 6). As an individual gene can be regulated by multiple miRNAs and individual miRNAs can target more than a 100 genes, miRNAs can be viewed as regulators that orchestrates expression changes in many different genes simultaneously. miRNAs are therefore essential in complex processes such as differentiation, metabolism, apoptosis and proliferation (5, 7). In the cardiovascular field, miRNAs seem central in remodelling of the heart (8) and several miRNAs are associated with atherosclerotic disease (9). Abnormal tissue-expression of miRNAs has also been described in conditions such as atrial fibrillation, hypertension and aortic stenosis (10, 11),

## miRNAs in plasma

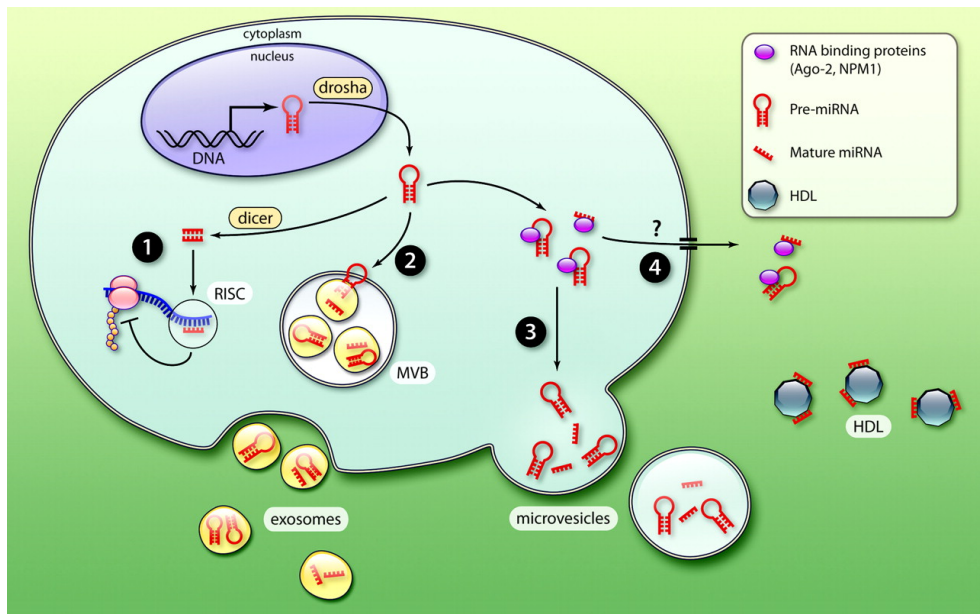
After being discovered 1993 in multicellular organisms, 15 years elapsed before miRNAs were found in plasma (12). This surprising discovery, in combination with the fact that miRNAs are stable in the circulation and a realisation that cells release or secrete miRNAs as a response to injury, has led to an insight that plasma miRNAs might be suitable biomarkers. So far, distinct miRNA signatures in plasma has been found for several diseases such as type 2 diabetes (13), myocardial infarction (14), heart failure (15) and hypertension (16). However, although more than 500 miRNAs can be found in plasma, only ten of them seem to constitute around 90 % of the total plasma miRNA pool in healthy volunteers (17). Due to the abundance of RNases in plasma, free miRNAs in plasma are rapidly degraded. However, by being associated with proteins (mainly ago2) and/or high-density lipoproteins (HDL) and/or located inside extracellular vesicles (EVs), they seem to be protected from degradation (18, 19).

Apart from conferring protection from RNases, EVs might also be central in a rather new field – the role of miRNAs in cell-to-cell communications. It was long assumed that cell-to-cell communication were restricted to protein-based signalling systems. However, although its functional significance is still to be proven *in vivo* (20), miRNA-containing EVs seem to be internalised by recipient cells followed by down-regulation of specific genes (21, 22). One of the advantages with an EV-based inter-cellular communication system is that it can interact with the target cell on multiple intracellular levels simultaneously. Hence, EVs have emerged as potential carriers of therapeutic agents *in vivo* (23, 24).

The EVs do not only contain miRNA but can harbour mRNA, DNA and/or proteins as well. EVs are present in most body fluids such as the blood, urine, cerebrospinal fluid, breast milk and saliva (25). Interestingly, the interior of EVs do not seem on the molecular level represent the cytoplasm/endosomal compartment of the mother cell. Instead, the content of EVs might be formed by an active selective process where certain molecules are packed inside the vesicle (26). These vesicle “signatures” potentially adds another dimension in miRNA biomarker research, especially in the cancer field since tumour cells are prone to producing EVs (27).

The nomenclature for EVs is rather confusing and no standard exists to date. However, it is common to distinguish three different groups - exosomes,

microvesicles and apoptotic bodies, based on their size, origin and membrane composition (28). Exosomes are only 30-100 nm in diameter and are formed through inward budding into late endosomal compartment that fuse with the cell membrane (29). Microvesicles are particles that are created through budding from the cell membrane with a size around 50-1000 nm (30). Apoptotic bodies are released from the plasma membrane during apoptosis (25) (figure 2).



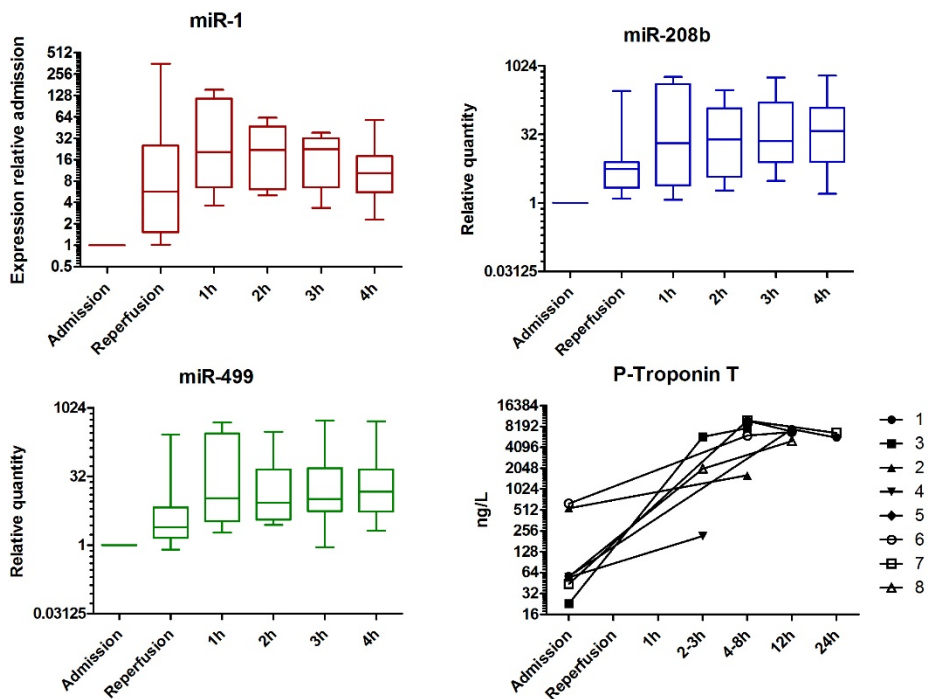
**Figure 2.** Release mechanisms and extracellular compartmentalisation of miRNAs. Reprinted with permission from Wolters Kluwer Health, Inc. *Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?* *Circ Res.* 2012 Feb 3;110(3):483-95.

In the circulation, most miRNAs are found both in the non-vesicle fraction (bound to proteins) and in the vesicle fraction (inside EVs). However, some miRNAs, like the liver-specific miR-122 investigated in paper III and IV, seem to compartmentalise exclusively in one of the two fractions, at least among healthy controls (miR-122 is found only in the non-vesicle fraction) (19, 31). Due to its liver specificity, miR-122 has been extensively evaluated in various forms of liver disease and is a sensitive marker liver damage due to drugs (32) and hepatitis B and C (33, 34). Due to the essential role for miR-122 in the replication of the hepatitis C virus, antisense-miR-122 (miravirsin) delivery has proven to successfully repress

the hepatitis C virus in a phase II trial (35). Importantly, no serious side effects were observed.

miR-124-3p (paper I and II) is the most abundant miRNA in the human brain, but miR-124-3p can also be found in plasma where it, in contrast to miR-122, seems to be abundant in the vesicle fraction (36, 37). In the brain, miR-124-3p has a central role in neural differentiation and suppresses the expression of non-neural genes, thereby maintaining a neuronal phenotype (38). Although by many regarded as brain-specific, miR-124-3p is also detectable in other human tissues such as the gastrointestinal tract and kidneys, albeit at much lower levels (39). After stroke, miR-124-3p is elevated in plasma and might predict neurological outcome (40).

In the cardiovascular field, cardiac-specific miRNAs are elevated in plasma after acute myocardial infarction (AMI) (14), but they do not seem to outperform hs-TnT or give any additional prognostic or diagnostic information (41, 42). Some have proposed that cardiac-enriched miRNAs may rise earlier in plasma than hs-TnT, but this remains to be confirmed (10). Unpublished data from our group including 8 STEMI patients admitted to the cath-lab indicates that hs-TnT is more sensitive than cardiac-specific miRNAs before reperfusion. However, especially the cardiac-enriched miR-1 rise rapidly at reperfusion and seem to peak earlier than hs-TnT after restoration of coronary blood flow (figure 3).



**Figure 3.** Graphs representing the release pattern of cardiac-enriched miRNAs and hs-TnT in 8 patients before and after coronary reperfusion. The expression is normalized against the synthetic spike-in Cel-miR-39. Box-and-whiskers plots and error bars representing the 95% CI are presented in the figure (Gilje P, Gidlöf O, Erlinge D. Unpublished data).

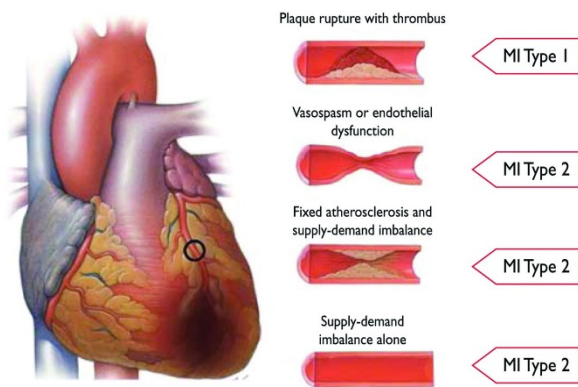
In contrast to the production of miRNAs, the extracellular elimination has not yet been characterized in detail (26). However, administration of exosome miR-155 mimic in a mouse model had a short half-life of around 5 minutes with the highest accumulation of miR-155 in the liver, followed by adipose tissue, lung and muscle and lowest in the kidney. Moreover, the organ signal from miR-155 vanished after 30-40 min indicating rapid degradation (28). After myocardial infarction, cardiac specific miRNAs have been found in the urine but it is not clear whether or not this is related to an active secretory mechanism or a passive secretion due to overload of reabsorbing mechanisms in the kidney (14).

## High-sensitivity Troponin T

Troponin T is a sarcomere protein that is a part of the contractile apparatus in the myocyte. Due to its high specificity, troponin T and I are some of the most commonly used biomarkers in the clinic as a reflection myocardial damage. During recent years, high sensitive assays has been developed which has fundamentally changed how positive troponins are interpreted (43, 44). Traditionally, elevated Troponin T has been closely related to the diagnosis of AMI due to plaque rupture and troponin T is a strong prognostic marker after both ST-elevation myocardial infarction (STEMI) as well as in non ST-elevation myocardial infarction (NSTEMI) (45). However, due to the high sensitivity of the modern high-sensitivity assays, troponin elevations can nowadays be detected in a large group patients without myocardial infarction, especially among the elderly (46). This poses challenges in how to interpret positive troponins, especially in the emergency department. Elevated troponin is also associated with mortality and morbidity in several acute non-cardiac conditions, such as stroke (47), sepsis (48), pulmonary embolism (49) and hip fracture (50). Interestingly, elevated troponins are also associated with increased mortality and structural heart disease among asymptomatic individuals (51).

More is needed for the diagnosis of AMI than elevated troponins. Importantly, there also has to be a dynamic troponin release pattern with at least one value above the 99<sup>th</sup> percentile reference value in combination with symptoms and/or ECG changes indicating ischemia and/or image evidence indicating a new wall motion abnormality (52). However, with the hs-TnT assays, dynamic troponin release patterns are observed in various acute conditions distinct from myocardial infarction due to plaque rupture. This has led to differentiation between different types of AMI depending on the cause of ischemia. The two most clinically relevant is the type 1 AMI which is due to plaque rupture and the type 2 AMI which is secondary to supply-demand imbalance of oxygen in the myocardium, usually due to factors such as hypoxemia, tachyarrhythmia and anemia (figure 4) (52, 53). Since shock and myocardial infarction are frequently encountered among survivors of out-of-hospital cardiac arrest (OHCA), we hypothesized in paper V that hs-TnT might contribute to additional prognostic information.





**Figure 4.** Illustrating the mechanisms behind different kinds of AMI. Reprinted with permission from European Heart Journal

## Current concepts in prognostication after out-of-hospital cardiac arrest (OHCA)

OHCA is a cause of substantial mortality and morbidity. Patients that are successfully resuscitated but remain unconscious have a poor prognosis and approximately half of the patients die within weeks to months due to irreversible neurological damage and multi-organ failure (MOF) (54, 55). After return of spontaneous circulation (ROSC), the post CA syndrome ensues. This syndrome is characterized by systemic inflammation, ischemia/reperfusion injury and encompasses organ dysfunction throughout the body (54). In this respect the post CA syndrome resembles the clinical picture of shock of other aetiologies, such as septic shock and cardiogenic shock (54). Due to the heterogeneous presentation of the post CA syndrome, it poses a challenge to the clinician in order to optimize care for the individual patient.

Early reliable prognostication after CA is important, since it guides the clinician in the decision to withdraw life-sustaining therapies (56). Due to the risk of false-positive rates, prognostication should never be based on a single indicator. Instead, contemporary prognostication involves an integrative approach involving several modalities, such as clinical examination, neurophysiological tests, biomarkers and imaging. According to the latest guidelines, the absence of pupillary and corneal reflexes, and/or bilaterally absence of short-latency somatosensory evoked

potentials at 72 h after CA indicates a poor outcome with a high degree of certainty (false positive rate <5% with narrow 95% CI) (57). Other signs such as high-levels of neuron-specific enolase (NSE), status myoclonus and imaging evidence of diffuse post-anoxic brain injury at 48 h-72 h after CA were also identified as useful tools for prognostication.

## **Biomarkers in OHCA**

Due to the complex prognostication process among patients that remain unconscious after OHCA, a reliable biomarker that early after CA identifies all patients with a poor prognosis would be of great clinical use. Unlike the clinical examination and neurophysiological tests, biomarkers have the advantage of not being affected by sedatives (57). However, the disadvantage of biomarkers in prognostication is the difficulty in finding cut-offs that excludes false positives while maintaining a relevant sensitivity. Moreover, brain-derived biomarkers do not identify CA-patients that die from other causes than brain damage. To date, two brain-enriched biomarkers have been extensively evaluated as prognostic markers after CA – NSE and S-100 $\beta$  (58). S-100 $\beta$  is less well investigated than NSE and no specific recommendations for its use can be found in the latest guidelines (57). Hence, only NSE is described below.

### *NSE*

NSE is an enzyme involved in glucose metabolism in the neuron. Although NSE is enriched in neurons, it can also be found in platelets and erythrocytes and haemolysis might lead to falsely elevated levels (59). Elevated levels of NSE can be detected in blood after CA and high levels have been shown to predict a poor outcome (59). Although there has previously been some controversy concerning the influence of therapeutic hypothermia treatment on NSE-levels (60), the recent NSE-sub study of the TTM-trial showed similar NSE-levels between 33 and 36 degrees hypothermia treatment (61). The same study confirmed that NSE at 48 h after OHCA offers a high specificity of a poor outcome while maintaining a clinically relevant sensitivity (at 48h: AUC=0.85). Although its clinical utility is still a subject of debate (62), the latest guidelines support the use of NSE as a part of the multimodal approach for prognostication after OHCA (57).

# Cardiogenic shock

Cardiogenic shock (CS) is characterized by end-organ hypoperfusion due to cardiac failure and occurs in approximately 8% of patients with ST-elevation myocardial infarction (STEMI) (63). AMI accounts for approximately 80% of cases and mechanical complications, decompensated valvular heart disease and arrhythmias are less frequent causes (64). The clinical picture is consistent with persistent hypotension in combination with signs of hypoperfusion due to severe reductions in cardiac index despite elevated cardiac filling pressures. (65). The effects of hypoperfusion compromise organ function throughout the body and leads to a vicious circle with further reduction in coronary perfusion, decreased cardiac function and a poor prognosis. Acute revascularization is of outmost importance since it seems to reduce mortality after CS (66). Other initial treatment options are inotropic drugs to achieve a sufficient perfusion pressure to vital organs and percutaneously implanted ventricular assist devices (PVADs). Acute echocardiography is also essential in order to detect mechanical complications and to assist in the general assessment of the patient.

## Biomarkers in cardiogenic shock

Despite the aggressive treatment described above, the prognosis of cardiogenic shock remains poor with a mortality of approximately 40-50% (64, 67). Due to the central role of organ hypoperfusion in the development of cardiogenic shock, biomarkers that rapidly respond to organ perfusion would be useful. In addition to hemodynamic factors, inflammation plays a key role during CS and there are many similarities between sepsis shock and both CS and the post cardiac arrest syndrome with regard to systemic inflammation and vasodilation (68). Hence, several inflammatory markers have been evaluated in CS. High IL-6 is associated with progression of MOF among patients with CS (69) and IL-6, -7, -8 and -10 have all been associated with mortality (70). Impairment of microcirculation is another characteristic of CS and markers of vascular integrity (angiopoietin -1 and -2) as well as creatinine are prognostic markers in CS (71, 72). Lactate production is closely related to hypoperfusion (73) and increased lactate levels at admission for CS are associated with increased mortality (74).

With the aim of finding new biomarkers of tissue injury and hypoperfusion during cardiogenic shock, the release pattern of a cardiac-specific and a liver-specific miRNAs were evaluated in paper III. To date, this is the only miRNA-paper

investigating plasma miRNAs during cardiogenic shock. However, a study assessing the prognostic value of plasma miRNAs in patients with STEMI and cardiogenic shock is ongoing.

# Aims and hypotheses

The overall objective of this thesis was to find new biomarkers for organ dysfunction and assessment prognosis in OHCA and cardiogenic shock.

The specific aims and hypotheses of the included papers were:

**Paper I:** to evaluate the impact of the cardiac arrest syndrome on the plasma levels of selected tissue-specific miRNAs and to assess their ability to accurately prognosticate death and neurological disability.

**Paper II:** to address the prognostic properties of circulating miRNAs for neurologic outcome and mortality after OHCA in a larger cohort compared to paper I.

**Paper III:** to evaluate the effects of ischemic-induced cardiogenic shock on the release pattern of the cardiac-specific miR-208b-3p and the liver-specific miR-122-5p and to assess the effect of hypothermia initiated after reperfusion on their respective plasma levels.

**Paper IV:** to extend the findings in paper III to initial survivors of OHCA and characterize plasma miR-122-5p during the first 72h after OHCA. We hypothesised that miR-122-5p would be acutely elevated after OHCA and that the levels of miR-122-5p would correlate to markers associated with liver hypoperfusion and be associated with shock at admission. We also hypothesized that miR-122-5p would be associated with all-cause mortality 180 days after OHCA.

**Paper V:** to characterize hs-TnT during the first 72 h after OHCA. We hypothesized that hs-TnT would be an independent prognostic marker of all-cause mortality as well as death due to cardiovascular causes or MOF at 6 months after OHCA. Furthermore, the relationship between hs-TnT and cerebral causes of death as well as targeted temperature management level, 33 °C vs 36 °C, was assessed.

# Material and methods

In this section, I give an overview of the cohorts and methods that have been used throughout the papers. For details regarding the individual studies, please refer to the individual papers.

## Population and cohort descriptions

Paper II, IV and V consists of patients included in the TTM-trial. Before the end of the TTM-trial, biomarker sub studies were pre-specified, including prognostic evaluation of the brain-specific miR-124-3p investigated in paper I and II and hs-TnT investigated in paper V. In paper V, the whole TTM-biomarker cohort of 699 patients was included. In paper II, patient dying before 48h after admission were excluded from the biomarker cohort which left 579 patients eligible for the study. Finally, paper IV consists of the 167 Danish patients that entered the TTM biomarker sub study. In contrast to the remaining TTM-biomarker cohort, the Danish patients had additional blood samples collected at admission which was essential for paper IV.

## Assessment of neurological outcome

Paper I and II used the five-point Cerebral Performance Category (CPC) Scale to assess neurological outcome : CPC score 1: good cerebral performance, CPC score 2: moderate cerebral disability, CPC score 3: severe cerebral disability, CPC score

4: coma and CPC score 5: death (75). A CPC score of 1 or 2 at 6 months after CA was considered a good outcome.

## miRNA extraction

Extraction of miRNAs from plasma is a delicate process. Due to the small amount of miRNA in plasma and several processing steps, initial insignificant errors can accumulate and produce invalid results in the end. It is therefore of outmost importance that several controls are used in order to produce valid results. The extraction of miRNA from plasma in this thesis were done in three different labs – the BMC lab in Lund (paper I and III, dr Yvan Devaux's lab in Luxembourg (paper II) and Exiqon Services in Vedbæk, Denmark (Paper IV). All three labs have much experience in miRNA extraction from plasma and all analyses have been performed according to the best standards that were available at the time of analysis. I will outline our protocol for preparation of RNA, cDNA-synthesis and Quantitative Real-Time PCR that we have used in Lund (paper I and paper III). In Luxembourg and at Exiqon, different quantification and normalization methods have been used and these will also briefly be described below.

### **Preparation of RNA and cDNA-synthesis (paper I and III)**

Plasma was mixed with TRIzol LS in a 3:1 ratio in order to denature proteins and lipids, before preparing total RNA (including miRNA) using the miRNeasy Kit (Qiagen, Hilden, Germany). In paper I, the yields of total RNA were assessed using Nanodrop. However, it was later published that the yields of miRNA from plasma are insufficient for quantification of plasma RNA with NanoDrop (76) and we therefore used equal volumes of RNA-preparation, rather than equal RNA amounts, as input in the cDNA-synthesis in paper III. A no-reverse transcriptase controls were run for each set of samples to rule out genomic contamination in the RNA preparations. cDNA was synthesized with the miRCURY LNA Universal RT cDNA synthesis kit (Exiqon).

## **Quantitative Real-Time PCR (qRT-PCR) (paper I and III)**

qRT-PCR was run using primer sets according to the protocol of the manufacturer (Exiqon) on the StepOne Plus Real-Time PCR System. All samples were run in duplicate. Melt curves were included in each run in order to ensure that no unspecific PCR-products were produced.

In paper I, custom qRT-PCR panels including 20 pre-specified miRNAs, were used for screening purposes according to the manufacturer's instructions (Exiqon, Vedbaek, Denmark).

## **RNA-sequencing (paper II)**

In paper II RNA-sequencing was used to quantify the brain-enriched miR-124-3p that we previously had found to be a prognostic marker after OHCA using qRT-PCR. The RNA-sequencing technique has many applications since it can assess the transcriptome from both in a qualitative and a quantitative manner. Not only does it measure RNA-levels with high accuracy, but it can also characterize previously unknown sequences of interest. In that respect, the RNA-sequencing technique is rapidly replacing microarrays in many labs (77). However, qRT-PCR is still the gold standard due to its relative robustness and high sensitivity and specificity and should be used to confirm findings from RNA-sequencing (78). In paper II, the results of the RNA-sequencing is expressed as tags per million, which is the number of reads for a particular miRNA divided by the total number of mapped reads and multiplied by 1 million.

## **The importance of monitoring the miRNA-extraction**

Due to the small amount of input RNA in several steps in the extraction process, initial small errors will amplify through the laboratory process. Moreover, the biological fluids contain small amounts of RNA relative to cells and tissues making the extraction process sensitive to contamination (79). The miRNA-extraction is also sensitive to other factors such as PCR-inhibitors and nucleases.

To address these issues and assure the highest quality of the miRNA-quantification, several control measures must be taken. All analyses in this thesis contain inter-run calibrators to correct technical variations between the PCR runs and negative controls for each primer on each plate to detect contamination. In order to monitor the miRNA-extraction, one should optimally use both external as well endogenous controls. The external controls are synthetic miRNAs that are added to the samples before the RNA-extraction to compensate for technical variability. There are several



potential sources for this variability and include differences in reaction efficiency between samples in the RNA-extraction, cDNA-synthesis and qRT-PCR (80). Endogenous controls are stable miRNAs in plasma and are therefore affected by the same pre-analytic and analytic factors as the miRNA of interest. By using an endogenous control it is possible not only to detect extraction problems during the miRNA extraction, but also detect differences in sample quality which might result in differences in RNA-load between samples. In that respect, the external and endogenous controls complement each other. Some groups have developed potential reference endogenous controls in specific disease states, but these suffer from lack of generalisability to other cohorts. In study IV, an endogenous control in the form of a global mean approach was used (described below).

### **The importance of proper normalisation of miRNA-data**

miRNA-research in plasma is usually performed using relative quantification, i.e. the target-miRNA is expressed relative some sort of normaliser. This is contrasted to absolute quantification (described below) that uses a standard curve to quantify the miRNA in absolute terms. The aim of the normalisation process is to highlight true biological changes while trying to eliminate the variability induced by the experimental setup (80). During recent years, the normalisation process has gradually evolved and the papers included in this thesis is a reflection of this development. However, in contrast to miRNA-extraction from tissue samples where several validated endogenous housekeeping genes are available the field of plasma miRNA still suffers from a solid and common normalisation strategy (80, 81). In the first paper (paper III), no normalization was performed – instead raw Ct-values were presented and analysed. With hindsight, this approach is not optimal since it does not control for factors that might interfere with the miRNA-extraction. In paper I and paper II, we used an external synthetic control for normalization to compensate for technical variation. This compensates for differences in extraction efficiency between the samples, and is a common normalisation approach (82).

Finally in paper IV, a global mean approach of normalization was applied. By combining the expression level of several stable endogenous controls, this normalisation procedure seems to increase the accuracy of the quantification (83). In paper IV, this was performed by using the average of the miRNAs that were detected in all samples and most stably expressed. The Normfinder software was used for this purpose and hsa-miR23a-3p and has-miR-103a-3p were selected for the normalisation procedure.

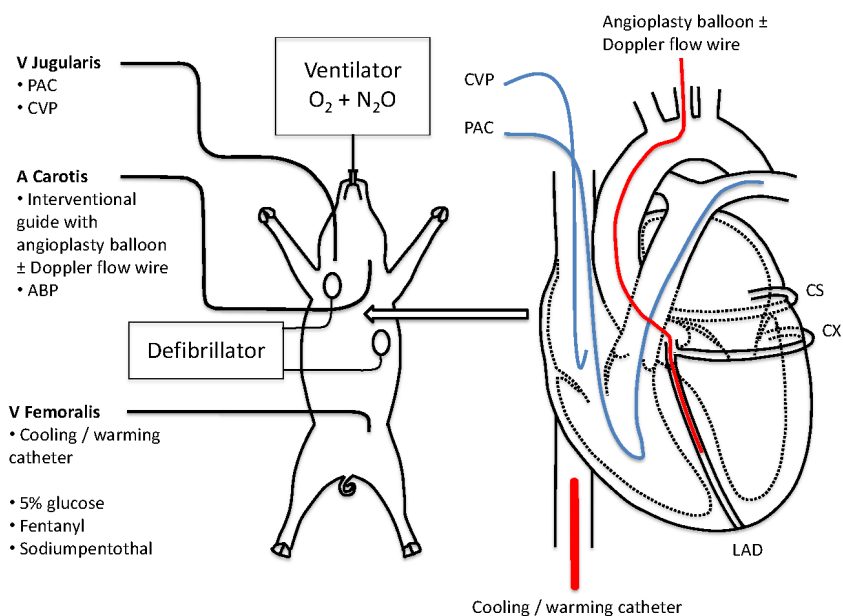
Whenever possible, the results are expressed relative both to baseline and the normalizer using the  $2^{-\Delta\Delta C_t}$  method. However, when we analysed the difference in miRNA levels at admission, we expressed the results only relative to a normalizer using the  $2^{-\Delta C_t}$  formula.

## **Absolute quantification**

In paper II, absolute quantification was used whereas relative quantification was used in paper I, III and IV. Although relative quantification is sufficient for most research studies, absolute quantification has some advantages (82). One is that the specified miRNA can be directly quantified and expressed in the unit copies/ml. It also provides a linear response to the number of copies of the miRNA of interest, allowing small differences between samples to be detected (84, 85). Moreover, in contrast to relative quantification absolute quantification is not as dependent on a normaliser thereby avoiding some of the methodological problems described previously in this section. However, absolute quantification is more technical demanding compared to relative quantification and there is also a risk of miRNA-contamination when producing the standard curve.

## **The porcine model (paper III)**

A schematic overview of the porcine model used for the cardiogenic shock experiments in paper III is presented in figure 5.



**Figure 5.** A schematic overview of the porcine model. Adapted from AB Ericsson, Cardioplegia and Cardiac Function, PhD Thesis, Karolinska Institute, Sweden, 2000.

Pigs were premedicated with ketamine. After intravenous induction of anesthesia with thiopental the animals were orally intubated. Balanced anesthesia was started with a slow intravenous infusion of fentanyl and thiopental that was titrated against animal requirement. The adequacy of anesthesia was assessed by continuous monitoring of intra-arterial blood pressure and heart rate. The animals were ventilated with a mixture of nitrous oxide (70%) and oxygen (30%). Blood gases were analysed every 30 min throughout the experiment. A cooling catheter was placed in the inferior vena cava to keep a constant body temperature and an angioplasty balloon was positioned in the proximal left anterior descending (LAD) artery. Ischemia was induced by inflating the balloon for 40 min followed by reperfusion. Total occlusion of the LAD artery during inflation of the balloon and restoration of blood flow was verified with angiograms. The animals that met with the inclusion criteria, i.e., were in cardiogenic shock before reperfusion, were randomized either to hypothermia ( $n = 6$ ) or to normothermia ( $n = 6$ ). The hypothermia group had their body temperature lowered to 33 °C throughout the experiment. In the normothermia group, the body temperature was maintained at 38 °C. The body temperature was measured using a temperature probe in the distal esophagus. Blood samples were obtained before occlusion and every 30 min for a

total of 150 min after inflation of the balloon. All pigs were killed after 4 h by explantation of the hearts. The study conforms to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health and was approved by the local animal research ethics committee. A detailed report of the procedure can also be found in the paper from Götberg et al. (86)

Porcine tissue (spleen, lung, intestine, liver and heart) were homogenized and miRNA was extracted in order to confirm the cardiac tissue-specificity of miR-208b-3p and the liver specificity of miR-122-5p.

### **Incubations of HepG2-cells**

HepG2 cells were incubated overnight. The following day the medium was changed, and the cells were in triplicates exposed either to hypoxia (2.5% oxygen), acidosis (pH 7.0), or to a physiologic environment used as a negative control. Samples from the medium were collected at baseline and at 15, 30, 45, 60, 180, and 360 min. Each sample was centrifuged at 13,000g for 2 min followed by aspiration of the supernatant, which was quickly frozen and stored at -80°C, followed by RNA isolation and cDNA synthesis.

### **High-sensitivity troponin-T analysis**

The Roche Cobas e601, high-sensitivity Troponin T assay (Basel, Switzerland) was used for the troponin analyses in paper V. This assay has a detection limit of 5 ng/l and a 99th percentile of 14 ng/l. The troponin data were not available to the treating physicians.

## Statistical methods

All statistical analysis in paper I, II, III and V were performed either in IBM SPSS Statistics (version 21 and 22) (Foster City, USA) or GraphPad Prism (version 6 and 7) (La Jolla, USA). In paper IV, the SigmaPlot software (version 12.3) (San Jose, California) and R version 2.15.2 with the ROCR and rms packages were used.

### Paper I

The two-tailed Mann–Whitney test was used to detect differences in miRNA levels with regard to outcomes. Categorical variables were analysed using Fisher’s exact test. The ability of miR-124-3p to discriminate between good vs poor prognosis at 24 h and 48 h was assessed with receiver operating characteristic (ROC) curve analysis. Correlations were performed using Spearman’s correlation test. Bonferroni corrections were applied to the P-values.

### Paper II

The Chi Square test or the Fisher exact test was used to compare categorical clinical characteristics. Differences in miRNA-levels between the groups was assessed using the Mann-Whitney test. Effect of sampling time on miR-124-3p levels was evaluated using 1-way repeated measures analysis of variance on ranks. The area under the receiver operating characteristic curve (AUC) was used to estimate prediction ability of multivariable models. The incremental prognostic value of miR-124-3p levels to a clinical model that included demographic and arrest related factors and NSE levels was evaluated by computation of the integrated discrimination improvement. Logistic regression was used to assess the potential association of miR-124-3p levels after log transformation with neurologic outcome. Kaplan-Meier survival analysis and Cox regression were used to evaluate the association between miR-124-3p levels and survival. In Kaplan-Meier analysis, expression levels of miR-124-3p were divided into quartiles. In Cox regression, the association of miR-124-3p and survival was assessed after adjustment with clinical variables using 10-fold multiple imputation.

### Paper III

Friedman test followed by Dunn post-test was used to compare the levels of ALT and miRNA between the time points. A two-tailed Mann-Whitney U test was performed to test for any difference in mean miRNA levels between the groups and

to investigate hemodynamic differences between the groups before randomization and at 150 min. Correlations between the levels of mir-122-5p and hemodynamic parameters were performed using Spearman rank correlation coefficient. Statistical significance was accepted when  $P < 0.05$ . All error bars represent SEM.

## **Paper IV**

### *Pilot trial*

The Wilcoxon matched-pairs signed rank test were used for comparisons between paired groups. Comparisons between non-paired groups were performed using the Mann-Whitney test.

### *Main trial*

Missing data was handled with 10-fold multiple imputation (MCMC procedure). Due to the multiple imputation analysis, a complete case analysis was performed which showed adequately similar results. Continuous variables with normal distribution were compared using ANOVA. In order to compare pooled data from multiple imputation datasets, non-parametric continuous data were log-transformed before analysed using ANOVA. Differences between repeated measurements of parametric groups were assessed using repeated measures ANOVA with the Sidak correction. Pearson's chi Square test was used for comparing differences between categorical data. Correlations between continuous data were assessed using Pearson's and Spearman correlation coefficients for parametric and non-parametric data, respectively. Associations between miR-122-5p and other parameters were investigated by multiple linear regression and logistic regression analysis. In order to meet the requirement of homoscedasticity and normality of residuals, miR-122-5p at admission and ALT at admission was log2-transformed before being entered as a dependent variable in the multiple linear regression model. In order to make the interpretation of the odds ratio more intuitive, miR-122-5p at admission was log2-transformed before entered as an independent variable in the logistic regression model. The assumption of linearity between independent variables and the log odds in the logistic regression was fulfilled as assessed by the Box-Tidwell test. The variables included in the regression models were those that were significantly associated with the dependent variable in the univariate analyses together with other variables that were deemed clinically relevant. There was no multicollinearity between the independent variables.

## **Paper V**

Missing values were replaced with 5-fold multiple imputations using the MCMC method. Continuous variables with normal distribution were compared using ANOVA. Non-parametric continuous data were compared using the Mann-Whitney U test. Pearson's chi Square test with the z-test as post-test were used for comparing differences between categorical data. The overall ability of hs-TnT to discriminate between outcomes was described by receiving operating characteristic (ROC) curves. Unadjusted mortality between quartiles of hs-TnT was compared using the Kaplan-Meier estimator with the log rank test for significance testing. Troponin T at 24 h, 48 h and 72 h were added to a logistic regression model containing temperature allocation, age, sex, bystander CPR, first prehospital rhythm, time from CA to ROSC, pH at admission and shock at admission as covariates. There was no multicollinearity as assessed with condition indices. As hs-TnT was expressed in the log2 scale, the odds ratio corresponds to a 2-fold increase in hs-TnT. When the logistic regression model was used to analyse specific causes of death, competing risk of dying was handled by censoring patients with other causes of death

# Results

## Paper I

This paper assessed the impact of the CA syndrome on the plasma levels of 20 selected tissue-specific miRNAs. Of those, miR-124-3p was found to be the most promising marker and the prognostic properties of miR-124-3p was evaluated in the whole cohort. The initial cohort consisted of 92 patients. The treatment protocol of this study has been described in detail elsewhere (59). We excluded 25 patients due to missing samples, and 2 patients were excluded because of unknown outcomes and/or missing patient data. In the end, 35 patients with favourable outcomes (CPC score 1 or 2) and 30 with poor outcomes (CPC scores 3 to 5) with plasma samples obtained at baseline and 24 hours and 48 hours after CA were entered into the study. The endpoint was neurological outcome 180 days after OHCA (CPC 1-2 vs 3-5). After a review of the literature, 20 miRNAs were screened using custom quantitative polymerase chain reaction qPCR-panels. Thereafter the brain-enriched miR-124-3p, miR-9 and miR-128; the inflammation-associated miR-146a; the liver-specific miR-122-5p; the cardiac tissue-specific miR-208b-3p; and the apoptosis inhibitor/angiogenesis-associated miR-21 were analysed at the 48 h time point in the entire cohort. However, at 48 h only miR-124-3p differed significantly between the outcome groups.

### *miR-124-3p after OHCA*

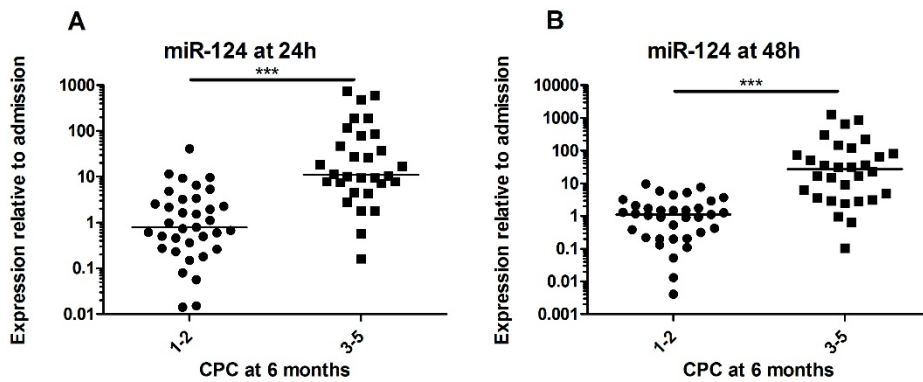
At admission within 6 hours after CA, there were no significant differences in miR-124-3p between the outcome groups but miR-124-3p was already 10-fold elevated at admission in patients compared with age- and sex-matched controls.

At 24 hours after CA, miR-124-3p was 14-fold elevated in the poor outcome group compared to the good outcome group ( $P < 0.0001$ ) (figure 6a). Analysis of ROCs for miR-124-3p at 24 h revealed an AUC of 0.87 (95% CI = 0.79 to 0.96) for poor neurological outcome (figure 7a). A cut-off of 44 yielded 100% specificity and 30% sensitivity at 24 hours for a poor neurological outcome. However, a cut-off of 12



increased sensitivity to 53% while maintaining a specificity of 97% for a poor neurological outcome.

At 48 hours after CA, miR-124-3p was 24-fold elevated in the poor outcome group compared to the good outcome group ( $P < 0.0001$ ). (Figure 6b).

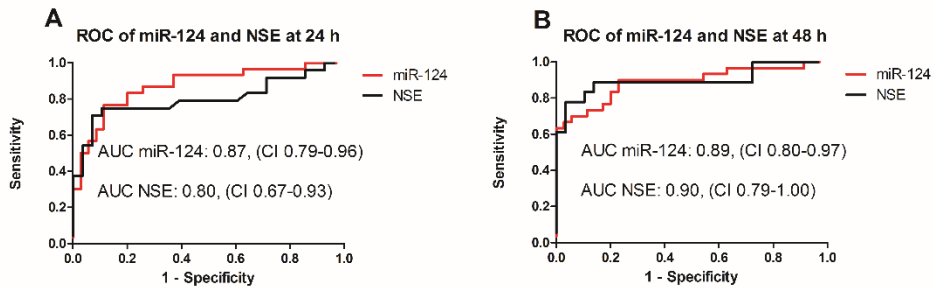


**Figure 6a.** At 24 h after CA, the median level of miR-124-3p was 14-fold elevated in the poor outcome group compared to the good outcome group ( $p < 0.0001$ ). **Figure 6b.** At 48 h after CA, the median level of miR-124-3p was 24-fold elevated in the poor outcome group compared to the good outcome group ( $p < 0.0001$ ).

Analysis of ROCs for miR-124-3p at 48 h demonstrated an AUC of 0.89 (95% CI = 0.80 to 0.97) (figure 7b). A cut-off of 12 yielded 100% specificity and 63% sensitivity at 48 hours for a poor neurological outcome.

### *Comparison between miR-124-3p and NSE after OHCA*

Analysis of ROC:s for NSE revealed AUCs of 0.80 (95% CI = 0.67 to 0.93) at 24 hours and 0.90 (95% CI = 0.79 to 1.00) at 48 hours, which were similar to the results for miR-124-3p (Figure 7a and b). Combining miR-124-3p and NSE did not improve diagnostic accuracy (AUC = 0.90 (95% CI = 0.80 to 1.00) at 24 hours and AUC = 0.93 (95% CI = 0.84 to 1.00) at 48 hours).



**Figure 7a.** ROC-curves for NSE and miR-124-3p at 24h after OHCA. **Figure 7b.** ROC-curves for NSE and miR-124-3p at 48h after OHCA.

Levels of miR-124-3p correlated only moderately to NSE ( $r = 0.51$  at 24 hours and  $r = 0.49$  at 48 hours).

## Paper II

Similar to paper I, this study evaluated plasma miR-124-3p as a prognostic marker after OHCA, but in a larger cohort. The study included the TTM biomarker sub study cohort of the TTM-trial including 699 patients (as in paper V), but patient dying before 48h after admission were excluded. In total, 579 patients entered the study and neurologic outcome 180 days after OHCA was chosen as primary endpoint. Survival at the end of the trial was chosen as secondary endpoint.

Fifty of the patients (25 in each outcome group) entered a discovery phase with RNA-sequencing. Thereafter, a validation phase including 529 patients was initiated (279 patients with CPC1-2 and 250 patients with CPC 3-5 at 180 days).

### *Discovery phase*

Two hundred thirty-six miRNAs were detected in all 50 samples with tags per million greater than or equal to 1. The brain-enriched miR-124-3p was the most differentially expressed miRNA (46-fold change; false discovery rate,  $2 \times 10^{-11}$ ;  $p = 7 \times 10^{-14}$ ).

### *Validation phase*

In the validation cohort, 231 patients (43.7%) were allocated to 33°C treatment and 298 (56.3%) were allocated to 36°C treatment. In concordance with the main TTM-trial, there was no difference in outcome between the temperature groups.

The serum levels of miR-124-3p at 48 h after ROSC did not differ between temperature groups. Patients with a poor outcome (CPC 3-5) had higher levels of miR-124-3p than patients with a good outcome, with an AUC of 0.77. Patients with high levels of miR-124-3p were at high risk for a poor neurologic outcome (univariate OR, 6.72; 95% CI, 4.53-9.97). In a multivariable analysis, levels of miR-124-3p were independent predictors of neurological outcome (OR 1.62 (95% CI, 1.13-2.32). Age, NSE levels, and first monitored rhythm also had a significant prognostic value.

In concordance with paper I, miR-124-3p did not provide any prognostic information when added to NSE as assessed with integrated discrimination improvement. When NSE levels were omitted from the prediction models, miR-124-3p levels provided a most prominent improvement of prognostic ability, with an AUC of 0.78 and 0.84 for the models without and with miR-124-3p levels, respectively ( $P < .001$ ).

### *Survival analysis*

In a Cox regression model, miR-124-3p levels had significant prognostic value for a shorter survival, as did age, first monitored rhythm, initial serum lactate levels, and NSE levels. In Kaplan-Meier analysis, miR-124-3p levels were strongly associated with shorter survival. Overall, patients with high levels of miR-124-3p 48 hours after ROSC were at high risk for death.

## **Paper III**

This paper addressed the effects of ischemic-induced cardiogenic shock on the release pattern of cardiac-specific miR-208b-3p and liver-specific miR-122-5p and assessed the effect of therapeutic hypothermia initiated after reperfusion on their respective plasma levels. The animals that met with the inclusion criteria, i.e., were in cardiogenic shock, were randomized either to hypothermia ( $n=6$ ) or to normothermia ( $n = 6$ ). The hypothermia group had their body temperature lowered

to 33°C throughout the experiment. In the normothermia group, the body temperature was maintained at 38°C. Before reperfusion, there were no differences in hemodynamic parameters between the groups (Table 1)

**Table 1.** Hemodynamic and blood gas variables before randomisation and reperfusion. Data are presented as mean (SEM).

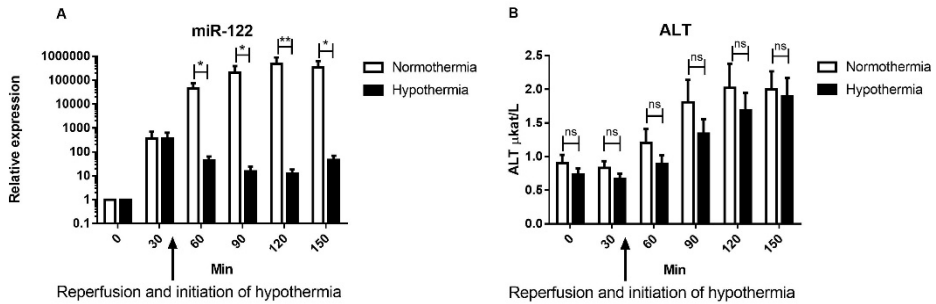
	<b>Hypothermia (n=6)</b>	<b>Normothermia (n=6)</b>	<b>p-value</b>
MAP (mm Hg)	85.8±6.3	76.0±6.4	ns
Stroke volume (ml)	22.8 ± 2.4	21.0 ± 1.9	ns
Heart rate (bpm)	90.7 ± 5.0	116.7 ± 21.6	ns
Cardiac output (l/min)	2.0 ± 0.2	2.3 ± 0.2	ns
CVP (mm Hg)	11.2 ± 2.3	13.3 ± 2.3	ns
Arterial pH	7.44 ± 0.03	7.40 ± 0.05	ns
Arterial pO <sub>2</sub> (kPa)	20.8 ± 2.3	17.7 ± 1.4	ns
Temperature (°C)	38	38	ns

miR-122-5p was found to be highly liver specific, whereas mir-208b-3p was expressed exclusively in the heart. In the control group, ischemic cardiogenic shock induced a 460,000-fold increase in miR-122-5p ( $p<0.05$ ) (figure 8a) and a 63,000-fold increase in miR-208b plasma levels of miR-122-5p ( $p<0.05$ ). Therapeutic hypothermia significantly diminished the increase in miR-122 compared with the normothermic group ( $p<0.005$ ) (figure 8a). In our model, hypothermia was initiated after coronary reperfusion and did not affect myocardial damage as assessed by the plasma level of mir-208b-3p. Hemodynamic and blood gas variables at 150 min (110 min after reperfusion) are presented in table 2.

**Table 2.** Hemodynamic and blood gas variables at 150 min (110 min after reperfusion). Data are presented as mean (SEM).

	<b>Hypothermia (n=6)</b>	<b>Normothermia (n=6)</b>	<b>p-value</b>
MAP (mm Hg)	87,5±6,6	48,8±10,9	<0.05
Stroke volume (ml)	25.0±1,3	15,5±2,3	<0.05
Heart rate (bpm)	86,5±3,4	114,2±10,1	<0.05
Cardiac output (l/min)	2,2±0,1	1,7±0,2	ns
CVP (mm Hg)	11,3±3,0	12,8±2,5	ns
Arterial pH	7,50±0,02	7,38±0,03	$p<0.005$
Arterial pO <sub>2</sub> (kPa)	24,33±3,11	16,31±5,74	$p<0.05$
Temperature (°C)	33	38	<0.05

The massive increase in miR-122-5p led us to measure ALT in plasma, an enzyme found in various tissues but clinically mostly associated with liver damage (26, 27). However, whereas miR-122-5p increased 460,000-fold, ALT increased less than 3-fold, indicating that minor tissue necrosis and hypothermia had no significant effect on its release (figure 8b)



**Figure 8 a and b.** Plasma release of miR-122-5p and ALT at normothermia (38 °C) vs hypothermia (33 °C) after induction of CS.

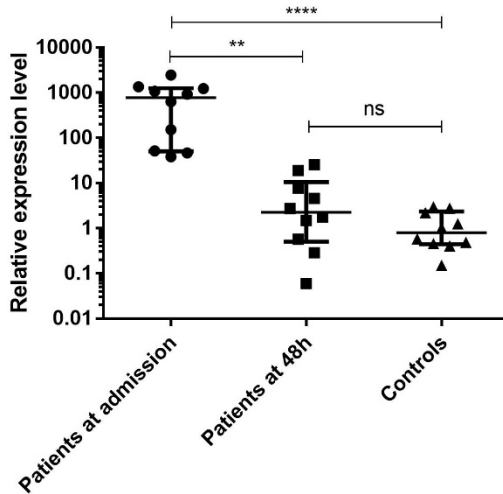
To characterize the release of miR-122-5p further, cultured hepatocytes were exposed to either hypoxia or acidosis. Hypoxia, but not acidosis, resulted in a 2-fold increase in miR-122-5p which did not reach statistical significance.

Peak Ct values of miR-122-5p at 150 min correlated significantly to mean arterial pressure ( $p < 0.05$ ,  $r = 0.59$ ), stroke volume ( $p < 0.02$ ,  $r = 0.67$ ) and pH ( $p < 0.05$ ,  $r = 0.66$ ). No correlations were found between the peak level of miR-208b-3p and hemodynamic parameters or between miR-208b-3p and miR-122-5p.

## Paper IV

Due to the findings in paper III, we decided to characterize the plasma release of miR-122-5p during the first 72 h after OHCA. Moreover, the associations between miR-122-5p and clinical variables as well as all-cause mortality were assessed.

Initially a pilot trial was conducted in a small OHCA cohort (from paper I) to evaluate the plasma levels of miR-122-5p after OHCA and to compare it to baseline levels among sex and age matched controls. At admission, the median plasma level of miR-122-5p was 404-fold (IQR 2298) elevated in OHCA-patients compared to controls ( $p < 0.001$ ). In contrast, at 48h after OHCA there was no significant difference between patients and controls (figure 9).



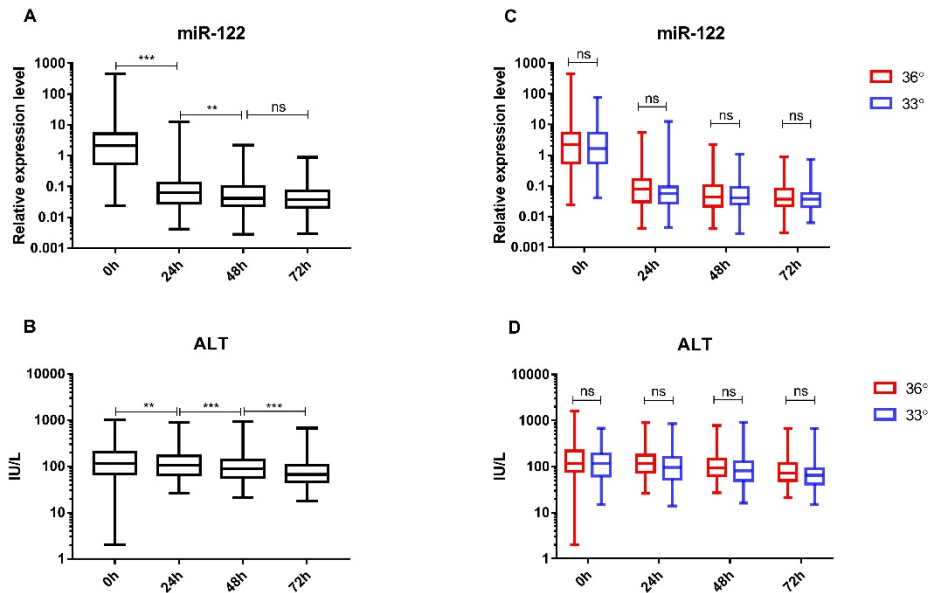
**Figure 9.** miR-122-5p at admission and at 48h after OHCA compared to sex and age matched controls.

#### *Release pattern of miR-122-5p after OHCA*

The main trial consisted of 167 patients. The primary outcome was all-cause mortality at 180 days. Shock status at admission was chosen as a pre-specified subgroup. miR-122-5p was available for 161 patients (96%) at admission, 151 patients (90%) at 24h, 145 patients (87%) at 48h and 140 patients (84%) at 72h. One hundred nine patients (65%) were alive 180 days after OHCA. In concordance with the pilot trial, miR-122-5p peaked at admission and was thereafter in decline before levelling out at 48h (figure 10 a). The median/mean plasma level of miR-122-5p was 57-fold/140-fold elevated at admission compared to the 72 h time point.

### *miR-122-5p according to targeted temperature*

Temperature assignment (33°C vs 36°C) was not associated with miR-122-5p at any time point and there was no interaction between temperature allocation and miR-122-5p at any time point (figure 10 b).



**Figure 10 a-d.** Plasma levels of miR-122-5p and ALT after admission for OHCA. Temperature assignment (33°C vs 36°C) was not associated with miR-122-5p or ALT at any time point.

### *Factors associated with miR-122-5p*

In the multilinear regression analysis ( $r^2=0.23$ ,  $p<0.001$ ), lactate at admission, bystander CPR and left ventricular internal diameter end diastole (LVIDD) at admission were significant predictors of miR-122-5p at admission (table 3).

**Table 3.** Multiple linear regression analysis for prediction of plasma log<sub>2</sub> miR-122-5p at admission. Lactate at admission, bystander CPR and LVIDD at admission were independent predictors. (CI: confidence interval; MAP: mean arterial pressure; LVIDD: left ventricular internal diameter end diastole; CA: cardiac arrest; ROSC: return of spontaneous circulation; CPR: cardiopulmonary resuscitation).

	$\beta$	95% CI	p-value
Lactate at admission (mmol/l)	0.130	0.03, 0.23	<0.01
LVIDD (cm)	-0.757	-1.25, -0.26	<0.01
Bystander CPR (yes)	1.092	0.16, 2.04	<0.05
Age (years)	-0.031	-0.06, 0.00	0.06
MAP at admission (mm Hg)	-0.022	-0.05, 0.01	0.09
Creatinine at admission (mg/dl)	-0.004	-0.01, 0.00	0.11
Sex (male)	0.755	-0.36, 1.87	0.18
Time CA to ROSC (min)	0.006	-0.02, 0.03	0.65
Heart rate at admission (bpm)	-0.006	-0.03, 0.02	0.58
Shock at admission (yes)	0.064	-1.23, 1.36	0.92

#### *miR-122-5p and liver enzymes*

The release pattern of ALT can be seen in figure 10 c. ALT did not differ between the outcome groups or between different causes of death ( $p=ns$ ). miR-122-5p at admission was significantly correlated with ALT at all time points (at admission  $r=0.59$  ( $p<0.001$ ), at 24h  $r=0.59$  ( $p<0.001$ ), at 48h  $r=0.57$  ( $p<0.001$ ) and at 72h  $r=0.55$  ( $p<0.001$ )). In the multivariate regression analysis ( $r^2=0.44$ ,  $p<0.001$ ) including, creatinine at admission, lactate at admission, bystander CPR, shock at admission, age and sex, miR-122-5p at admission remained independently associated with ALT at admission ( $\beta=0.29$ , 95% CI: 0.21-0.37,  $p<0.001$ ). There were no significant associations between ALT at admission and invasive hemodynamic parameters or between ALT at admission and temperature assignment (33°C vs 36°C) at any time point (figure 10 d).

#### *miR-122-5p and shock at admission*

Seventeen patients (10%) had shock at admission whereas 144 (90%) did not. Compared to the whole cohort, there was a similar release pattern of miR-122-5p regardless of shock status at admission. The median/mean plasma level of miR-122-5p at admission among patients with shock at admission was 1.8-fold/2.1-fold elevated compared to the ones without shock, but this did not reach statistical significance ( $p=0.14$ ). In a logistic regression model including lactate at admission and time from CA to ROSC, miR-122-5p at admission was not associated with shock at admission (OR: 1.04, 95% CI: 0.83-1.30,  $p=0.76$ ).



### *miR-122-5p and all-cause mortality*

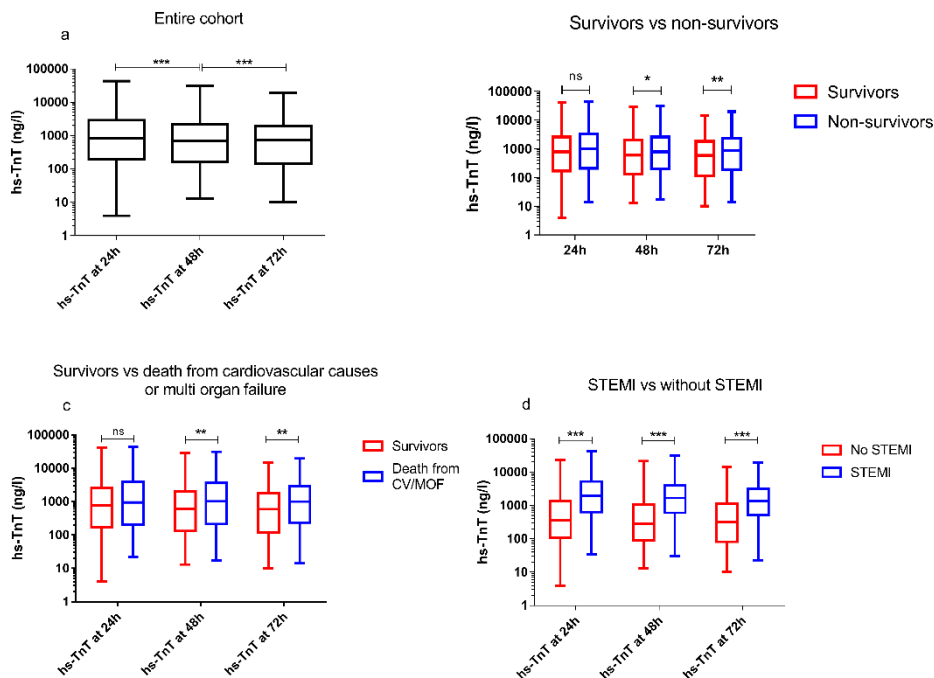
There were no differences in miR-122-5p at any time point with regard to survival status at 180 days after OHCA. In a logistic regression analysis, miR-122-5p at admission was not associated with all-cause mortality. miR-122-5p at admission could not at any time point differentiate between cerebral and cardiovascular/multi-organ failure causes of death ( $p=ns$ ).

## Paper V

In paper V, the prognostic properties of hs-TnT after OHCA were assessed. Of the 939 patients in the TTM-trial, biomarker data was available for 699 patients and blood samples were obtained at 24, 48 and 72 h after ROSC. Hs-TnT was available for 646 patients (92%) at 24h, 618 patients (88%) at 48h and 573 patients (82%) at 72h. Subgroups were pre-specified based on the initial ECG after ROSC (STEMI vs all other ECG presentations). Primary outcome was all-cause mortality at 180 days after OHCA. Secondary outcome was death due to cardiovascular causes or MOF.

Hs-TnT peaked at 24 h after admission (848 ng/l (IQR 2685 ng/l). At 48 h and 72 h, the median values were 695 ng/l (IQR 2039 ng/l) and 756 ng/l (IQR 1786 ng/l), respectively (figure 11 a). Hs-TnT was significantly higher among non-survivors compared to survivors at 48 h ( $p<0.05$ ) and 72 h ( $p<0.01$ ) after ROSC (figure 11 b).

Hs-TnT was independently associated with all-cause mortality which was driven by death due to cardiovascular causes or multi-organ failure and not cerebral causes (at 48h: OR 1.10, CI 1.01-1.20,  $p<0.05$ ) (table 4). Hs-TnT was also an independent predictor of death due to cardiovascular causes or multi-organ failure (at 48h: OR 1.13, CI 1.01-1.26,  $p<0.05$ ) (figure 11 c). In patients with STEMI, hs-TnT was independently associated with death due to cardiovascular causes or multi-organ failure (at 48h: OR 1.47, CI 1.10-1.95,  $p<0.01$ ) (figure 11 d).

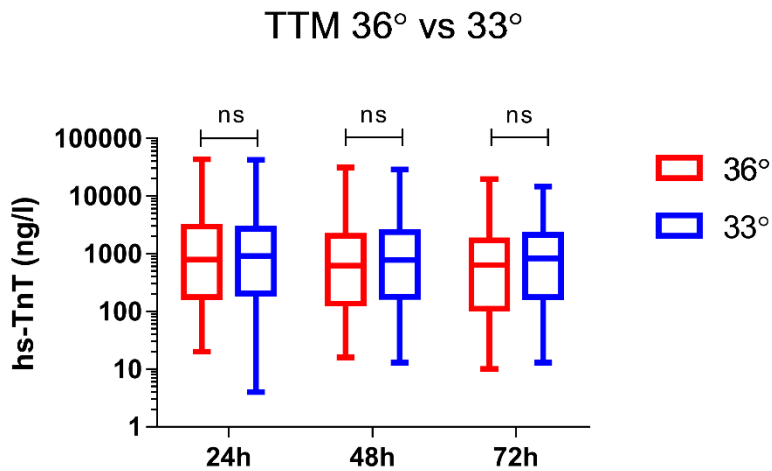


**Figure 11 a.** hs-TnT values at 24h, 48h and 72h after ROSC ( $p < 0.001$  for differences between timepoints). **Figure 11 b.** hs-TnT at 48h and 72h were significantly elevated among non-survivors compared to survivors (at 48h:  $p < 0.05$ ; at 72h:  $p < 0.01$ ). **Figure 11 c.** hs-TnT was significantly elevated among patients that died due to cardiovascular causes and multi-organ failure compared to survivors (at 48h and 72h:  $p < 0.01$ ). (CV, cardiovascular; MOF, multi-organ failure). **Figure 11 d.** hs-TnT was significantly elevated among patients with STEMI compared to patients without STEMI ( $p < 0.001$  for differences between groups).

**Table 4.** Logistic regression analysis with hs-TnT and clinical variables to predict all-cause mortality. Hs-TnT at 48 h and 72 h were independent predictors. (CPR: cardiopulmonary resuscitation; PEA: pulseless electrical activity; CA: cardiac arrest; ROSC: return of spontaneous circulation)

<b>At 24h</b>	<b>Odds ratio (95% CI)</b>	<b>p-value</b>
Temperature group (36°C)	1.23 (0.85-1.77)	ns
Age	1.07 (1.05-1.09)	<0.001
Sex (male)	0.75 (0.47-1.20)	ns
Bystander CPR (yes)	0.64 (0.42-0.97)	<0.05
Asystole/PEA (yes)	6.77 (3.91-11.73)	<0.001
CA to ROSC (min)	1.03 (1.02-1.04)	<0.001
Shock at admission (yes)	1.42 (0.81-2.50)	ns
pH	0.12 (0.03-0.46)	<0.01
hs-TnT at 24h	1.07 (0.99-1.17)	ns
<b>At 48h</b>		
Temperature group (36°C)	1.25 (0.87-1.80)	ns
Age	1.07 (1.05-1.09)	<0.001
Sex (male)	0.73 (0.45-1.17)	ns
Bystander CPR (yes)	0.65 (0.43-0.98)	<0.05
Asystole/PEA (yes)	6.80 (3.94-11.71)	<0.001
CA to ROSC (min)	1.03 (1.02-1.04)	<0.001
Shock at admission (yes)	1.39 (0.79-2.44)	ns
pH	0.12 (0.03-0.49)	<0.01
hs-TnT at 48h	1.10 (1.01-1.20)	<0.05
<b>At 72h</b>		
Temperature group (36°C)	1.26 (0.87-1.81)	ns
Age	1.07 (1.05-1.09)	<0.001
Sex (male)	0.72 (0.45-1.17)	ns
Bystander CPR (yes)	0.65 (0.43-0.99)	<0.05
Asystole/PEA (yes)	6.82 (3.95-11.58)	<0.001
CA to ROSC (min)	1.03 (1.02-1.04)	<0.001
Shock at admission (yes)	1.38 (0.78-2.44)	ns
pH	0.13 (0.03-0.50)	<0.01
hs-TnT at 72h	1.11 (1.02-1.21)	<0.05

Targeted temperature management at 33 °C was not associated with hs-TnT compared to 36 °C (figure 12)



**Figure 12.** Temperature allocation did not influence the levels of hs-TnT. The error bars represent the 95% CI.

# Discussion

Survivors that remain unconscious after OHCA and patients in cardiogenic shock have a poor prognosis. Both patient groups are challenging to the clinician and biomarkers that are able to reflect and detect organ dysfunction and guide treatment efforts are needed. In this thesis, we have identified biomarkers that can predict prognosis and detect organ dysfunction among these critically ill patient groups.

## *miRNAs as biomarkers*

Plasma miRNAs have several characteristics that make them suitable as biomarkers. Not only are they stable in the circulation and during multiple freeze-thaw cycles, but they are also simple in their structure making it easy to design probes with high specificity. They are devoid of post-processing modifications and several are regarded as tissue-specific. Moreover, they generally seem to have a rapid release pattern following tissue injury. For example in a rat model, miR-122 can be detected already 1h after acetaminophen induced hepatotoxicity and already at admission after OHCA (paper IV). Indeed, the most promising candidate for future clinical use is probably miR-122 as a marker of acetaminophen hepatotoxicity. This is an intoxication that only give mild or no symptoms during the first 10h after ingestion, the critical time window when treatment with acetylcysteine must be administered to avoid liver failure (32). Hence, biomarkers that increase early after ingestion would be of clinically useful. Among patients, more studies are needed, but data are promising (87).

Among the disadvantages with plasma miRNAs as biomarkers might be high intra individual variation, although more research is needed in this field. However, in two

healthy volunteers, some miRNAs seem to have very stable levels with coefficient of variation (CV) < 10% (1-5% of plasma miRNAs), whereas others (~20% of plasma miRNAs) have unstable plasma levels (CV>100%) (88). High intra individual variation might not pose a problem for using plasma miRNA among critically ill populations, such as cardiogenic shock and OHCA studied in this thesis due to a clearly defined start of disease that enable standardized sample time points. Furthermore, these conditions have a profound effect on vital and metabolic parameters which results in relatively large elevation in miR-122-5p and miR-124 studied in this thesis. However, if plasma miRNAs are to provide clinically meaningful information among broader and more stable patient groups with small differences in miRNA-levels between health and disease, it will probably require knowledge of both intra and inter individual variability. In addition analytical imprecision must be as low as possible and validated controls must be used to monitor the extraction process. Another major issue before plasma miRNAs can enter the clinic is the issue of normalization. Although miRNAs are very stable molecules in serum or plasma, differences in normalization strategies may have a profound influence on the result (89). It is not a controversial view that the best data normalization strategy is one that employs a combination of endogenous and exogenous control miRNAs, but this is rarely the case. Hence, before prospective plasma miRNA-studies are being conducted, effort should be made to characterize the intra and inter individual variability of the target miRNA and to validate endogenous controls.

Currently, RNA-extraction followed by cDNA-synthesis and qRT-PCR is the gold standard for miRNA quantification. Although qRT-PCR has advantages such as high sensitivity, high specificity and being relatively inexpensive, the process is time consuming which might pose a problem when time is a crucial factor. However, due to its robustness and well-established technology, automated PCR-based miRNA-extraction workflows are probably going to be the first technique to become routinely used for plasma miRNA analyses in the clinic. However, new tools for quantification of miRNA in plasma are currently being developed. One of the interesting techniques is single-molecule counters, such as the Nanopore technique, which uses voltage to move miRNAs through nanoscale pores in a membrane (90). The target miRNA binds complimentary to a probe added to the sample and when this miRNA-probe complex passes through the pore, an electrical signature is produced which enables quantification. A major advantage with this technique is that there is no need for amplification of the miRNA-target which reduces the analytical imprecision.

Most studies to date have ignored the fact that miRNA in plasma is compartmentalized into either a vesicle fraction or a non-vesicle fraction. However, information on whether abnormal levels of serum or plasma miRNAs are due to changes in the vesicle- or the non-vesicle associated pool might provide additional diagnostic and prognostic information (91, 92). For example, one could hypothesise that a plasma increase in the non-vesicle associated fraction might be due to a passive release process, whereas an increase in the vesicle fraction might reflect an active release, thereby reflecting different physiological processes. Indeed, as described below for miR-122-5p there are some evidence that differences kinds of injury trigger different miRNA-reactions in the vesicle fraction compared to the non-vesicle fraction (92). Hence, investigators should put effort into separating the vesicle and non-vesicle fractions in future publications.

#### *miR-122-5p as biomarker*

In both a porcine model (paper III) and in patients (paper IV), plasma levels of miR-122-5p were associated with markers of hypoperfusion. In addition, the association between miR-122-5p at admission and bystander CPR in paper IV indicates that some of the release of miR-122-5p might be secondary to traumatic chest and liver compression. This potential traumatic liver injury might also explain the elevated ALT at admission. Since intracellular miRNA is mainly bound to proteins (ie the non-vesicle fraction), one might speculate that mechanical trauma to the liver, such as inadequately deployed chest compressions, might cause passive release of non-vesicle associated miR-122-5p from injured cells. Hence, to further characterize plasma mir-122-5p after OHCA, future studies should focus on separating the vesicle fraction and the non-vesicle fractions before assessing prognostic and diagnostic implications of miR-122-5p.

In most studies to date, miR-122-5p has been found to be a more sensitive and more dynamic marker of liver damage compared to ALT. This has been observed after chemically induced hepatocellular injury in the rat as well as among patients with acetaminophen hepatotoxicity (87, 93). In concordance, the release of miR-122-5p observed after OHCA in paper III and IV is so rapid that it probably starts before tissue necrosis and apoptosis ensues. Indeed, in a rat model of liver ischemia, only 2% of hepatocytes showed signs of apoptosis directly after 60 min of ischemia followed by 60 min of reperfusion (94). Therefore, miR-122-5p release might not be a reflection of liver necrosis or apoptosis but rather of a pre-apoptotic state, or

“tissue stress”, due to acidosis, hypoxia and hypoperfusion in the liver. This kind of cell stress is a documented trigger for increased vesicle production (30, 31). Active release of miRNAs from cells into the blood is well-established and many cell types communicate with other cells by miRNA-release (30, 31). One might therefore speculate that the rapid increase of miR-122-5p in the blood during circulatory collapse is an active secretory process to communicate/orchestrate either a local or a systemic stress response. Indeed, some evidence indicate that increased plasma levels of miR-122-5p initiate the production of complement factor B, a well characterized initiator and modulator of the immune response, in a sepsis animal model (32).

Among patients with STEMI, conflicting results exists regarding miR-122-5p. D'Alessandra et al observed a downregulation of miR-122-5p at admission and at 5 and 30 days compared to healthy controls (95). Others have observed an increase in plasma miR-122-5p already at admission and throughout the first 72 h after STEMI compared to controls (96). These contradictory results are hard to explain. miR-122-5p is 3000 times more abundant in the human liver compared to the heart (97), but one study reported an approximately 10-fold upregulation of miR-122-5p in infarcted human myocardium compared to remote myocardium (98). If miR-122-5p is released after AMI, it remains to be elucidated whether the elevation of miR-122-5p originates from the liver or from the heart. In concordance with paper IV, miR-122-5p at admission among STEMI-patients does not seem to be associated with hemodynamic parameters (96). As discussed above, separating the vesicle and non-vesicle fractions before analysing miR-122-5p might in the future lead to further insights into the pathophysiological role of miR-122-5p.

#### *miR-124-3p as biomarker*

Compared to miR-122-5p that exhibit an extremely rapid release pattern after CA (paper IV), miR-124-3p peaks around 48h after CA (paper II). Moreover, while miR-122-5p was 400-fold elevated at admission compared to controls (paper IV), miR-124-3p was only 10-fold elevated at admission (paper I). Since the brain is extremely sensitive to ischemia, this is somewhat contra intuitive. However, the blood-brain barrier is surrounding the brain and prevents access of most molecules to the central nervous system (99) and in animal experiments, the blood-brain barrier seems to be intact just after CA (100). Although exosomes have been shown to cross the blood-brain barrier, it consists of several layers that might prevent rapid plasma release and this might explain the differences in release pattern between miR-122-5p and miR-124-3p after OHCA (101). Alternatively, the rapid elevations in miR-



122-5p could be due to active release whereas miR-124 is passively released from necrotic neurons. More research is needed to identify the release mechanism of miR-124.

With regard to prognostication after CA, NSE is a relative good prognostic marker with an AUC for a poor neurological outcome of 0.85 at 48 h in a recent TTM-sub study. The use of NSE is recommended in the latest guidelines, but it has some disadvantages. First, even invisible haemolysis may increase NSE levels (102), but this can be tested for spectrophotometrically. Second, there has long been controversies regarding optimal NSE cut-off for predicting a poor prognosis. This might partly be due to different cohorts but it highlights the problem of finding a cut-off that can be applied to the individual patient. Third, there have been disagreements on the influence of therapeutic hypothermia on NSE-levels after CA. However, after the TTM-trial, 36 °C-treatment has mainly become the treatment of choice and a sub study of the TTM-trial could not detect any differences in NSE-levels between the 33°C and 36°C hypothermia groups. Fourth, there is a lack of standardization of the NSE-analysis and available tests show variability of 40% on NSE levels from the same sample (62, 103). Hence, with regard to these shortcomings there might be room for additional prognostic biomarkers after CA.

As a prognostic marker after CA, miR-124-3p has now been clinically validated in two retrospective analyses with clinically relevant endpoints (paper I and II). Moreover, the studies have been performed in two different but comparable patient populations and in two different labs. In the two studies, miR-124 at 48 h has had AUC:s of 0.87 and 0.77, which in paper I was comparable to NSE. However, when added to NSE, miR-124 did not provide any additional prognostic information (paper I and II) and this is consistent with the fact that NSE and miR-124-3p levels both reflect cerebral damage. To be of clinical use, miR-124 must be further evaluated in a prospective study with pre-defined cut-offs. However before a prospective study is conducted, several issues should be assessed. First, miR-124 should be characterized in the vesicle fraction and in the non-vesicle fraction separately, as this might provide additional prognostic information and lead to further insights regard release mechanisms. Second, a prospective study must include a method for miRNA-extraction that is easy to use, have validated controls, is relatively fast and that can be easily incorporated into the clinical setting. Hence, rather than conducting a prospective study using the contemporary technique for miRNA-extraction, new analysing methods, such as the Nanopore technique, should be evaluated.

### *High-sensitivity troponin-T and OHCA*

Hs-TnT as a prognostic marker after OHCA has previously been investigated in a study including 155 OHCA-patients with VT/VF as primary rhythm and was found not to be independently associated with outcome (104). In contrast, paper V found in a larger cohort of OHCA-patients that hs-TnT was independently associated both with all-cause mortality as well as with death due to cardiovascular causes or multi-organ failure. Moreover, our study included a more heterogeneous OHCA cohort as both patients with shockable and non-shockable rhythms were included. Interestingly, targeted temperature management at 33 °C did not reduce hs-TnT compared to 36 °C.

Our results in paper V harmonizes with data from other cohorts, including both healthy controls as well as patients with various illnesses. Although the results are statistically significant and theoretically plausible, the clinical impact on the risk of death of elevated hs-TnT is modest and there exists a considerable overlap between the outcome groups. In the whole cohort, a doubling of hs-TnT at 48 h corresponded to an increase in the odds of death of 10%.

# Limitations

Although we have tried to design the papers to the best of our ability, some issues can be raised concerning the methods and the designs:

- In paper III, no normalization of the miRNA-levels was performed.
- The PCR-reactions should have been performed in triplicates instead of duplicates.
- With the exception of paper IV, we did not use an endogenous control in the miRNA-extraction. There are currently no validated endogenous controls for miRNA-extraction from plasma after OHCA or CA.
- We did not characterize the vesicle fraction and the non-vesicle fraction separately, which might have provided additional information.
- Markers for the detection of hemolysis were not used in the pcr-reaction. However, miRNA-markers of hemolysis were omitted since miR-122-5p and miR-124-3p never have been documented in the erythrocyte (105).
- Although miR-124-3p at 24 h after OHCA had an AUC of 0.87 (95% CI 0.79-0.96) in paper I, this time point was not evaluated in paper II. Since the sensitivity of NSE is too low to be clinically useful at 24 h after OHCA, the 24 h time point is interesting from a miR-124-3p-perspective.
- Since all the papers are either experimental or retrospective, the results can only be regarded as hypothesis-generating.

# Conclusions

- Plasma miR-124-3p is a prognostic marker of neurological outcome marker after OHCA that combine a high specificity while maintaining a clinically relevant sensitivity.
- Therapeutic hypothermia treatment at 33 °C after OHCA does not influence plasma miR-124-3p compared to 36 °C.
- Hypothermia initiated after coronary reperfusion does not influence the plasma levels of the cardiac-specific miR-208b-3p.
- miR-122-5p is acutely elevated after circulatory collapse and might be a marker of liver hypoperfusion and traumatic chest compressions.
- After OHCA, therapeutic hypothermia treatment at 33 °C does not influence plasma miR-122-5p compared to 36 °C. However, 33 °C hypothermia treatment *during* cardiogenic shock in the pig attenuates the increase of miR-122-5p compared to 38 °C.
- Hs-TnT is an independent prognostic marker after OHCA with regard to all-cause mortality as well as death due to cardiovascular causes or multi-organ failure.
- Therapeutic hypothermia treatment at 33 °C after OHCA does not influence hs-TnT compared to 36 °C.

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