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Circulating microRNAs as biomarker for cardiovascular risk prediction within the project of BiomarCaRE
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Evaluation of methodical approaches

Dissertation

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1 Introduction

1.1 Definition and risk prediction of cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and include the following:

- Coronary heart disease such as angina and myocardial infarction - a disease of the blood vessels supplying the heart muscle
- Cerebrovascular disease or stroke - a disease of the blood vessels supplying the brain
- Peripheral arterial disease - a disease of blood vessels supplying the arms and legs
- Rheumatic heart disease - damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria
- Congenital heart disease - birth defects that affect the normal development and functioning of the heart caused by malformations of the heart structure from birth
- Deep vein thrombosis and pulmonary embolism - blood clots in the leg veins, which can dislodge and move to the heart and lungs.

Worldwide CVDs remain the leading cause of death (WHO, 2020). There are many approaches to disease prevention by primary and secondary risk reduction. In clinical routine, risk estimation scores (Table 1) are used to define the target group for intervention by defining a 10-year risk of coronary events. Risk factors as gender, age, cholesterol-levels, smoking, blood pressure, diabetes and body mass index (BMI) are in the focus. Despite all affords, CVD incidents remain high and in a subset of individuals, despite low calculated risk in currently available CVD risk scores, a substantial amount of people develop CVD and suffer adverse cardiovascular events. With the current criteria, scores remain incomplete and are not able to include all patients at risk (Assmann, 2002; Conroy, 2003; D'Agostino et al., 2008; Goff et al., 2014; Khot, 2003a; SCORE2 working group and ESC Cardiovascular risk collaboration et al., 2021). To improve risk estimation in the population and to enhance the specificity of the scores, new approaches are necessary.

Table 1 Risk prediction scores for coronary heart disease and variables used for risk calculation to define a 10-years risk. Scores applicable for patients with no prior history of coronary heart disease. FRS, Framingham Risk Score (D'Agostino et al., 2008); PROCAM, Prospective Cardiovascular Münster Study (Assmann, 2002); ACC/AHA, American College of Cardiology / American Heart Association (Goff et al., 2014); SCORE, Systematic COronary Risk Evaluation (Conroy, 2003); SCORE2, Systematic COronary Risk Evaluation 2 (SCORE2 working group and ESC Cardiovascular risk collaboration et al., 2021).

FRS	PROCAM	ACC/AHA	SCORE	SCORE2
-----	-----	Caucasians vs. non-Caucasians	Race	Four risk regions in Europe
Gender	Gender	Gender	Gender	Gender
Age	Age	Age	Age	Age
Total cholesterol, HDL	LDL, HDL, triglycerides	Total cholesterol, HDL	Total cholesterol	Total cholesterol, HDL
Smoking	Smoking	Smoking	Smoking	Smoking
Systolic blood pressure	Systolic blood pressure	Systolic blood pressure	Systolic blood pressure	Systolic blood pressure
-----	Diabetes mellitus	Diabetes mellitus	-----	-----
Treatment for hypertension	Family history of premature MI	Blood pressure treatment	-----	-----

1.2 Biomarkers in risk stratification

Biomarkers have become an integral part of clinical practice in diagnosis and prognosis of disease, response to therapy but also in risk prediction and decision-making. Novel biomarkers are of great interest and harbor the potential to play an important role in prediction and discrimination of cardiovascular risk (Blankenberg et al., 2010; Schulte and Zeller, 2015). Risk assessment in cardiovascular disease is important because many decisions in prevention and therapy depend on these evaluations. According to clinical guidelines drug treatment can be started for high blood pressure or LDL-reduction based on the level of CVD risk (Visseren et al., 2021). At the same time, as Ford et al. describe, it is unclear why some individuals with high cholesterol levels do not and other individuals with low cholesterol levels do develop atherosclerosis and therefore have a considerably high risk of CVD (Fernández-Friera et al., 2017).

Despite the availability of scores, prediction is incomplete and a considerable number of patients at risk go unidentified based on traditional risk factors alone.

Khot et al. have reported that 62 % of patients with MI present with none or only one risk factor and less than 10 % show three or four risk factors (Khot, 2003b). Overall, decision-making in health care and justification of starting drug-therapy, long term consultation and supervision as well as medical interventions can be easier and more precise with new measurable risk factors.

Criteria to establish new prognostic biomarkers in cardiovascular diseases include the following:

1. Biomarkers should allow early identification of individuals at risk for adverse clinical outcome.
2. Results should be relatively easy to measure and provide accuracy and generalizability.
3. Costs should be acceptable in relation to the benefit.
4. Reference limits must be clearly defined.
5. Biomarker should provide new information beyond status quo (Ahmad et al., 2014).

In terms of biomarkers conventional risk score for cardiovascular risk prediction commonly include levels of cholesterol, LDL, HDL and triglycerides. Nevertheless, an increasing number of biomarkers to predict risk have been identified and the number of pathways known contributing to cardiovascular risk is expanding (Ferrucci and Fabbri, 2018; Gibb and Hill, 2018). For example, three widely studied cardiac biomarkers, N-terminal pro-brain natriuretic peptide (NT-proBNP) associated with left ventricular wall stress, C-reactive protein (CRP), a marker of inflammation and cardiac troponin I (cTnI), associated with myocardial damage, have shown correlations. The Framingham population study demonstrated the association of NT-proBNP level and risk of CVDs (Wang et al., 2004). Within the BiomarCaRE project NT-proBNP, C-reactive protein, and cTnI were shown to be persistently associated with cardiovascular events and not one single biomarker alone but the panel of these three markers significantly improved risk assessment (Blankenberg et al., 2010). Moreover, in a large epidemiological study, involving 40 cohorts of general population with over 95000 subjects, the ability of NT-proBNP plasma level to significantly improve prediction of heart failure, stroke and ischemic heart disease when added to C-reactive protein and HDL-cholesterol level could be shown (Willeit et al., 2016). Up to now biomarkers for CVD risk are based on a wide range of individuals summarized in

cohort studies, not allowing the available data to be broken down to a specific individual's characteristics.

1.3 MiRNAs as biomarker

Micro ribonucleic acids (miRNAs) comply with many important criteria as biomarkers. They are surprisingly stable in circulation through association with lipids, proteins, or microparticles and their expression is tissue-/disease-specific. Opposed to their cellular origin, miRNAs are well-known as extracellular messengers and detectable in various body fluids, including peripheral blood, plasma, serum, blood cells, urine and bile. Although miRNAs originate from specific cells or tissue types, their release into extracellular compartments, and especially into the blood stream has presented the possibility to relatively easy detect them. They are detectable using sequence-specific amplification methods and overall it can be suggest that microRNAs can serve as reliable biomarkers for specific disease (Condrat et al., 2020; Karakas et al., 2016; Mitchell et al., 2008b). In contrast to the evaluation of single biomarkers, miRNA sets are in the focus to improve models of CVD risk prediction. It makes miRNAs ideal candidates to detect subtle differences in biological functions and early stages of pathological processes to improve the sensitivity of detecting subtle differences between high- and low-risk individuals (Schulte and Zeller, 2020).

1.4 MiRNAs biogenesis and function

MiRNAs are short non-coding strands of approximately 19-25 nucleotides (Ranganathan and Sivasankar, 2014). They are encoded in the genome of the nucleus and first are transcribed into primary miRNAs (pri-miRNAs). A nuclear RNase III Dicer, Dosha, transforms primary miRNAs into miRNA precursors (pre-miRNAs). Pre-miRNAs are exported by the enzyme Exportin 5 to the cytosol where the RNase III Dicer processes them to miRNAs. Mature miRNAs, bound to ribonucleoproteins containing Argonate (Ago), are essential parts of the RNA-induced silencing complex (RISC).

The function of RISC is to regulate mRNA at a posttranscriptional level. It can turn off (gen-knockout) or turn down (gen-knockdown) the production of specific proteins by degrading the coding messenger (mRNA) or inhibiting the translation. MiRNAs function as guides that lead RISC to its targets. They are complementary to the mRNAs and bind through Watson-Crick base pairing to the 3' untranslated

region (3'UTR) of the mRNA. The binding activates the Ago of the RISC, which then dictates the mechanism of gene regulation of either degrading mRNA or inhibiting translation (Bartel, 2004; McManus and Sharp, 2002; Pratt and MacRae, 2009).

Besides their role in gene expression, transcription, metabolism and stability within the cell, miRNAs are also secreted from cells and play a role in cell-to-cell communication by delivering gene silencing signals to recipient cells (Iguchi et al., 2010).

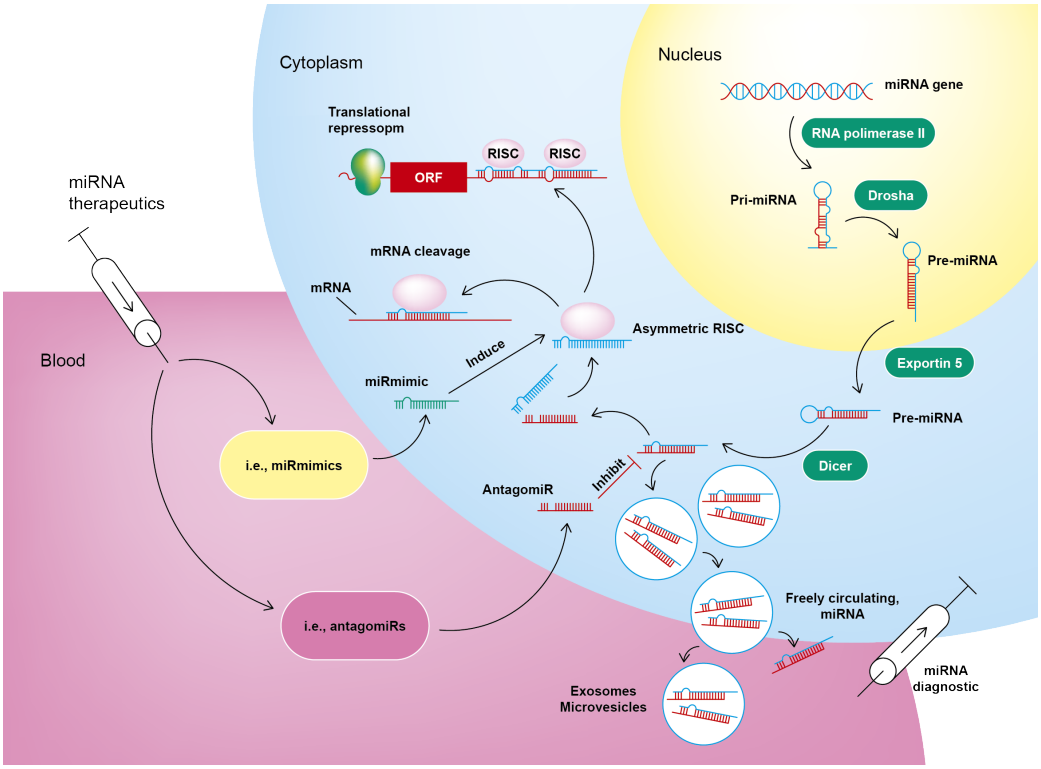

Figure 1 Biogenesis and function of miRNA. In the nucleus pri-miRNA is generated by RNA polymerase II and transformed by Dosha to pre-miRNA. GTP-dependent Exportin 5 then transports pre-miRNA to the cytoplasm. Processed by Dicer a short double stranded miRNA is generated, from which one single-stand is incorporated into RISC. Depending on complementarity of the miRNA, the RISC is guided to its target mRNA. After binding to mRNA translational repression or cleavage is induced (Schulte et al., 2015b).

1.5 MiRNAs in cardiovascular diseases

In cardiovascular diseases the role of miRNAs in the regulation of a variety of pathophysiological processes is important. It is known that miRNAs influence the development and progression of CVDs. In these cases, the expression pattern of miRNAs and therefore the regulatory influence are altered (Small and Olson, 2011). Apart from an altered miRNA expression in case of illness within the cell there is strong evidence that miRNA can serve as stable, circulating biomarker

for various cardiovascular diseases in acute events and in risk prediction (Nair et al., 2014; Silva et al., 2017). Compared to other types of RNA, miRNAs are remarkably stable in circulation even though they are not intrinsically resistant to plasma RNase (Mitchell et al., 2008b). Bound to lipoprotein complexes or transported in membrane-derived vesicles miRNAs are protected from degradation (Schulte et al., 2020).

1.5.1 MiRNAs in acute myocardial infarction

MiRNAs are expressed in a tissue and cell-type specific or enriched way. In the myocardium much examined are miR-1, miR133a, miR-208a/b and miR-499 as abundantly expressed and key player in cardiogenesis, heart function and pathology. Muscle-enriched miR-1 and miR-133a predominantly support commitment of cardiac-specific muscle lineage from embryonic stem cells and mesodermal precursors in the early stages of cardiogenesis. They are involved in cardiac conductance and regulation of cardiac action potential. Cardiac-enriched miR-208 and miR-499 are involved in the late cardiogenic stages mediating differentiation of cardioblasts to cardiomyocytes and fast/slow muscle fiber specification. They are located in introns of the heavy chain myosin genes and regulate expression of sarcomeric contractile proteins (Chistiakov et al., 2016; Xiao et al., 2019).

All four show potential as biomarkers in the diagnostics of acute myocardial infarction but findings vary in improvement of diagnostic power compared to cTnI and cTnT. Cardiac-enriched miRNA are elevated in case of myocardial injury only while muscle enriched miRNA can rise in different cardiac pathologies. Wang et al. found that miR-208a showed 90.9% sensitivity and 100% specificity for AMI diagnosis, representing a more advantageous biomarker than even cTnI (Wang et al., 2010). Schulte et al. showed that the muscle-enriched miRNAs are more readily detected at baseline while the cadiac-enriched ones reach detectable levels only but correlate best with hs-cTnT and predict myocardial injury better in one cohort and as good as hs-cTnT in another cohort. Nevertheless they point out that miRNA detection in general is inferior to high-sensitivity protein assays (Schulte et al., 2019).

1.5.2 MiRNAs in heart failure

For heart failure (HF) and its leading processes there is a wide range of miRNAs analysed and found to be altered (Schulte, 2015). Matcovich et al found 444 miRNAs to be altered in failing hearts tissue (Matkovich et al., 2009). Tijsen et al. found elevated levels of miR-423-5p, miR-18b, miR-129-5p, miR-622, miR-654–3p and miR-1254 in the plasma of patients with HF (Tijsen et al., 2012). MiR-29a was found to correlate with left-ventricular hypertrophy and fibrosis. By analysing patients with hypertrophic cardiomyopathy with and without HF symptoms it was found that miR-29a is upregulated in patients without HF symptoms leading to the assumption that miR-29a has a protective effect for remodelling processes (Roncarati et al., 2014; Vegter et al., 2016). In terms of implementation to clinics, there is a first-in-human phase 1b study in patients with HF receiving an antisense therapy targeting miRNA-132 indicating efficiency of this drug (Täubel et al., 2021).

1.5.3 MiRNAs in atrial fibrillation

Correlation between incidences of atrial fibrillation (AF) and specific miRNAs is also object of research. AF is a supraventricular tachyarrhythmia, the most common type of arrhythmia in clinical practice, where abnormal electrical impulses are promoted by altered electrophysiological abnormalities in atrial tissue. In many studies patients with AF show divergent levels of miRNAs compared to controls. Elevated levels miR-9, -19, -20a-5p -133a, -146, -152, -374a, -454, -634, -664, -483-5p and -4798 and lower levels of miR-19a, -146a, -150, -375, -29b, - 21, -1, -145, -162, -222, -328, -432, -493b are described (Dawson et al., 2013; Liu et al., 2012; Lu et al., 2015; Menezes Junior et al., 2023; Nishi et al., 2013). Within the studies there is little consistency of results.

1.5.4 MiRNAs in cardiovascular risk prediction

Valuable data of circulating miRNAs for prognostic purposes are underrepresented. So far there are only six prospective population/community-based cohort studies examining the potential of miRNAs in cardiovascular risk prediction, one, the JACC study, with a combined endpoint of premature death due to cancer and CVD and the SAFEHEART study examining the risk of familial hypercholesterolemia patients suffering a cardiovascular event (Table 2).

Between them there is no comparability neither in their selection of miRNAs nor in their set endpoints or findings.

Table 2 Studies investigating role of miRNAs in cardiovascular risk prediction. Bruneck study; Framingham Offspring study; HUNT (Trøndelag Health) study; DETECT (Diabetes and Cardiovascular Risk Evaluation: Targets and Essential Data for Commitment of Treatment) study; Stockholm study; China-Cardiovascular Disease study; JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) study; SAFEHEART (Spanish Familial Hypercholesterolemia Longitudinal Cohort) study.

Study	Size	Follow-up time and endpoints	Findings	Validation
Bruneck (Zampetaki et al., 2012)	820 patients	10 years follow-up, myocardial infarction	Analysed 19 candidate miRNAs, found 3 miRNAs consistently and significantly related to incident of myocardial infarction: MiR-126 showed a positive association whereas miR-223 and miR-197 were inversely associated with the risk of MI.	
Framingham Offspring (McManus et al., 2014)	2445 patients	5.4 years median follow-up, analysis was separated into pre-existing AF and no prior AF, AF	Analyzed 385 miRNAs, several miRNAs including miR-328, -150-5p, -331-3p, -28-5p were negatively associated with prevalent AF but only miR-328 was statistically significant after adjustment for age, sex, and technical covariates and weakened after adjusting to clinical risk factors. For new-onset AF there was no significant association to miRNAs.	
HUNT (Bye et al., 2016)	112 patients	10 years follow-up, fatal AMI	Found that adding a panel of 5 miRNAs (miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p) enhances AMI risk prediction.	
DETECT (Keller et al., 2017)	178 patients	5 years follow-up, overall mortality and cardiovascular events	Analysed miRNA panel of 5 circulating miRNAs (mi- R-34a, miR-223, miR-378, miR-499 and miR-133). The panel of the five miRNAs was able to improve risk stratification with respect to mortality beyond the Framingham or the SCORE risk score.	SHIP study as validation cohort with 129 patients and a follow-up of 12 years
Stockholm Study (Gigante et al., 2020, 2012)	100 patients	11-year follow-up, major adverse cardiovascular events (MACE), defined as MI, angina, or sudden cardiac death	Screened a panel of 754 miRNAs, identified nine miRNAs potentially associated, miR-145-3p with the largest increase of estimated risk, miR-720 with reduced MACE risk. Further Identified 16 interacting miRNA pairs associated with an increased probability of an event, miR-320b was present in all interacting miRNA pairs. Performing target prediction three of these clusters (cluster 1: miR-320b plus miR-145-3p, miR-128a, miR-548d-3p; cluster 2: miR-320b plus let-7g-5p, let-7d-5p, let-7e-5p, miR-196b-5p, miR-191-5p, miR-324-3p; and cluster 4: miR-320b plus miR-301b,miR-340- 3p, miR-376a) were linked with cardiovascular development and function, and CVDs.	58 patients with incident MI and 60 sex-matched controls from the HUNT study. Although not significant, they observed a similar pattern of association with the risk of MI, and a trend in a progressive increase in MI risk estimates for miRNAs from clusters 2 and 4.

China-Cardiovascular Disease Study (X. Wang et al., 2020)	2812 patients	6.0 years median follow-up, more than 50% diameter stenosis in at least 1 of the 3 major coronary arteries in angiography	Out of the 48 miRNA candidates, miR-423-3p was able predict CAD events.	
JACC (Yamada et al., 2021)	1224 patients	20 years follow-up, premature death due to cancer or CVD	Analyzed 3 miRNAs (miR-21, -29a, -126), high circulating miR-21 and miR-29a were associated with higher risk of total death, cancer death, and CVD death, low circulating miR-126 levels were associated with significantly higher risk of total death.	
SAFEHEART (Escate et al., 2021)	72 patients	8 years follow-up, familial hypercholesterolemia patients with cardiovascular event, sudden death, fatal and non-fatal MI, unstable angina and cerebrovascular accident	Analyzed 10 miRNAs in patients with genetic diagnosis of familial hypercholesterolemia, miR-133a in circulation anticipate those familial hypercholesterolemia patients that are going to present a clinical CVE within the next 2 years (average).	285 patients, 8 years follow-up

1.6 Methodical approaches for miRNA measurement

MiRNAs are surprisingly stable in circulation through association with lipids, proteins, or microparticles protecting them from RNase degradation (Cui et al., 2019; Mitchell et al., 2008b). Their resistance to degradative factors such as long-term storage, physical disturbance and freeze-thaw cycles provide a good base for the potential as biomarkers (Glinge et al., 2017). To accurately interpret and compare study results and to identify miRNAs acting as novel specific and sensitive biomarkers, standardized and consistent methods need to be applied at many levels, from whole blood collection to plasma/serum preparation, handling and banking to miRNA extraction and quantification. Table 3 shows the diversity of miRNA quantification technologies.

Table 3 MiRNA quantification technology (Krepelkova et al., 2019; Moody et al., 2017)**.**

Technology	Advantages	Limitations
qPCR	• Current gold standard for sensitivity and specificity	• No genome-wide coverage
Microarray	• Commercially available reagents • Genome-wide coverage	• Specific probes • Specialized equipment • Lack of reproducibility between

		platforms • Difficult data normalization
NGS	• Genome-wide coverage • Multiple samples may be run in parallel • Promotes novel miRNA discovery • Can detect polymorphisms	• Complicated, non-standardized data analysis
Immunoassay miREIA	• No need for RT or amplification • Quantification of generated DNA/RNA hybrids	• No genome-wide coverage
SplintR-qPCR	• RT is replaced by enzymatic ligation of two DNA oligonucleotides splinted by target miRNA • qPCR as the visualization system	• No genome-wide coverage
Isothermal amplification	• No need for thermocycling equipment • Can improve existing qPCR, microarray, and NGS methods	• Disadvantages are technique-specific (see below)
• Exponential amplification	• High sensitivity	• May require a nicking enzyme, which complicates primer design
• Rolling circle amplification	• 1 primer • Can be optimized for linear or exponential amplification	• Requires 2 enzymes (polymerase and ligase) • Initial denaturation not performed at room temperature
• Duplex-specific nuclease signal amplification	• High specificity	• Enzyme is not readily available
• Hybridization chain reaction	• No polymerase	• Linear amplification only
Near-infrared technology	• No autofluoresence • Minimal photobleaching • No tedious treatment of sample before or after the test	• Lanthanide probes are not yet commercially available and must be optimized

Reverse transcription of miRNA to complementary DNA (cDNA) template followed by quantitative PCR (RT-qPCR) is the main and most common approach for measuring a selection of only a few miRNAs. The reverse transcription is conducted by reverse transcriptase synthesis of single stranded target miRNA to double stranded cDNA. Different techniques for priming the target miRNA are used. One common method is the use of stem-loop structures specific to the miRNA sequence (Chen, 2005). The primer consists of a short single stranded sequence that anneals to the complementary sequence at the 3′end of the target miRNA. The primer also includes a double stranded part (the stem) and a loop, resulting in a small stem-loop structure (Figure 2, top left). Another widely used method is based on poly(A) polymerase to lengthen the mature miRNA by adding an adenosine tail, poly(A) tail, to the 3´end. The poly(A) tail makes it possible for oligo-dT primers to reverse transcribe the miRNA into cDNA (Figure 2, bottom left). Unlike the stem-loop primer, this reaction is universal to all miRNAs in the sample.

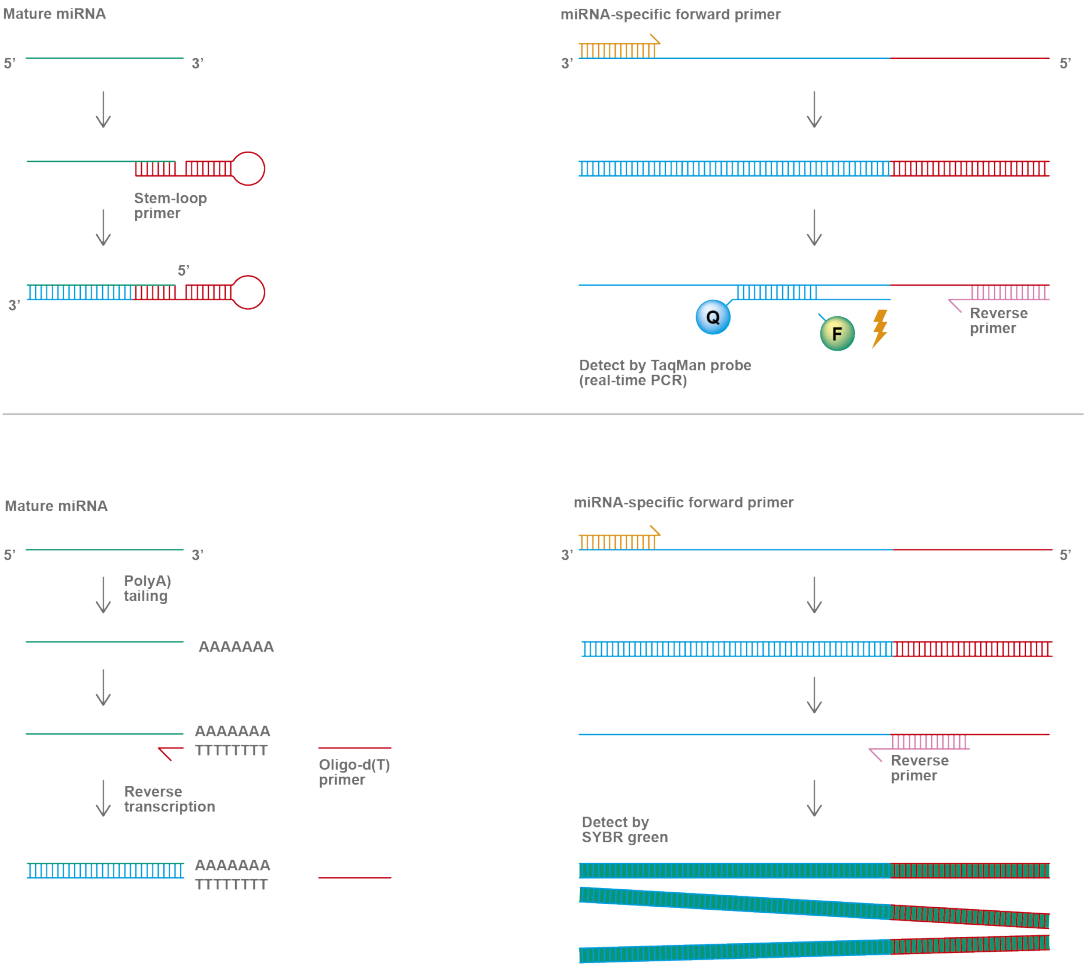

Figure 2 The most common approaches for RT-qPCR for miRNA detection and quantitation. For the RT-reaction, stem–loop primers that are specific to the 3′ end of the miRNA (top left) or polyadenylation by poly(A) polymerase and reverse transcription by oligo-dT primers (bottom left), are the most used methods. For the qPCR-reaction miRNA specific primer are mostly used, and detection can be performed by TaqMan probes (top right) or SYBR® green fluorescent dye (bottom right) (Pritchard et al., 2012).

The qPCR method is based on the knowledge of the process where the cDNA template is amplified to multiple copies. The amplification is generally repeated for 40 cycles and is composed of different phases (Figure 3). When the amplification reaches the exponential phase, the template will increase exponentially as the reagents in the reaction are unlimited. The exponential increase of template is only reached by optimal reaction efficiency of 100 %. Then, when the linear phase is reached, the template will increase linear as the reagents have become limited. The plateau is reached when the reagents are consumed, in this phase the template amount will not increase.

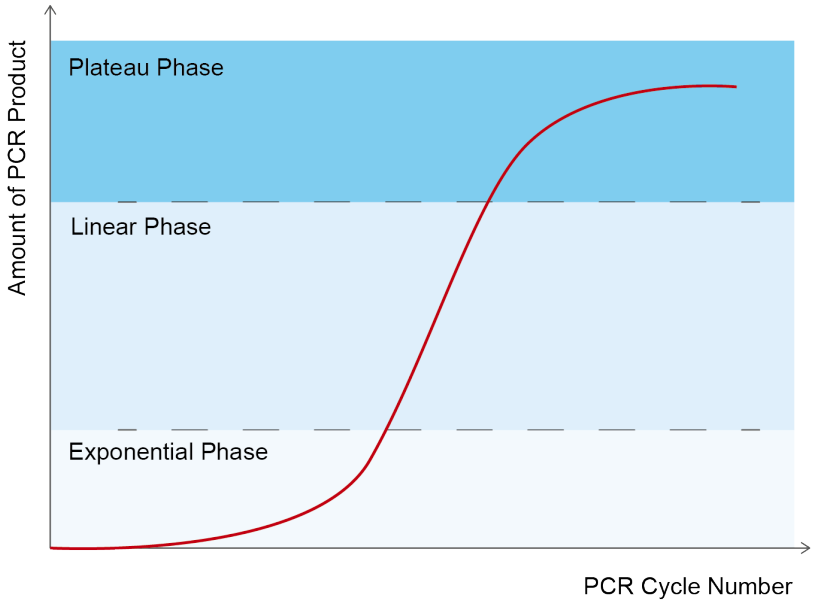

Figure 3 The different phases of the qPCR amplification.

In the phase where the template increases exponentially, a threshold cycle is set based on which level the template reaches a defined threshold. The threshold can be determined by two methods. The baseline threshold method considers all amplification curves in the reaction setup collectively to determine the threshold. The cycle where a selected curve hits the baseline threshold is called quantification cycle (Cq) or threshold cycle (Ct). The relative threshold method determines a threshold for each amplification curve individually. This second method uses the reaction efficiency to estimate the threshold based on the shape of each separate amplification curve and uses this to determine the relative threshold.

Specific primers, complementary to the target cDNA sequences, are used for the amplification, but the quantitative detection can be performed by several different methods. There are especially two common methods for detecting the amounts of template. One method is based on non-specific fluorescent dye that intercalate with all double-stranded DNA. The fluorescent dye SYBR® green is the most popular (Figure 2, bottom right). The other method is based on sequence-specific probes. The probes consist of oligonucleotides that are labelled with a fluorescent reporter that hybridizes to a complementary sequence of the target. The most common used are the TaqMan probes (Figure 2, top right). The TaqMan probes consist of a reporter dye at the 5´end and a quencher at the 3´end. The quencher suppresses the fluorescence of the reporter dye when the probe is intact, but when annealed to 13 target the encounter with the DNA polymerase results in

cleavage of the probe. The cleavage separates the reporter and the quencher, and the fluorescence of the reporter is emitted. The sequence-specific probes have greater specificity than the non-specific fluorescent dye, as they hybridize to target-specific sequences.

As shown in table 4, not only the choice of detection platform but in every step from sample choice to miRNA measurement and data analysis, there is a heterogeneity in methods available and standardised protocols are needed to obtain reproducible, comparable miRNA profiles. Up to now there is little consensus in procedures even though there are already several approaches implementing results of research into clinical practise.

Table 4 Factors influencing results when measuring miRNAs by qPCR.

Sample material	Plasma, serum, blood cells, urine, bile, tissue
Sample processing	Choice of anticoagulant, centrifugation, storage time and temperature
Extraction methods	Phenol-based, phenol/chloroform, phenol-free
Normalization	Exogenous and endogenous controls vs. study-specific using algorithms as geNORM, NormFinder or BestKeeper vs. cycle threshold average of all measured miRNA
Patient related factors	Cancers, inflammatory and autoimmune diseases, medication, as heparin or anti-platelet therapy, physical activity, diet, fasting or non-fasting, age

Hypothesis and aims

Circulating miRNAs are potential disease-specific biomarkers for risk prediction of CVD. Methods of miRNAs handling and measurement vary enormously in terms of collection of material, RNA isolation and detection methods, normalisation and harmonisation of results. To implement miRNAs as new biomarkers for risk prediction of CVD it is inevitable to evaluate methodical approaches and develop a standardised protocol.

Therefore, the aims of this doctoral thesis were:

1. Evaluation of detectability of circulating miRNAs
2. Evaluation of new PCR-based detection methods
3. Improvement of the protocol and elaboration of current possibilities of miRNA measurement for application in large population based cohorts as in the project of BiomarCaRE.

2 Materials and methods

Materials

2.1 Devices

7900HT Fast Real-Time PCR System	Applied Biosystems, Darmstadt
Heraeus Fresco 21 Centrifuge	Thermo Fisher Scientific, Schwerte
Pipettes Eppendorf Research	Eppendorf, Hamburg
Thermocycler GeneAmp PCR System 9700	Applied Biosystems, Darmstadt
Vortex Genie 2	Scientific Industries, Darmstadt
Centrifuge 5810	Eppendorf, Hamburg

2.2 Chemicals and reagents

Chloroform	AppliChem, Darmstadt
Ethanol	AppliChem, Darmstadt
Isopropanol	Sigma-Aldrich, Taufkirchen
PBS (Phosphate-Buffered Saline)	Gibco, Darmstadt
RNase-free water	Gibco, Darmstadt
TRIzol LS Reagent	Thermo Fisher Scientific, Schwerte

2.3 Consumables

Adhesive qPCR seal	Sarstedt, Nümbrecht
Falcon Tube 15 ml	Corning Inc., Kaiserslautern
PCR plate (96-well)	Bio-Rad, München
PCR plate, Multiply® (348-well)	Sarstedt, Nümbrecht
Pipette filter tips, Biosphere®	Sarstedt, Nümbrecht
Tube 0,5/1,5/2 ml	Eppendorf, Hamburg

2.4 Kits, assays and miRNAs

TaqMan™ Advanced miRNA cDNA Synthesis Kit	Applied Biosystems™, Darmstadt
TaqMan™ miRNA cDNA Synthesis Kit	Applied Biosystems, Darmstadt
miRCURY® SYBR® Green PCR Kit	Qiagen, Hilden

Syn-Cel-miR-39-3p miRNScript miRNA mimic	Qiagen, Hilden
TaqMan™ Advanced miRNA Assay	Applied Biosystems, Darmstadt
TaqMan™ MicroRNA-Assay	Applied Biosystems, Darmstadt
TaqMan™ Fast Advanced Master Mix	Applied Biosystems, Darmstadt
miRCURY® LNA® miRNA PCR Assay	Qiagen, Hilden
miRCURY® SYBR® Green Master Mix	Qiagen, Hilden

Methods

2.5 BiomarCaRE

The Biomarker for Cardiovascular Risk Assessment in Europe (BiomarCaRE) consortium assesses the value of established and emerging biomarkers for cardiovascular risk prediction in a European wide research project and aims to establish innovative technology in detection methods. The project is a multi-modular designed study subdivided in three main modules. Module one's goal is to select biomarkers, established as well as novel ones according to their association of CVD risk, based on pre-existing non-publically available datasets and develop assays and protocols. In module two biomarkers are measured and statistically analysed. Module three assesses clinical translation and economical effectiveness.

The collaborative BiomarCaRE consortium of over 30 academic institutions and five small/medium-sized, research intensive enterprises incorporate 21 well-established European population-based cohort studies, most of which were previously harmonised in the MORGAM project (Evans, 2004), four cohorts of disease cohorts for secondary prevention and four clinical trials comprising over 300,000 participants with a follow up in the range from 2.5 to 25 years for fatal and non-fatal acute coronary events, stroke and death (Zeller et al., 2014). Population-based and disease cohorts are analysed separately in the project.

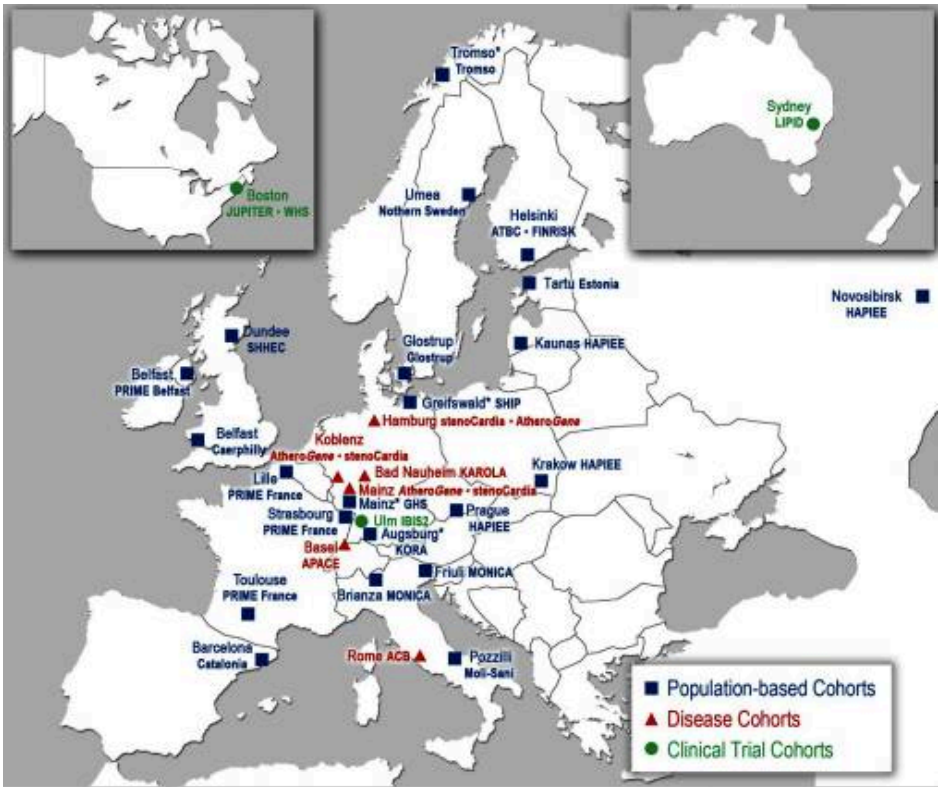

Figure 4 BiomarCaRE collaborations. A general survey of collaborating countries and cohorts (Zeller et al., 2014).

For the purposes of primary prevention and prediction of first occurrence of cardiovascular events, measurements of established biomarkers are performed in population based cohorts (n = 300,000). The cohorts comprise 171,000 men and 129,000 women. The mean age at baseline is 51.2 (SD 12.7) years. To assess novel, molecular biomarkers such as metabolites and RNA molecules the BiomarCaRE case cohort set has been designed. In a case cohort study, cases are defined as those participants of the cohort who developed the disease of interest, but controls are identified before the cases develop. This means that controls are randomly chosen from all cohort participants regardless of whether they have the disease of interest or not, and that baseline data can be collected early in the study (Kirch, 2008). The full case cohort set of BiomarCaRE consists of 11,360 individuals from the following population based cohorts: FINRISK97 (Finland), Monitoring of Trends and Determinants in Cardiovascular Diseases/Cooperative Health Research in the Region of Augsburg (MONICA/KORA; Germany), MONICA-Brianza and Moli-sani (Italy), DanMONICA (Denmark), and the Scottish Heart Health Extended Cohort (United Kingdom). The cohorts followed-up the participants for a range of 2.5 to 25 years for acute coronary events, stroke, heart failure, atrial fibrillation and type 2 diabetes. The harmonised baseline variables include systolic and diastolic blood

pressure, blood lipids, body-mass-index, and questions on smoking, disease history, and medication for hypertension, dyslipidaemia and diabetes.

Acute coronary events are harmonized to categories: definite or possible myocardial infarction or coronary death, unstable angina pectoris, cardiac revascularization, or unclassifiable death, where the data are insufficient for the other coronary diagnoses and there is no evidence of other causes. The categories can be combined to define different endpoints for analysis. Stroke has been characterized as ischaemic stroke, intracerebral haemorrhage, and subarachnoid haemorrhage, although reliable diagnostic information for the subtyping is not always available, in particular for the early years of follow-up. Follow-up for heart failure, atrial fibrillation, type 2 diabetes were usually based on linkage with national hospitalization registers and other administrative registers (Zeller et al., 2014).

MiRNA measurements within the BiomarCaRE case cohort set

For miRNA measurements within the BiomarCaRE project, serum samples from four case cohorts are available. Table 5 shows the number of individuals according to center, the availability of samples for miRNA information as not all had enough sample volume, the time of collection and the number of incident events. The reduced sample number can be explained by the fact that already various measurements of established and new promising biomarkers have been done by the BiomarCaRE consortium. For this thesis the Brianza cohort was selected to evaluate methodical approaches of miRNA measurement in large cohorts for subsequent application in the overall BiomarCaRE case cohort set.

Table 5 Selected cohorts for miRNA measurement.

Center	Country	Cohort size (available)	Years of examination	Mean follow-up time (years)	Incident events
Selected for evaluation of methodical approaches					
Brianza	Italy	4932 (694)	1986-1994	21	355
Included for the BiomarCaRE project					
DanMONICA	Denmark	7582 (4364)	1982-1992	23	2897
Moli-sani	Italy	24325 (2325)	2005-2010	4	1525
SHHEC	Scotland	16000 (3977)	1984-1995	21	5001

The final selection of miRNAs in the BiomarCaRE project had already been done by the consortium. The selection was literature-based in terms of miRNAs as well as a suggested association of miRNAs and clinical endpoints complemented by pilot miRNAs detection in small subsets within BiomarCaRE. Fourteen miRNAs were selected: miR-423, -21, -150, -133a, -3135, -210, -16, -223, -451, -718, -378, -499, -34, -133b as shown in table 6.

Table 6 Selected miRNAs in the BiomarCaRE project in different cardiovascular pathologies and risk assessment. ACS, acute coronary syndrome; AF, atrial fibrillation; AMI, acute myocardial infarction; CAD, coronary artery disease; HF, heart failure; NT-proBNP, n terminal prohormone of brain natriuretic peptide; UAP, unstable angina pectoris.

MiRNA	Cardiovascular end point association / Cardiovascular risk prediction studies
miR-423	Up-regulated in HF (Visseren et al., 2021), improvement of diagnostic accuracy of NT-proBNP in HF (Topkara and Mann, 2011)
miR-21	Up-regulated in ACS (Wang et al., 2010)
miR-150	Down-regulated in UAP (D'Alessandra et al., 2013), up-regulated in future cardiovascular death (Bye et al., 2016), down-regulated in AF (Adam et al., 2012), negatively associated with risk of AF (McManus et al., 2014)
miR-133a	Up-regulated in AMI (Kuwabara et al., 2011), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017)
miR-3135	Up-regulated severe hypertension (Shi et al., 2021)
miR-210	Up-regulated in future cardiovascular death (Bye et al., 2016)
miR-16	Down-regulated in atherosclerosis and CAD (M. Wang et al., 2020)
miR-223	Cardiovascular death in patient cohort with CAD (Schulte et al., 2015a), up-regulated in AMI (Luo and Zhang, 2016), up-regulated in future cardiovascular death (Kiechl et al., 2002), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017), inversely associated with the risk of MI (Zampetaki et al., 2012)
miR-451	Down-regulated in hypertrophic cardiomyopathy (Song et al., 2014), up-regulated during myocardial infarction and cardiac hypertrophy (Taverner et al., 2021)
miR-718	Down-regulated in aortic dilation and involved in the regulation of vascular remodelling (Martínez-Micaelo et al., 2017)
miR-378	Down-regulated in left ventricle hypertrophy (Chen et al., 2014), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017)
miR-499	Up-regulated in ACS and AMI (Wang et al., 2010) (Luo and Zhang, 2016), up-regulated in infective carditis (Zile et al., 2011), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017)
miR-34	Up-regulated in the aging heart (Boon et al., 2013), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017)

miR-133b	Down-regulated in ACS (Kumar et al., 2020), improve risk stratification with respect to overall mortality and cardiovascular events (Keller et al., 2017)

Cel-miR-39 as exogenous spike-in control was additionally introduced during the RNA extraction process. It serves as exogenous control being a reference for the efficiency of the transcription reaction and the technical variability of the following experimental process and was measured additionally.

RT-qPCR technologies for quantitative miRNA detection

Three different assays were used for miRNAs measurement for troubleshooting and miRNAs analysis (Table 6).

Table 7 Comparison of different RT-qPCR technologies for quantitative miRNA detection.

	Provider	Input total RNA	Reverse transcription	qPCR primers	Detection
TaqMan™ Advanced miRNA	Thermo Fisher Scientific	1 ng - 10 ng	Polyadenylation by poly(A) polymerase at the 3´end, ligase and universal RT primers, miR-Amp reaction	miRNA specific primers	TaqMan™ MGB probes
TaqMan™ MicroRNA	Thermo Fisher Scientific	1 ng - 10 ng	miRNA specific stem-loop primer transcription	miRNA specific primers	TaqMan™ MGB probes
miRCURY® LNA® miRNA®	Qiagen	10 pg - 200 ng	Polyadenylation by poly(A) polymerase at the 3´end universal RT primers	LNA optimized miRNA specific primers	SYBR® Green I fluorescent dye

2.6 Extraction of miRNAs

For extraction of miRNA, 300µl of TRIzol was added to 100µl of serum. Smaller amounts of serum were replenished to 100µl by PBS buffer. After mixing, the sample was incubated 10 min at room temperature to inhibit RNase activity during sample homogenization. As synthetic miRNA, cel-miR-39 (syn-Cel-miR-39-3p, miRNScript miRNA mimic cat no MSY0000010 QIAGEN) was added as spike-in control after 10 min incubation with TRIzol (2µl 100nM cel-miR-39 diluted in water (1:200)). 80µl Chloroform was added to the solution including synthetic miRNA and the samples were mixed thoroughly. After mixing, the sample was centrifuged 15 min at 14,000 rpm at 4°C. The upper aqueous phase was transferred into a new reaction tube and RNA was precipitated by adding 0.66

volume Isopropanol to the aqueous layer. Samples in Isopropanol were stored at -80°C until further processing of sample (RT-qPCR).
Before RT-qPCR, samples were centrifuged for 15 min at 13,000 rpm at 4°C and RNA was washed by adding 70% EtOH and centrifugated for 5 min at 13,000 x g at 4°C to remove impurities, dried and then resuspended in 30µl H_2O for use in RT-qPCR.

2.7 TaqMan™ Advanced miRNA

2.7.1 Reverse Transcription

Before performing qPCR miRNAs had to be reversed transcribed to cDNA. TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied biosystems by Thermo Fisher Scientific) was used first. Total miRNA was modified by extending the 3' end of the mature transcript through poly(A) addition. For each reaction 2 µl of total miRNA was mixed with the poly(A) reaction cocktail containing 0.5 µl of 10x Poly(A) Buffer, 0.5 µl of ATP, 0.3 µl of Poly(A) Enzyme and 1.7 µl of RNase-free water. Poly(A) tailing reaction was performed in a thermal cycler by polyadenylation at 37°C for 45 min and stop reaction at 65°C for 10 min. Immediately after, ligation reaction was performed. For each reaction 10 µl of ligation reaction cocktail containing 3 µl of 5x DNA Ligase Buffer, 4.5 µl of 50% PEG 8000, 0.6 µl of 25x Ligation Adaptor, 1.5 µl of RNA Ligase and 0.4 µl of RNase-free water was used. In the thermo cycler adaptor ligation was performed by lengthening the 5' end at 16°C for 60 min. Consecutively the reverse transcription reaction followed. 15 µl of RT reaction cocktail containing 6 µl of 5x RT Buffer, 1.2 µl of dNTP mix (25mM each), 1.5 µl of 20x Universal RT primer, 3 µl of 10x RT Enzyme Mix and 3.3 µl of RNase-free water were added to each well and incubated in the thermal cycler for 15 min at 42°C for reverse transcription and 5 min at 85°C to stop the reaction.

2.7.2 MiRNA-Amplification

For miRNA-Amplification a new reaction plate with 45 µl of miRNA-Amp reaction mix in each well, containing 25 µl of 2x miR-Amp Master Mix, 2.5 µl of 20x miR-Amp Primer Mix and 17.5 µl of RNase-free water was prepared. After adding 5 µl of the RT reaction product each, incubation in the thermal cycler was performed with the following settings using MAX ramp speed: Enzyme activation at 95°C for 5 min for one cycle, denaturation at 95°C for 3 sec and anneal/extend at 60°C for

30 sec alternately for 14 cycles and stop reaction at 99°C for 10 min for one cycle. The cDNA template was then ready for quantification by qPCR.

2.7.3 qPCR

For measurement of miRNA, TaqMan™ Advanced miRNA Assay was used.

On a 384-well plate, selected miRNAs were measured. Cel-miR-39 as exogenous control was measured on a separate plate. Using two different TaqMan™ reporter with distinct fluorescent spectra (FAM™ and VIC®) should enable the amplification and quantification of two targets in a single reaction well.

Table 8 Reporter dyes FAM™ and VIC®. Both can be excited at a single wavelength (488 nm) but emit at distinct different wavelength. *E*: extinction coefficient; Φ = quantum yield; τ = fluorescence lifetime.

Reporter	λmax/nm (absorption)	λmax/nm (emission)	E at λmax	Φ	τ/ns
FAM™	494	518	68000	-	-
VIC®	538	554	-	-	-

To each specific primer on its 5'end either FAM™ or VIC® was linked. If one or both target miRNAs were present, the specific reporter linked respectively. In the phase of amplification, the reporter was spitted off by 5'-3' exonuclease activity of Taq-DNA-Polymerase and the quencher is liberalized leading to the emission of a measurable fluorescence-signal (Figure 5). The two different fluorescent dyes were detected independently on qPCR with excitation sources and emission filters in the respective wavelength.

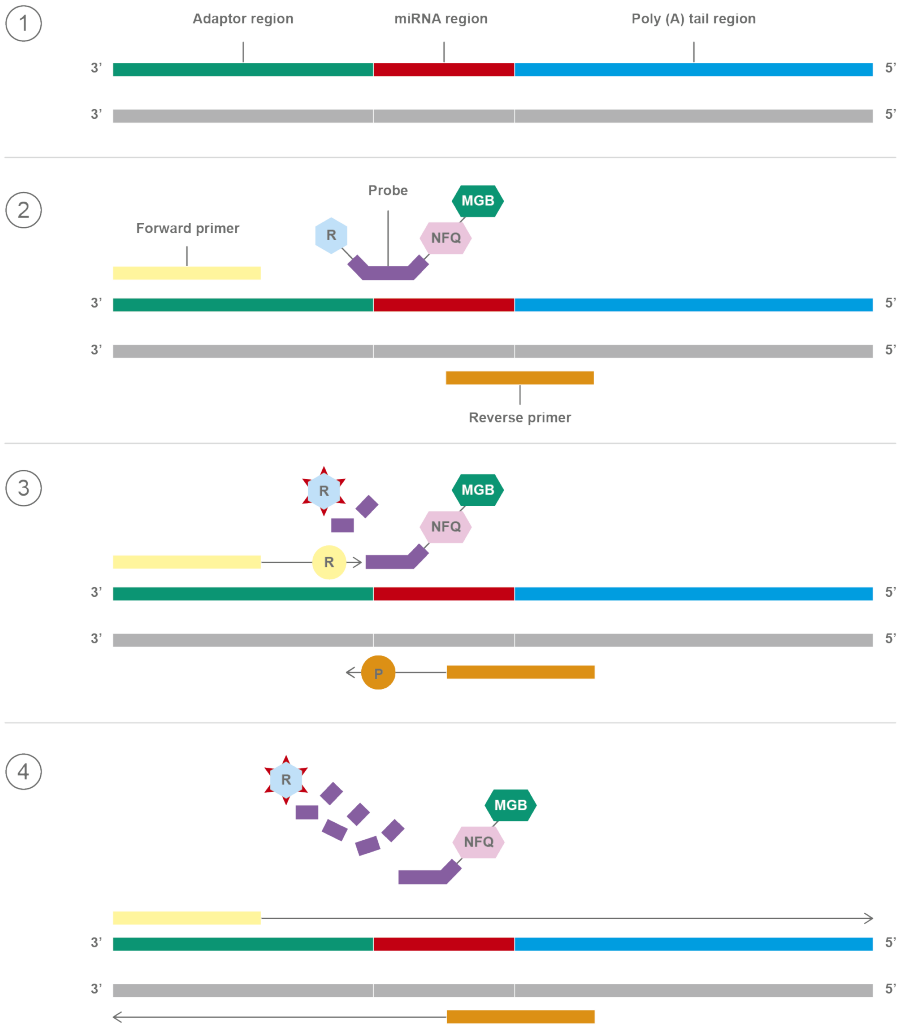

Figure 5 qPCR detection by using TaqMan™ probes.

In case of duplex reactions and measurement, it was necessary to test reciprocal effects (inhibition) of the reporter by measuring the cT-values separately and combined. The cT-values had to be the same when combined two miRNA-measurements in one well as measuring each miRNA in a separate reaction. After testing clear signalling, it was planned to measure miRNA-423 and -21, -150 and -133a, -3135 and -210, -16 and -223, -451 and -718 as well as -378 and -499 together in a duplex reaction. MiRNA-34 and -133 should be measured separately because in the combined testing they showed inhibition in qPCR (Table 9).

Table 9 MiRNAs and reporter dyes. MiRNAs in the first column were marked with FAM™, in the second column with VIC® reporter dye. MiRNAs in the same row could be measured in one duplex reaction. The last two, miR-34 and -133b should be measured separately.

FAM™	VIC®
miR-423	miR-21
miR-150	miR-133a
miR-3135	miR-210
miR-16	miR-223
miR-451	miR-718
miR-378	miR-499
miR-34	-
-	miR-133b

To prepare the qPCR reaction, a 1:10 dilution of cDNA template was made. To the pre-coated plates of Thermo Fisher with 5 µl of the diluted cDNA template was added. For cel-miR-39 4.5 µl of cDNA template was added to 10 µl of reaction mix containing 5 µl of 2x TaqMan™ Fast Advanced Master Mix and 0.5 µl of TaqMan™ MicroRNA Assay (20x). Using the 7900 HT Fast Real-Time PCR System thermal cycling starts with 50°C for 2 min for one cycle, enzyme activation at 95°C for 20 sec starts and alternately for 40 cycles denaturation at 95°C for 3 sec and anneal/extend at 60°C for 30 sec follows.

2.8 Qiagen miRCURY® LNA® miRNA ®

The kit was used to measure cel-miR-39 (spike-in), miR-103a-3p, miR-16-5p and miR-425-5p. For reverse transcription it uses polyadenylation by poly(A) polymerase at the 3´end universal RT primers. In qPCR miRNA specific primers are locked nucleic acid (LNA) enhanced. LNA oligonucleotides give more thermal stability when hybridized to a complementary DNA or RNA strand. For each incorporated LNA monomer, the melting temperature (Tm) of the duplex increases by 2–8°C. In addition, LNA oligonucleotides can be made shorter than traditional DNA or RNA oligonucleotides and still retain a high Tm. This is important when the oligonucleotide is used to detect small or highly similar targets, as miRNAs.

2.8.1 Reverse Transcription

For each reaction 6.5 µl of total miRNA was mixed with the Mastermix containing 2 µl of 5x miRCURY® Reaction Buffer and 1 µl of 10x miRCURY® RT Enzyme mix.

RT-reaction was performed in a thermal cycler for 60 min at 42°C, followed by 5 min at 95°C to inactivate the Reverse Transcriptase Enzyme and immediately cooling to 4°C to stop reaction.

2.8.2 qPCR

For measurement of miRNA, miRCURY® SYBR® green qPCR Assay (Qiagen) was used. On a 384-well plate, 4 different miRNAs (cel-miR-39 (spike-in), miR-103a-3p, miR-16-5p, miR-425-5p) were measured. To prepare the qPCR reaction, a 1:30 dilution of cDNA template was made. To 3.5 µl of reaction Mastermix containing 2.5 µl of miRCURY® SYBR® Green Mastermix, 0.025 µl of ROX reference dye, 0.5 µl of PCR primer mix and 0.475 µl of RNase-free water, 1.5 µl of the diluted cDNA template was added.

Using the 7900 HT Fast Real-Time PCR System thermal-cycling starts with enzyme activation at 95°C for 2 min and alternately for 40 cycles denaturation at 95°C for 10 sec and anneal/extend at 56°C for 60 sec follows. The fluorescent dye, SYBR® green I, is used to detect the miRNAs. SYBR® green I emits a fluorescent signal when intercalating between the bases of all double-stranded nucleic acids (Figure 6).

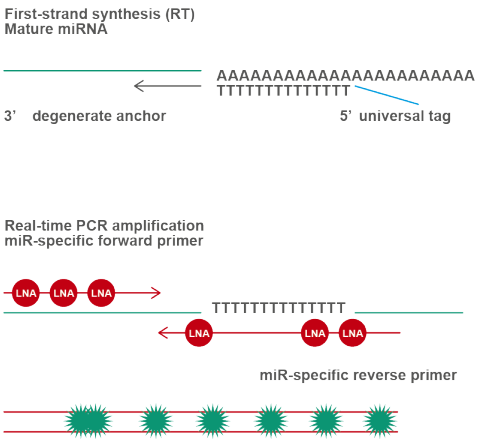

Figure 6 RT-qPCR method using miRCURY® technology.

2.9 TaqMan™ MicroRNA

The kit was used to measure cel-miR-39 (spike-in), miR-103a-3p, miR-16-5p and miR-425-5p. For reverse transcription it uses miRNA specific stem-loop primer as shown in figure 7. For qPCR miRNA specific primers are used.

2.9.1 Reverse Transcription

For reverse transcription reaction 5 µl of total miRNA was mixed 3 µl of RT primer and 7 µl of Reaction mix containing 1.05 µl of 10x RT buffer, 0.105 µl of dNTPs with dTTP (100mM), 0.133 µl of RNase Inhibitor, 2.912 µl of RNase-free water, 2.1 µl of RT Primer and 0.7 µl of Multiscribe RT. RT-reaction was performed in a thermal cycler for 30 min at 16°C and 30 min at 42°C, followed by 5 min at 85°C to inactivate the Reverse Transcriptase Enzyme and immediately cooling to 4°C to stop the reaction.

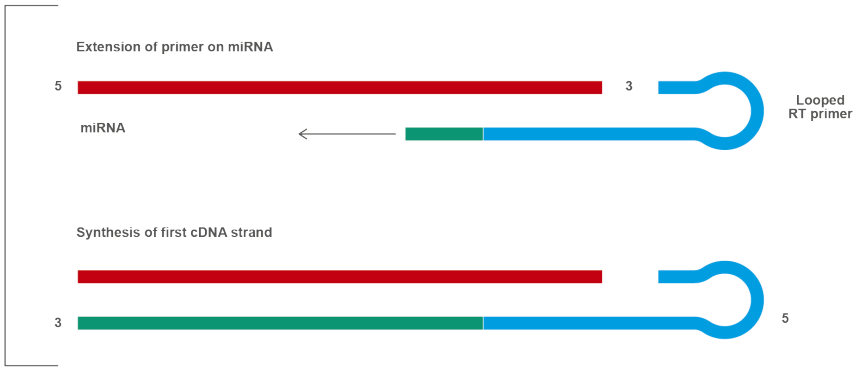

Figure 7 TaqMan™ MicroRNA RT based on miRNA specific, stem-loop RT primers.

2.9.2 qPCR

For qPCR 4.5 µl of cDNA template was added to 10 µl of reaction mix containing 5 µl of 2x TaqMan™ Master Mix and 0.5 µl of TaqMan™ MicroRNA Assay (20x). Using the 7900 HT Fast Real-Time PCR System thermal cycling starts with 50°C for 2 min for one cycle, enzyme activation at 95°C for 20 sec starts and alternately for 40 cycles denaturation at 95°C for 3 sec and anneal/extend at 60°C for 30 sec follows.

2.10 qPCR date analysis

The statistical analyses were performed using the Thermo Fisher Scientific Analysis Software. All raw real-time PCR data were imported into the Thermo Fisher Scientific Analysis Software. cT values over 40 were defined as undetermined values.

3 Results

3.1 Evaluation of detectability of circulating miRNAs

Three miRNAs where included in order to test detectability with TaqMan™ Advanced miRNA Assay. First, the spike-in cel-miR-39 was selected as artificially added normalization control. Further, two endogenous miRNAs miR-150 and miR-133a were selected as they could be measured in one well on the pre-coated plates of Thermo Fisher (VIC and FAM coated). In case of the duplex reactions and measurement, reciprocal effects (inhibition) of the reporter were tested beforehand by measuring the cT-values separately and combined, as described in 2.8.3. The cT-values had to be the same when combined two miRNA-measurements in one well as measuring each miRNA in a separate reaction. The Brianza cohort, as the smallest of the four population-based cohorts in BiomarCaRE was selected to perform the first measurements. As shown in table 10, detectability of miR-150 and miR-133a was poor. Furthermore, cel-miR-39 could not be detected by qPCR at all. MiR-150 and miR-133a showed 77.3% and 66.7% undetermined values, respectively.

Table 10 Cel-miR-39, miR-150 and miR-133a in the Brianza cohort using TaqMan™ Advanced miRNA.

MiRNA	Reporter dye	N total	N undetermined	% undetermined
Cel-miR-39	FAM	444	444	100.0
miR-150	FAM	444	343	77.3
miR-133a	VIC	444	296	66.7

Figure 8 compares the qPCR amplification curves of the three measured miRNAs to an ideal amplification curve with a true exponential phase underlining the non-detectability of the three measured miRNAs. Views of the qPCR amplification curves show for cel-miR-39 only a baseline pattern with unspecific amplification by the end of 40 cycles. This is typical for cases in which no amplification takes place. For miR-150 (FAM coded) and miR-133a (VIC coded) the curves show high baseline noise and a late amplification curve after 30 cycles with a rising baseline plus amplification. This could be caused by interactions in duplex reactions but also by a poor cDNA synthesis and consequently limited amount of amplification template.

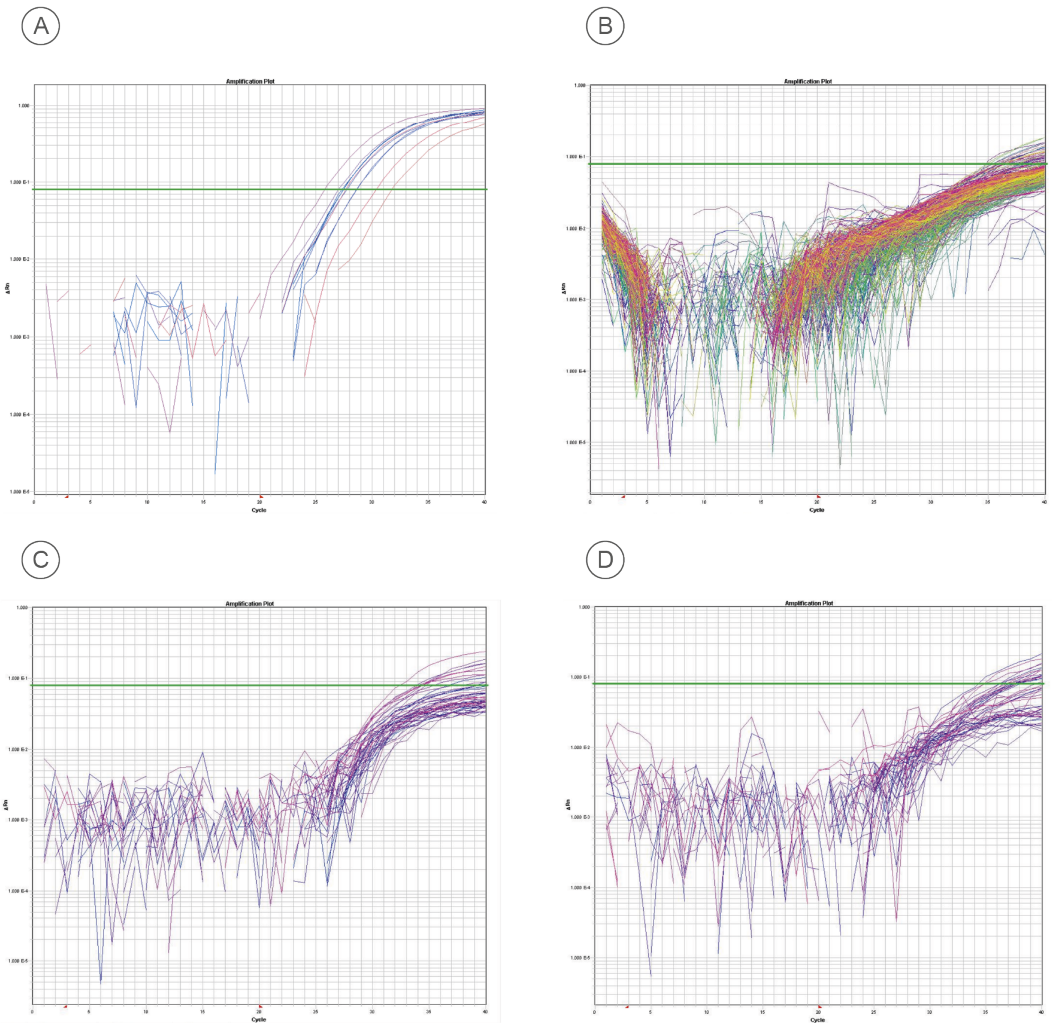

Figure 8 qPCR amplification curves. A comparison between an ideal amplification curve with a true exponential phase (A) and the amplification curves of cel-miR-39 (B) with high baseline noise with no amplification curve but unspecific rise by the end of 40 cycles, and miR-150 (C) and miR-133a (D) with high baseline noise and late amplification curve after 30 cycles with little samples reaching the threshold after 35 to 40 cycles measured with TaqMan™ Advanced miRNA from Brianza samples.

3.2 Evaluation of new PCR-based detection methods

Based on the non-detectability of the three tested miRNAs with TaqMan™ Advanced miRNA assays, a troubleshooting was performed.

Several reasons could cause this phenomenon:

1) Material does not contain any transcripts
2) Transcripts not amplified in cDNA synthesis
3) Amplified transcripts not detectable in qPCR

The first focus of the remeasurements was cel-miR-39 because the non-detectability was especially remarkable. Cel-miR-39 is not an endogenous

miRNA but instead was spiked-in during the RNA extraction procedure and should provide good detectability independently of the age of the sample or its handling (i.e. freeze-thaw cycles).

Table 11 Troubleshooting for measurement of the original spike-in cel-miR-39. Step 1: qPCR was repeated with new assay (TaqMan™ microRNA). Result: No detection of cel-miR-39. Step 2: reverse transcription (rt) of RNA into cDNA was performed anew before repeating qPCR with new assays (TaqMan™ microRNA and the miRCURY® LNA® miRNA). Result: Detectable cel-miR-39.

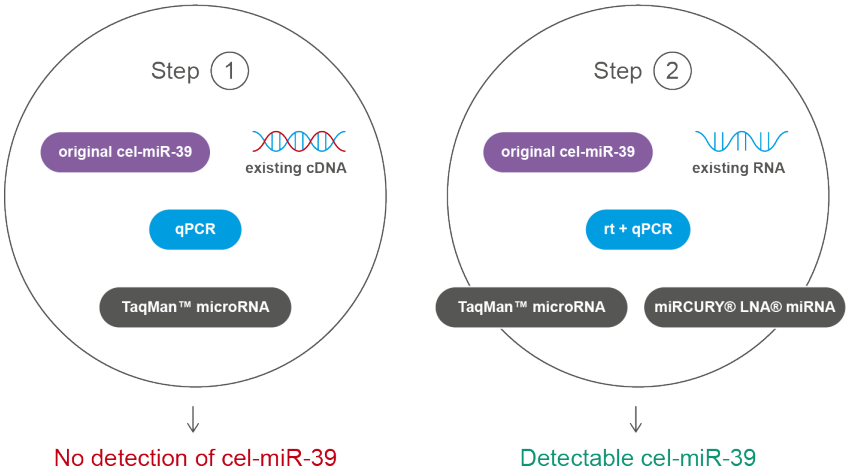

Table 11 shows the troubleshooting in two steps. First, only the qPCR was repeated with a new assay, TaqMan™ microRNA and existing cDNA but again, in all samples cel-miR-39 was not detectable. Second, reverse transcription and qPCR were performed anew from the existing RNA with two new assays. With the TaqMan™ microRNA and the miRCURY® LNA® miRNA assay, cel-miR-39 was finally detectable (Figure 9). As the main change was related to the RNA transcription to cDNA, it could be concluded that the reverse transcription step was the cause of miRNAs non-detectability.

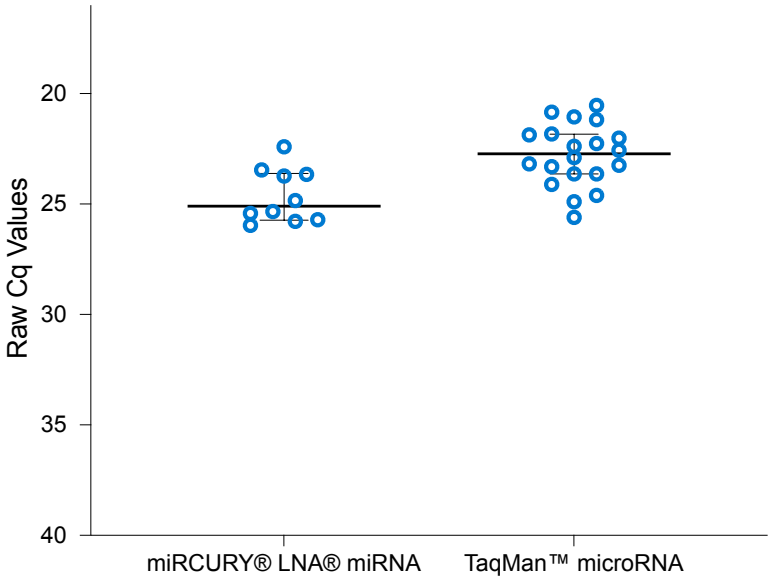

Figure 9 Cel-miR-39 remeasurement with TaqMan™ microRNA and miRCURY® LNA® miRNA. Original RNA and spike-in, new rt and qPCR.

Inquiries about the assays used returned that the TaqMan™ Advanced miRNA kit needs transcripts to be 5'-phosphorylated in the RT-step in order to be amplified and detectable (Figure 10). This was not the case for the cel-miR-39 spike-in used in BiomarCaRE.

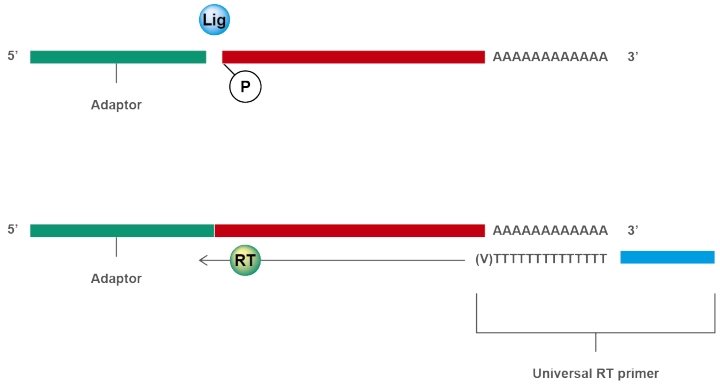

Figure 10 MiRNA with poly(A) tail undergoes adaptor ligation at the phosphorylated 5' end in the RT-step. The adaptor acts as the forward-primer binding site for the miR-Amp reaction.

In order to continue the project, three options were evaluated:

1) Using the existing RNA (containing the non-5`-phosphorylated cel-miR-39 spike-in) to make new cDNA and RT-qPCR with a different quantification system/kit
2) Using the existing cDNA with a different quantification system/kit (RT-qPCR only)
3) Extracting novel RNA from the original BiomarCaRE samples

Option 2 is not feasible, since the RT-step in the original reaction did not amplify cel-miR-39. Option 3 had to be excluded given the financial and logistic effort it would have taken to repeat the RNA extraction step. Therefore, option 1 was followed up on. In order to use the existing RNA with non-5`-physphorylated cel-miR-39, different amplification kits were necessary to be tested. Thermo Fisher TaqMan™ MicroRNA Assay as well as Qiagen miRCURY® LNA miRNA PCR Assay were chosen as two of the most widely used and most sensitive kits available (Jensen et al., 2011; Redshaw et al., 2013; Schulte et al., 2019).

When re-evaluating the protocol concerning cel-miR-39, another problem emerged. The spiked-in concentration of 2µl of 100nM cel-miR-39 diluted in water was approximately 1000-fold higher than recommended. Such high spike-in concentration can possibly be a reason for relatively low detectability of endogenous miRNAs as discussed further below. In order to test the cel-miR-39 concentration independent of degradation effects in older samples, fresh serum was used and new RNA extraction was performed using a) cel-miR-39 spike-in in the original concentration (old) and b) new spike-in in the currently recommended concentration of 1-10nM (new). Again, the two detection systems (TaqMan™ MicroRNA and miRCURY® LNA miRNA PCR) were used to assess inter-platform differences. Figure 11 shows very high cel-miR-39 raw Cq values with the original concentration for both platforms, while the new spike-in returns the expected Cq values around 15-20 cycles. In both cases the TaqMan™ MicroRNA Assay showed considerably better results.

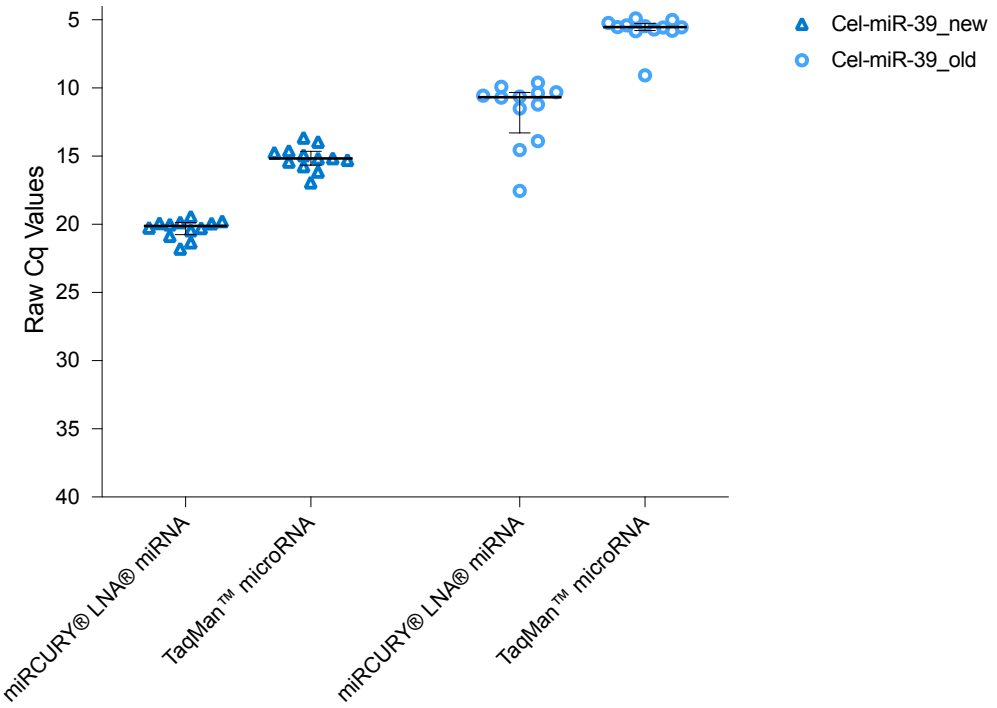

Figure 11 Comparison of TaqMan™ microRNA and miRCURY® LNA® miRNA. New serum, new RNA extraction, new vs. old concentration of Cel-miR-39.

In order to test if a very high concentration of cel-miR-39 interferes with the detectability of endogenous miRNAs, the following measurement was performed. Three endogenous miRNAs (miR-103a-3p, -16-5p, -425-5p) randomly selected were measured with the currently recommended cel-miR-39 spike-in concentration (new) and with the original concentration used (old) (Figure 12). No effect of high cel-miR-39 spike-in concentration on endogenous miRNAs could be detected, refuting the potential effect of high cel-miR-39 levels on detectability of endogenous miRNAs. Comparing the results of the two kits, the TaqMan™ MicroRNA Assay again showed considerably better results.

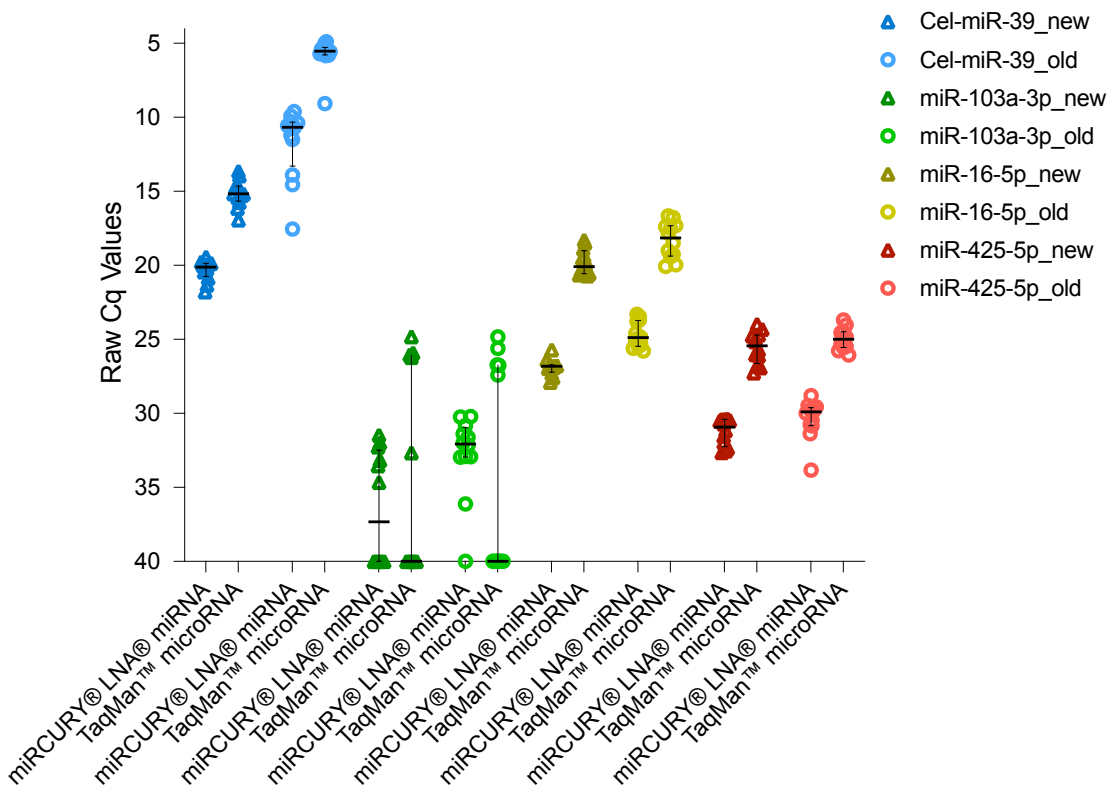

Figure 12 Comparison of TaqMan™ microRNA and miRCURY® LNA® miRNA. New serum, new RNA extraction, new vs. old concentration of cel-miR-39.

For elaboration of current possibilities of miRNA measurement for application in large population based cohorts as in the project of BiomarCaRE, cel-miR-39 was measured in all four cohorts with the Thermo Fisher TaqMan™ MicroRNA Assay. All samples showed a high variability of raw Cq values as it should not be the case for a spike-in of the same concentration in each sample. Possible interpretations of these results could be RNA degradation or suboptimal RNA extraction. Comparing different sub-cohorts, Brianza and Dan Monica showed a smaller variability than Moli Sani and SHHEC, arguing for differences in sample quality (Figure 13).

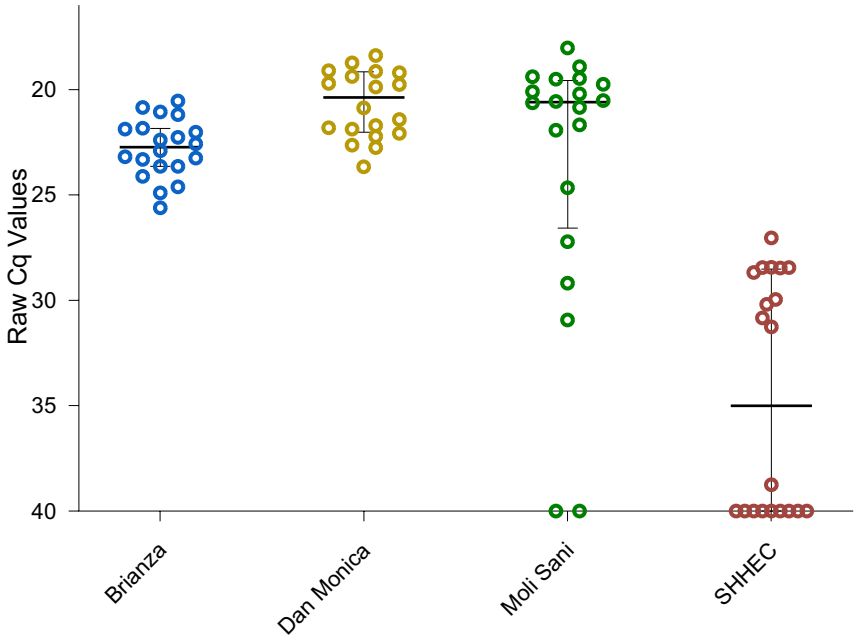

Figure 13 Cel-miR-39 remeasurement with TaqMan™ microRNA in all four cohorts. Original RNA and spike-in, new rt and qPCR.

4 Discussion

This thesis aimed to evaluate three PCR-based approaches to detect circulating miRNAs. In detail, this thesis starts with a test run in one population based cohort and the measurement of three out of fourteen miRNAs with the TaqMan™ Advanced miRNA Assay. Facing problems in detectability a review of the protocol had to be done. Using two new assays, the miRCURY® LNA® miRNA and TaqMan™ microRNA Assay simultaneously, allowed detectability but also a comparison of the two assays. This thesis showed that, for the measurement of selected miRNAs, the TaqMan™ MicroRNA Assay provides considerably better results and could be recommended for miRNA measurement in large population based cohorts as in the project of BiomarCaRE.

The necessity of a standardized protocol for miRNA detection

Circulating miRNAs are promising biomarkers for risk prediction of CVD. In order to make full use of these biomarkers, it is elementary to evaluate the detection methods and establish a sophisticated and standardised protocol. So far, in different publications there are major differences in methods of miRNA testing and analysis, in sample collection, isolation process, measuring, normalisation and harmonisation techniques (Felekkis and Papaneophytou, 2020; Lee et al., 2017; Wang et al., 2012a). Up to now there is no standardised workflow.

4.1 Factors influencing detectability and results of circulating miRNAs

Three miRNAs where included in order to test detectability with TaqMan™ Advanced miRNA Assay. The spike-in cel-miR-39 and two endogenous miRNAs miR-150 and miR-133a were selected. The two endogenous miRNAs were tried to be measured in one well on the pre-coated plates of Thermo Fisher (VIC and FAM coated). As all three miRNAs were undetectable, a review of single steps in the protocol was necessary to ensure a high quality of measurements in future applications.

Sample choice

For our measurements serum samples were used. Various studies have shown that sample choice affects the amount of measured miRNAs significantly. Comparisons between different plasma and serum protocols and their amount of circulating miRNA show contrary results. McDonald et al. reported that plasma

presents higher level of specific miRNAs than does serum (McDonald et al., 2011). In contrast, Mitchell et al. and Wang et al. described higher concentrations in serum and hypothesized that the higher miRNA concentration observed may be due to miRNA release from blood cells such as platelets during the coagulation process (Mitchell et al., 2008a; Wang et al., 2012b).

Moreover, the effect of centrifugation of the sample is highly related to haemolysis effecting the sample. Prolonged centrifugation at a high speed may cause haemolysis and therefore release of miRNAs from platelets while low speed and brief centrifugations may lead to poor separation of serum or plasma from cellular components (Lesche et al., 2016). In several studies it is demonstrated that cellular components as well as haemolysis can have a substantial impact on the miRNA content in plasma/serum. Especially miRNAs abundant in red blood cells vary on the degree of haemolysis. Concerning miR-16, frequently used as endogenous control in several studies and highly expressed in red blood cells, hemolysis contributes to a significant increase in concentration (Kirschner et al., 2013; McDonald et al., 2011; Vasilatou et al., 2010).

Isolation of RNA

A similar range of variability applies to RNA isolation. In literature, isolation of miRNAs is often described as challenging because of their low concentrations and contaminants from blood may affect the results. Further, different transport mechanisms in plasma or serum as within protein complexes or extracellular vesicles might possess different levels of resistance and vulnerability to particular isolation methods (Felekkis and Papaneophytou, 2020; Mitchell et al., 2008b; Moldovan et al., 2014).

In general, RNA extraction methods are divided into three major categories:

1. the phenol-based techniques that employ organic solvents, phase separation, while RNA is recovered by precipitation,
2. the column-based methods that initially use phenol/chloroform to isolate RNA from other biomolecules and subsequently a column for RNA adsorption and
3. the phenol-free methods that use a lysis buffer to release RNA in the solution and a column for RNA recovery (Felekkis and Papaneophytou, 2020; Moldovan et al., 2014).

To facilitate the process of RNA isolation there are several commercial kits available and several studies have investigated the effects of isolation methods and miRNA recovery. Up to now, there is no conclusion on which method is the best, there is a consensus that the different isolation methods provide different quantities of miRNA (Kroh et al., 2010; Li et al., 2015; Moldovan et al., 2014; Sourvinou et al., 2013; Tan et al., 2015).

Exogenous control

The most obvious problem appeared in the measurement of the exogenous control cel-miR-39 as it was added during the extraction process, thus definitely present in the sample, but not detectable in qPCR. After a test series with TaqMan™ microRNA and miRCURY® LNA® miRNA, it could be shown that the reverse transcription step in TaqMan™ Advanced miRNA was the problem. Further investigation revealed that the TaqMan™ Advanced miRNA kit could not detect non-5'phosphorilated miRNAs but new assays can. With the TaqMan™ MicroRNA Assay, the cel-miR-39 showed relatively best results for an exogenous normalization control with Cq values around 15-20 cycles. When measuring cel-miR-39 in all four cohorts with the TaqMan™ MicroRNA Assay, all cohorts showed a high variability of raw Cq values as it should not be the case for a spike-in of the same concentration in each sample. As the same concentration in each sample is spiked-in while performing the miRNA isolation, the variation of Cq values indicates suboptimal RNA extraction or RNA degradation. Comparing different sub-cohorts, Brianza and Dan Monica showed a smaller variability than Moli Sani and SHHEC, arguing for differences in sample quality (Figure 12). In this case precipitation was used for RNA extraction. If the extraction procedure is not optimal, also RNase activity might still be present and sensitivity especially of naked miRNA like exogenous cel-miR-39 is high. This might be one explanation for the variability in the measured concentrations of cel-miR-39. Interestingly and important for further measurements, endogenous miRNAs exist in a surprisingly stable form that is more resistant to RNase activity (Mitchell et al., 2008a).

In general, the necessity of an exogenous spike-in control is widely discussed as well as the choice of exogenous control if desired. Overall, these spike-ins are introduced during the RNA extraction process, usually before reverse transcription of RNA, they can only provide a reference for the efficiency of the

transcription reaction and the technical variability of the following experimental process. Spike-in controls neither correct for variability in sample collection nor in quality of the sample (McDonald et al., 2011). For implementation of a new protocol and new miRNAs as biomarkers, an exogenous control seems to be necessary and has shown to be very useful not least for quality control. It ensures that in large scale measurements where processes are repeated at different times and possibly by different people, results always remain reproducible and comparable.

The question of too high concentrations of cel-miR-39 as spike-in as a reason for relatively low detectability of endogenous miRNAs could be neglected. The concentration of spike-in was approximately 1000-fold higher than recommended in the first measurement with the TaqMan™ Advanced miRNA Assay. It could possibly be a reason for relatively low detectability of endogenous miRNAs as the components of the reaction cocktails are limited and for example the amount of dNTPs might be used up already for cel-miR-39 cDNA synthesis before all amplification cycles are finished. Comparing measurements of three endogenous miRNAs with the recommended versus 1000-fold higher concentrations of cel-miR-39 showed no effect on endogenous miRNAs concentration.

Endogenous control

Using qPCR as the current standard for miRNA quantification, normalization of the qPCR data is vital. For a long time it showed a wide consensus to use both endogenous and exogenous controls. As discussed above synthetic non-mammalian miRNA mainly derived from Caenorhabditis elegans (C. elegans) was introduced during the RNA extraction process as exogenous spike-in control. These molecules can provide a reference for normalization of the technical variability in RNA extraction.

For variables occurring before the RNA extraction as sample choice and handling, haemolysis and storage conditions an endogenous control is recommended but a universal applicable method for normalisation does not exist (Donati et al., 2019). Concerning the high variation in Cq values of the exogenous control in all four cohorts, an endogenous control can be recommended for normalisation to ensure comparability. As endogenous controls various endogenous miRNAs and small non-coding RNA molecules that are detectable

in all samples, show low dispersion of expression levels and no association with disease are used. One common one is miR-16. In literature there is also evidence that it is not independent of clinical phenotypes. It could be shown that miR-16 was downregulated in the cell, animal models of atherosclerosis and in CAD patients (M. Wang et al., 2020). Moreover, miR-16 is highly expressed in red blood cells and hemolysis contributes to a significant increase in its concentration in plasma and serum (Kirschner et al., 2011). It is certain that miR-16 as endogenous control must be carefully evaluated before classification as stable miRNA to serve as endogenous control or as fluctuant miRNA and possibly related to disease risk.

Another approach for normalization is to use other small non-coding RNA molecules (RNUs) as RNU6 genes RNU6A and RNU6B. However, RNU6 is not a miRNA, and, consequently, does not reflect the biochemical character of miRNA molecules in terms of their transcription, processing, and tissue-specific expression patterns. In addition, the efficiency of their extraction, reverse transcription, and PCR amplification may differ from that of miRNAs (Schwarzenbach et al., 2015). To overcome the problem of possibly unstable endogenous RNA controls and if numerous miRNAs and RNU6 are measured, stability assessment algorithms as geNORM, NormFinder or BestKeeper can then be used to identify stably expressed RNAs and combinations from the set that was measured (De Spiegelaere et al., 2015).

Several authors have concluded that a universal endogenous control is unlikely to be discovered and a suitable reference should be assessed every time, considering the different biological conditions of the samples, their nature and the disease for which the biomarker is being developed (Precazzini et al., 2021).

As an alternative for large scale miRNA expression profiling studies, where several hundreds of miRNAs are analysed global normalization is recommended and uses the calculated mean of all miRNAs in a given sample as the normalizer (Mestdagh et al., 2009).

Cut off in data analysis

In the present thesis, the predominant part of Cq values reached 40 and therefore were considered as undetectable in qPCR. The question of choosing a lower cut off value in this case has no effect. For further measurements, it is important to take into consideration that a Cq cut off for undetermined values set at 40 is

considerably high. In order to identify unreliable measurements of miRNAs a Cq threshold of 35 is recommended to exclude Cq values which follow uniform distribution and thus can be considered as random noise (Chen et al., 2009; Schulte et al., 2019).

4.2 Comparison of assays

Potential pitfalls in establishing a PCR-based approach for testing circulating miRNAs became obvious in the test run and showed the necessity of a step by step analysis of the protocol.

For the reverse transcription step, there are two distinct priming methods used among commercially available qPCR-based platforms. Some platforms make use of unique, sequence-specific RT primers for cDNA synthesis while others make cDNA using universal tailing primers. There are various studies comparing sensitivity, specificity and reproducibility of results. Chugh et al. describe that the specificity of the sequence-specific RT primers was greatly increased and yielded one prominent PCR product. This was evident for both highly abundant and moderately abundant miRNAs. The sequence-specific RT primer assay failed to yield a signal for low abundance miRNAs. By contrast, the universal tailing RT-primer-based assay yielded a quantitative signal, but most of this signal was due to non-specific amplification (Chugh and Dittmer, 2012). For this thesis and the planned measurements in BiomarCaRE universal tailing primers are used with the advantage that the cDNA transcripts can be used for all selected miRNAs measurements in one probe.

In this thesis, three assays were used and a comparison of the last two, miRCURY® LNA® miRNA and TaqMan™ microRNA, can be done. The first set of measurement carried out with the TaqMan™ Advanced miRNA kit could not detect the selected miRNAs. When even the exogenous control cel-miR-39 was not detectable, fundamental problems emerged. Cel-miR-39 was not phosphorylated. With the used kit it could not be detected in the RT-step and was therefore not amplified. This resulted in undetermined values in qPCR. Procedures of isolating and measuring miRNAs are long and complicated. The more steps it takes the more vulnerable it becomes for methodical mistakes. In this case the kit could not detect non-5'phosphorilated miRNAs. Finding this error by remeasuring cel-miR-39 with TaqMan™ microRNA and miRCURY® LNA® miRNA showed that different assays measure different amounts of miRNA and

vary in sensitivity. For the selection of assay and to review the effect of a very high concentration of spike-in cel-miR-39 another set of measurements was performed with the two assays where it could be shown, that the TaqMan™ microRNA Assay detects higher numbers of miRNAs. The miRCURY® LNA® miRNA assay had the advantage that with the LNA technique it does not require an amplification step. Such step can introduce inaccuracies. In other studies, both assays have shown good results in terms of sensitivity, specificity and reproducibility of results. An analysis of the differences in the results from TaqMan™ microRNA assay and miRCURY® LNA® miRNA assay found an effect of small RNA enrichment on the expression levels of miRNAs with these methods and that the variability was lower with the TaqMan™ platform (Redshaw et al., 2013). Another comparison of TaqMan™ and miRCURY® LNA® miRNA and their effects on quantification accuracy and repeatability showed that both assays displayed similar efficiencies but that the miRCURY® LNA® miRNA generated slightly more variable measurements (Redshaw et al., 2013).
Overall, miRCURY® LNA® miRNA and TaqMan™ microRNA would be good choices for re-measurements in BiomarCaRE. In this thesis it could be shown for cel-miR-39 that results measured with the TaqMan™ microRNA assay bring higher results.

4.3 Limitations

The oldest samples for miRNA measurement from the project of BiomarCaRE were collected and frozen 1984. The role of storage time and temperature could have an influence on the quality of the measurements and the miRNAs themselves. MiRNAs are regarded as surprisingly stable but damage of samples over time and alterations cannot be excluded as samples were frozen over years. In literature there is little evidence for the effect of such long storage times, but it is shown that there are only minimal to no differences between fresh and frozen samples, even after repeated freeze-thaw cycles (Cheng et al., 2013; Grasedieck et al., 2012; Page et al., 2013).

Further, miRNAs in the measurements with TaqMan™ Advanced miRNA Assay were partly different ones than with TaqMan™ microRNA and miRCURY® LNA® miRNA. A direct comparison can only be done for cel-miR-39 and the two assays TaqMan™ microRNA and miRCURY® LNA® miRNA. Moreover, the

measurements of endogenous miRNAs in this thesis give remarks on possible sources of error but do not further prove an approach for the testing of all 14 miRNAs selected for BiomarCaRE.

Besides, the planned study on miRNAs within BiomarCaRE shows a candidate gene approach. The pre-selection of miRNAs tested for this study is based on known association to CVD risk and pre-existing non-publically available datasets. New and yet unknown miRNAs are not taken into consideration. To avoid this bias and test the samples for all possible miRNAs, detection methods as next-generation sequencing are an alternative but for economic reasons not suitable for this dimension of sample size. Until today, qPCR is regarded as the “gold standard” in the quantitative analysis of defined sets of miRNAs (D’Alessandra et al., 2022; Git et al., 2010). NGS and microarray would be favourable for a screening approach. Novel promising strategies are immunoassays as miREIA, SplintR-qPCR, isothermal amplification and near-infrared technology (Krepelkova et al., 2019; Moody et al., 2017).

Further, baseline characteristics of selected population-based cohorts of BiomarCaRE are of a limited number and other patient-related factors are known to affect the results. Various cancers, inflammatory and autoimmune diseases along with many others are accompanied with a change in miRNA levels and might influence the results (Ardekani and Naeini, 2010). Further, medication, as heparin or anti-platelet therapy, physical activity, diet, fasting or non-fasting and age show altered levels of circulating miRNAs (Boeckel et al., 2013; Hackl et al., 2010; Noren Hooten et al., 2010; Polakovičová et al., 2016; Quintanilha et al., 2017; Willeit et al., 2013).

4.4 Outlook

For further analysis of circulating miRNAs as potential disease-specific biomarkers for risk prediction of cardiovascular diseases an improved protocol is elementary. In this thesis, TaqMan™ microRNA showed the best results and can be recommended for the measurements of miRNA in BiomarCaRE.

A strategy of handling the raw data is elementary. For normalisation cel-miR-39 should be remeasured with the new assay chosen as it can provide a reference for the efficiency of the transcription reaction and the technical variability of the

following experimental process. Besides, it is important to keep in mind that an endogenous control should be an option and can be evaluated by one of the stability assessment algorithms geNORM, NormFinder or BestKeeper as mentioned above. For this reason, miR-16 as well as RNU6 could be further integrated in new measurements to identify stably expressed RNAs or combinations that serve as endogenous controls.
It can be recommended to repeat measurements again in the Brianza cohort with a set of endogenous miRNAs and the above mentioned possible controls. With the results an evaluation in terms of detectability and raw qPCR results can be done to make sure the protocol can be approved also for the endogenous miRNAs and results can be used for normalisation of data. Alternatively, sequencing all non-coding RNA would eliminate the selection bias of qPCR and harbors the chance to discover new and not yet described biomarkers, miRNAs but also long non-coding RNAs (ncRNAs), described as promising biomarkers for CVD (Fang et al., 2020).

Up to now and to measure a defined set of miRNAs, qPCR is convincing as “gold standard” but for large cohorts as in the project of BiomarCaRE new approaches seem promising even though technical obstacles are still to overcome. Novel strategies as miREIA, SplintR-qPCR, isothermal amplification and near-infrared technology are not yet advanced but after all need less steps, therefore avoid sources of error and provide the opportunity to improve or replace existing technologies in the future. Also the droplet digital PCR as an absolute quantification method might be an alternative if it can be proved more accurate and reproducible in miRNA quantification (Hindson et al., 2013; Robinson et al., 2018).

In a large study as BiomarCaRE with collection of patients material over years, principle in sample collection and storage is not influenceable. Harmonisation of standards should be taken into consideration in future validation studies as well as for implementation in clinical practise.

5 Summary

This thesis evaluated which qPCR based method for miRNAs detection is most suitable for application in large population based cohorts such as the BiomarCaRE project and current possibilities and new approaches for circulating miRNA detection. Up to now, there is no standardised protocol for measuring miRNAs making comparability of study results difficult and clinical application of miRNAs as biomarkers impossible.

Besides, this thesis points out factors influencing the detectability of miRNAs and discusses the possibilities of qPCR data normalization in order to give a wider understanding of the results when measuring and analysing miRNAs.

For this thesis, samples from the BiomarCaRE case cohort set were available. For evaluation of detectability of circulating miRNAs, cel-miR-39, miR-150 and miR-133a were measured with TaqMan™ Advanced miRNA. Results showed that this assay was inadequate for the given samples. TaqMan™ microRNA and miRCURY® LNA® miRNA were tested as alternative assay approach.

In summary, using TaqMan™ microRNA Assay showed the best results and can be recommended for further measurements of miRNAs in large scale human cohorts. Under all conditions, an exogenous control such as cel-miR-39 should be used to compensate for differences in extraction efficiency between samples. Moreover, the implementation of an endogenous control is recommended for reliable miRNA analysis accounting for variables before extraction from sample choice and haemolysis to storage conditions.

Up to now a RT-qPCR based method for measuring miRNAs in large-scale human cohorts is the most commonly used method and comparably cost effective but also shows major disadvantages in terms of reliability and reproducibility of results. A standardisation is inevitable. Newer and not yet standardised methods raise high expectations for implementation in laboratory and clinics but must be further developed and confirmed in future studies.

6 Zusammenfassung

In dieser Arbeit wurde untersucht, welche qPCR-basierte Methode zum Nachweis von miRNAs am besten für die Anwendung in großen populationsbasierten Kohorten wie dem BiomarCaRE-Projekt geeignet ist, sowie aktuelle Möglichkeiten und neue Ansätze zum Nachweis zirkulierender miRNAs. Bisher gibt es kein standardisiertes Protokoll zur Messung von miRNAs, was die Vergleichbarkeit von Studienergebnissen erschwert und eine klinische Anwendung von miRNAs als Biomarker unmöglich macht.

Darüber hinaus zeigt diese Arbeit Faktoren auf, die die Nachweisbarkeit von miRNAs beeinflussen und diskutiert die Möglichkeiten der qPCR-Datennormalisierung, um ein breiteres Verständnis der Ergebnisse bei der Messung und Analyse von miRNAs zu ermöglichen.

Für diese Arbeit standen Proben aus den BiomarCaRE-Fall-Kohorten zur Verfügung. Zur Bewertung der Nachweisbarkeit zirkulierender miRNAs wurden cel-miR-39, miR-150 und miR-133a mit TaqMan™ Advanced miRNA gemessen. Die Ergebnisse zeigten, dass dieser Assay für die gegebenen Proben unzureichend war. TaqMan™ microRNA und miRCURY® LNA® miRNA wurden getestet, um die beste Alternative zu bewerten.

Zusammenfassend zeigte der TaqMan™ microRNA Assay die besten Ergebnisse und kann für weitere Messungen von miRNAs in großen humanen Kohorten empfohlen werden. Unter allen Umständen sollte eine exogene Kontrolle wie cel-miR-39 verwendet werden, um Unterschiede in der Extraktionseffizienz zwischen den Proben auszugleichen. Darüber hinaus wird die Implementierung einer endogenen Kontrolle für eine zuverlässige miRNA-Analyse empfohlen, die Variablen vor der Extraktion berücksichtigt, von der Auswahl des Probenmaterials über die Hämolyse bis hin zu den Lagerbedingungen.

Bisher ist eine RT-qPCR-basierte Methode zur Messung von miRNAs in großen humanen Kohorten die am häufigsten verwendete Methode und vergleichsweise kostengünstig, weist jedoch auch erhebliche Nachteile hinsichtlich der Zuverlässigkeit und Reproduzierbarkeit der Ergebnisse auf. Eine Standardisierung ist unumgänglich. Neuere und noch nicht standardisierte Methoden wecken hohe Erwartungen an die Implementierung in Labor und Klinik, müssen jedoch in zukünftigen Studien weiterentwickelt und bestätigt werden.

7 Abbreviations

Ago	Argonate
AF	atrial fibrillation
ATP	adenosine triphosphate
BiomarCaRE	biomarker for cardiovascular risk assessment in Europe
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
Ct	cycle threshold
CrT	relative cycle threshold
cTnI	cardiac Troponin I
cTnT	cardiac Troponin T
Cq	quantification cycle
hs-cTnT	high-sensitive cardiac Troponin T
CRP	C-reactive protein
CVD	cardiovascular disease
°C	degree Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
cDNA	complementary deoxyribonucleic acid
GTP	guanosine triphosphate
HDL	high-density lipoprotein
HF	heart failure
LDL	low-density lipoprotein
log	logarithm
MACE	major adverse cardiovascular event
MGB	minor groove binder
min	minutes
miRNA	micro ribonucleic acid
ml	millilitre
mRNA	messenger ribonucleic acid
ng	nanogram
NGS	next generation sequencing

NT-proBNP	N-terminal pro-brain natriuretic peptide
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
Pre-miRNA	precursor micro ribonucleic acid
Pri-miRNA	primary micro ribonucleic acid
qPCR	quantitative polymerase chain reaction
RISC	ribonucleic acid-induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
SD	standard deviation
sec	seconds
UTR	untranslated region
x g	times Earth's gravitational force

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10 Curriculum vitae

Lebenslauf aus datenschutzrechtlichen Gründen nicht enthalten.

11 Affirmation

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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