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Droplet digital PCR of serum miR-499, miR-21 and miR-208a for the detection of functionally relevant coronary artery disease☆

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ABSTRACT

Background: microRNAs (miRNAs) have shown promise as potential new biomarkers for myocardial injury and myocardial ischemia. New digital polymerase chain reaction (PCR) techniques allow for highly precise and reliable absolute direct quantification.
Methods: In this pilot study we used droplet digital PCR (ddPCR) to assess if miRNAs might be released into circulation in patients with functionally relevant coronary artery disease (CAD). Blood samples for measurement of high-sensitivity cardiac troponin I (hs-cTnI) and miRNAs were obtained before, immediately after peak stress, and 2 h after stress testing in a blinded manner in consecutive patients referred for rest/stress myocardial perfusion single-photon emission tomography/computer tomography (MPI-SPECT/CT). ddPCR was used to directly quantify the serum concentrations of miR-21, miR-208a, and miR-499 as potential markers of myocardial injury/ischemia. Functionally relevant CAD was determined by expert interpretation of MPI-SPECT/CT, coronary angiography and fractional flow reserve, if performed.
Results: Overall, 200 patients were included and functionally relevant CAD was detected in 85 of them (42%). Neither miR-21, miR-208a, nor miR-499 concentrations differed at rest, stress, or 2-h after stress when comparing patients with versus without functionally relevant CAD, while hs-cTnI concentrations were significantly higher in patients with functionally relevant CAD ($P < 0.001$). Exercise-induced changes in miRNA or hs-cTnI concentrations did not have diagnostic utility and were similar in patients with versus without functionally relevant CAD.
Conclusion: miR-208a, miR-21 and miR-499 concentrations at rest, after exercise and exercise-induced changes do not provide additional clinical value regarding the detection of functionally relevant CAD.

1. Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide [1,2]. Early clinical detection of the disease is therefore an important task for physicians to initiate appropriate treatment [3]. Exercise electrocardiography (ECG) is limited by major deficits in sensitivity and specificity. Gold standard tests such as coronary angiography and myocardial perfusion single-photon emission computed tomography (SPECT) have their own limitations including high costs, limited availability, exposure to contrast agents and radiation, and also at times false negative and/or false positive results if used in isolation [3,4]. While the use of blood biomarkers such as cardiac troponin T/I measured with high-sensitivity assays (hs-cTn) has become an indispensable tool in the early diagnosis of acute myocardial infarction (AMI) [3,5,6], it is largely unknown whether blood biomarkers could also provide diagnostic utility in the detection of stable CAD.

Recently, hs-cTnT/I concentrations at rest and after exercise, but not exercise-induced changes, have been shown to provide some incremental value to detect functionally relevant CAD [7–10]. However, the diagnostic accuracy achieved was only moderate as concentrations at rest and after

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exercise can also be chronically raised in patients with other structural cardiac disorders such as heart failure, valvular heart disease, cardiomyopathy, and myocardial hypertrophy [11].

The establishment of novel biomarkers for the detection of functionally relevant CAD is therefore an important aim of medical research. Recently, micro-RNAs (miRNAs) have been identified as promising markers for a number of diseases, including cardiovascular disease [12]. miRNAs are small non-coding single-stranded RNAs (~22 nucleotide-long) involved in the post-transcriptional regulation via alteration of messenger RNA translation, influencing gene expression [13]. miRNAs are released into the circulation in both physiological and pathological states. They are remarkably stable in serum and have become attractive candidates as novel biomarkers [14]. Of particular interest for this study are miR-21, miR-208a and miR-499.

Therefore, the aim of this study was to determine by ddPCR if miR-21, miR-208a and miR-499 are associated with functionally relevant CAD and are released into serum during exercise-induced myocardial ischemia.

2. Methods

This analysis is part of a large ongoing prospective diagnostic study (NCT01838148, clinicaltrials.gov) designed to advance the early detection of functionally relevant CAD [7,9,10,15,16]. The study was approved by the local ethics committee and was carried out according to the principles of the Declaration of Helsinki. All patients provided written informed consent.

2.1. Patient population

Patients referred to the University Hospital Basel, Switzerland for evaluation of functionally relevant CAD by myocardial perfusion single-photon emission tomography/computer tomography (MPI-SPECT/CT) were included consecutively. At this institution, MPI-SPECT/CT is the preferred imaging modality in patients with a wide range of pre-test probabilities for functionally relevant CAD. This study included 200 consecutive patients recruited between July 2010 and June 2011. Stress was induced by exercise only, whenever the patient was able to achieve the target heart rate by exercise. In all other patients, adenosine was added to achieve the target heart rate.

2.2. Blood sampling and laboratory methods

Venous blood samples were drawn at rest, immediately after peak stress, and 2 h after stress testing. Blood was then immediately processed and frozen at −80 °C, until it was assayed or underwent miRNA extraction. All hs-cTnI measurements were performed at Singulex, using the Erenna system. The Erenna system employs a micro-particle immunoassay and single-molecule counting in a capillary flow system and its methodology has been described in detail elsewhere [17]. This assay has been shown to outperform all other currently available hs-cTn assays in terms of analytical sensitivity [18], has been validated using Clinical Laboratory Standards Institute guidelines, and was performed in a Clinical Laboratory Improvement Amendments licensed and College of American Pathologists accredited clinical laboratory [17]. The assay has a limit of detection (LoD) of 0.1 ng/L, a limit of quantification of 0.3 ng/L (20% coefficient of variation [CV]), a total imprecision at 10% CV of between 0.8 and 1.6 ng/L, and a 99th percentile of 10.1 ng/L with <10% CV [19].

Serum samples underwent miRNA extraction according to the manufacturer's instructions (Qiagen miRNeasy Serum/Plasma Kit, ID: 217184). mirRNAs were isolated from 50 μL of serum. After the initial Qiazol denaturation step, all samples were spiked with 3.5 μL miRNeasy Serum/Plasma Spike-In Control (1.6×10^7 copies/μL, adjusted to decrease the count for ddPCR) *C. elegans*-miR-39 (5′-UCACCGGGUGUAAAUCAGCUUG-3′, Qiagen ID: 219610) to act as an exogenous control. microRNA was eluted from spin columns in 30 μL of nuclease-free water.

Each sample then underwent separate reverse transcription (RT) for *C. elegans*-miR-39, hsa-miR-21-5p, hsa-miR-208a-3p and hsa-miR-499-5p using 2.77 μL/well nuclease-free water, 1 μL/well RT buffer, 0.1 μL/well 100 nM dNTP, 0.13 μL/well RNAse inhibitor, 0,67 μL/well multi-scribe reverse transcriptase (TaqMan™ MicroRNA Reverse Transcription Kit, Applied Biosystems, Inc. ID: 4366596), and 2 μL/well specific RT primer (Applied Biosystems, Inc. ID: 000200, 000397, 000511, 001352). For the RT reaction 6,67 μL mastermix and 3,33 μL sample were combined and spun on a microplate centrifuge at 4 °C for 2 min at 2000*g* (Thermo Scientific). Samples underwent a 10 μL thermal cycling protocol using the C1000 Touch™ Thermal Cycler (BioRad) at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and held at 4 °C. The RT product was then further used for ddPCR, with a separate reaction for every target. For every sample 7.67 μL/well nuclease-free water, 10 μL/well ddPCR™ supermix for probes (no dUTP) (BioRad) and 1 μL/well 20× specific hydrolysis primer/probe for each miRNA analyzed (Applied Biosystems, Inc. ID: 000200, ID: 000397, 000511, 001352) were used, briefly centrifuged and partitioned into 18.67 μL/well with 1.33 μL/well RT product. A no template control (NTC) was included. Next, 20 μL of sample was pipetted into each well in an 8-well cassette with 70 μL droplet generation oil for probes (BioRad) and placed in the QX200™ Droplet Generator (BioRad). 40 μL of the droplet emulsion was pipetted into separate wells. Samples were transferred to the C1000™ Thermal Cycler (BioRad) and underwent the following thermal cycling protocol set for 40 μL: 95 °C for 10 min, then 40 cycles of 94 °C for 30 s and 60 °C for 60 s (2.5 °C/s ramp rate), followed by a final step at 98 °C for 10 more minutes before being held at 12 °C.

Final ddPCR concentrations for each miRNA were the results as calculated by the QuantaSoft™ (BioRad) software multiplied by the dilution factor of the template for the reverse transcription and ddPCR reactions. The data was normalised across samples using a median normalisation procedure (normalisation factor = median value of all *C. elegans*-miR-39 measurements/*C. elegans*-miR-39 value for the given sample) [20].

2.3. Adjudication of the presence of functionally relevant CAD

Details of the rest-stress MPI-SPECT/CT protocol have been described previously [7,9,10,15,16]. Summarized, perfusion images were scored semiquantitatively using a 17-segment model with a 5-point scale (0 = normal, 1 = mildly reduced tracer uptake, 2 = moderately reduced uptake, 3 = severely reduced uptake, and 4 = no uptake). Each segment represents 5% of the left ventricle. The determination of the presence or absence of functionally relevant CAD was based on expert interpretation of MPI-SPECT/CT images and invasive coronary angiography, and fractional flow reserve measurements whenever available. While previous studies used MPI-SPECT/CT as a sole reference [21–24], we added data from coronary angiography and fractional flow-reserve to further improve the accuracy of the diagnosis and to prevent the misclassification of exercise-induced myocardial ischemia not caused by obstructive CAD. Two independent cardiologists (one interventional cardiologist, one general cardiologist) who were blinded to biomarker results reviewed the case in instances where the findings from MPI-SPECT/CT and coronary angiography were equivocal. A positive perfusion scan was overruled when coronary angiography showed normal coronary arteries and a negative perfusion scan was overruled if a high-grade coronary lesion was found on coronary angiography within three months after MPI-SPECT assessment (except in the case of acute myocardial infarction).

2.4. Outcome definition

For the prognostic assessment, two endpoints were defined: first, all-cause mortality and second, major adverse cardiac events (MACE), the composite of AMI and cardiovascular death, during follow-up. Patients were contacted one, two and five years after the examination either by letter with a questionnaire or by telephone interview performed by trained researchers. Medical records and case files were collected from treating facilities or primary care physicians in case of an event or equivocal information from a patient.

2.5. Statistical analysis

The sample size (n = 200) was determined based on prior work establishing the diagnostic utility of hs-cTnI in this indication [7,15,16], and the assumptions that 1) the incidence of functionally relevant CAD would be about 40% and 2) at least one of the examined miRNAs would achieve a similar diagnostic accuracy as compared to hs-cTnI. Baseline characteristics were compared using the unpaired *t*-test, the Mann Whitney-*U* test or Fisher's exact test as appropriate. Comparisons within individuals were performed with the Wilcoxon Signed Rank Test. Concentrations of miRNAs and hs-cTnI were correlated using Spearman's rank correlation coefficient. Diagnostic accuracy for functionally relevant CAD was quantified by the area under the receiver operating characteristic curve (AUC). To assess if the miRNAs provide incremental information on top of hs-cTnI, logistic regression analysis was performed. The association of miRNAs and hs-cTnI concentrations with survival and occurrence of MACE, was evaluated using Cox regression analysis. All hypothesis testing was two-tailed, and a P-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 25.0 (IBM, Armonk, NY, USA) and R (version 3.3.3). Missing data was excluded from respective analyses. Predefined subgroup analysis was performed in patients without previously known CAD, as in these any interference of biochemical signaling related to chronic pre-existing CAD should be minimal as compared to the acute ischemic response during functional testing. A second subgroup analysis was performed in patients undergoing exercise stress only, as in these hs-cTnI—as the biochemical reference standard—performed best in previous work [7,15,16].

3. Results

3.1. Patient characteristics

Baseline characteristics of the 200 consecutive included patients are shown in Table 1. In total, 62 patients (31%) were female, 99 (50%) had previously known CAD and the mean age was 67 (±11) years. Functionally relevant CAD was determined to be present in 85 (42%) patients.

3.2. Diagnostic performance of miRNAs compared to hs-cTnI

miRNA and hs-cTnI concentrations before and after exercise testing stratified by the presence or absence of functionally relevant CAD are presented in Table 1A and Fig. 1. miRNA concentrations at baseline, immediately after stress and 2 h after stress were not significantly

Table 1
Patient characteristics and biomarker concentrations stratified by functionally relevant CAD.

	Overall, (n = 200; 100%)	No functionally relevant CAD, (n = 115; 58%)	Functionally relevant CAD, (n = 85; 42%)	P value
Age, years	67 ± 11	65 ± 11	69 ± 12	0.024
Sex, female	62 (31%)	46 (40%)	16 (19%)	0.002
Previously known CAD	99 (50%)	35 (30%)	64 (75%)	<0.001
History of AMI	71 (36%)	25 (22%)	46 (54%)	<0.001
Cardiovascular risk factors				
BMI, kg/m^2	27 ± 4	27 ± 4	28 ± 4	0.602
Diabetes	40 (20%)	17 (15%)	23 (27%)	0.048
Hyperlipidemia	140 (70%)	69 (60%)	71 (84%)	<0.001
Hypertension	153 (77%)	80 (70%)	73 (86%)	0.007
Tobacco use	109 (55%)	65 (57%)	44 (52%)	0.566
miR-21, copies/μL				
Rest (n = 200)	2798 [524–6798]	2991 [588–6854]	2639 [453–6778]	0.447
Stress (n = 189)	3623 [255–8427]	4016 [264–8715]	3556 [190–7136]	0.470
2 h after stress (n = 186)	3099 [472–6662]	3278 [278–7975]	2876 [680–5502]	0.765
ΔRest-stress	−114 [−2910–871]	−115 [−2586–1077]	−113 [−3047–634]	0.670
ΔRest-2 h after stress	28 [−2287–1646]	26 [−2292–1876]	29 [−2093–1447]	0.701
miR-208a, copies/μL				
Rest (n = 200)	4.4 [0.0–12.7]	4.6 [0.0–14.5]	3.7 [0.0–10.0]	0.262
Stress (n = 189)	3.7 [0.0–15.7]	4.5 [0.0–18.1]	2.3 [0.0–12.8]	0.272
2 h after stress (n = 186)	3.6 [0.0–15.4]	3.5 [0.0–12.5]	3.9 [0.0–19.4]	0.218
ΔRest-stress	0.0 [−6.7–4.0]	0.0 [−7.9–4.4]	0.0 [−6.4–3.0]	0.582
ΔRest-2 h after stress	0.0 [−5.7–4.6]	0.0 [−4.9–6.3]	0.0 [−9.4–4.6]	0.246
miR-499, copies/μL				
Rest (n = 200)	4.5 [0.7–11.9]	5.3 [1.1–12.2]	3.7 [0.3–11.3]	0.207
Stress (n = 189)	4.7 [0.0–14.0]	5.1 [0.1–12.3]	3.5 [0.0–14.2]	0.454
2 h after stress (n = 186)	4.1 [0.0–11.3]	4.1 [0.0–13.3]	4.2 [0.0–9.9]	0.411
ΔRest-stress	0.0 [−5.2–4.5]	0.0 [−4.1–5.4]	0.0 [−5.8–3.0]	0.489
ΔRest-2 h after stress	0.0 [−5.5–6.2]	0.0 [−6.9–6.1]	0.0 [−4.6–6.9]	0.589
hs-cTnI, ng/L				
Rest (n = 198)	3.4 [2.2–6.2]	2.7 [1.9–4.1]	5.2 [3.2–10.1]	<0.001
Stress (n = 193)	3.7 [2.3–5.8]	3.0 [1.9–4.3]	5.5 [3.3–10.2]	<0.001
2 h after stress (n = 190)	4.1 [2.5–6.4]	3.4 [2.2–4.7]	5.5 [3.4–10.6]	<0.001
ΔRest-stress	−0.1 [−0.6–0.1]	−0.2 [−0.5–0.1]	−0.1 [−0.6–0.2]	0.599
ΔRest-2 h after stress	−0.2 [−1.1–0.2]	−0.3 [−1.1–0.2]	−0.2 [−1.1–0.2]	0.336

Values are median [IQR] and number (percentage). AMI = acute myocardial infarction; BMI = body mass index; CAD = coronary artery disease; diabetes = insulin dependent and non-insulin dependent diabetes mellitus; tobacco use = current or previous tobacco use.

different between patients with versus without functionally relevant CAD. In contrast, hs-cTnI concentrations were significantly higher in patients with functionally relevant CAD versus those without functionally relevant CAD. When evaluating miRNA concentrations within a patient, only miR21 concentrations immediately after stress as compared to rest (3623 vs 2798 copies/μL, P = 0.009) differed significantly. However, ΔmiR-21 (stress-baseline) was similar in patients with versus without functionally relevant CAD (P = 0.670). The respective diagnostic accuracies of the miRNAs and hs-cTnI for functionally relevant CAD as quantified by the AUC is shown in Table 2A and Fig. 2. While the miRNAs concentrations showed significant correlation with each other (Table A.1), they did not correlate with hs-cTnI concentrations.

3.3. Prognostic performance of miRNAs compared to hs-cTnI

Median follow-up time was 1946 days (IQR 866–2213 days). In total, 42 (21%) of the patients died during the follow-up and 25 (13%) patients had a MACE (10 cardiovascular death, 15 AMI). There was no association between the assessed miRNAs and all-cause mortality or MACE, while hs-cTnI was a significant predictor for both (Table A.2).

3.4. Subgroup analysis: patients without previously known CAD

Overall, 101 (50%) of the 200 included patients had no prior history of CAD and in 21 (21%) of these patients functionally relevant CAD was detected. In this subgroup, miR-208a and miR-499 concentrations at rest were significantly higher in patients without functionally relevant CAD as compared to patients with functionally relevant CAD (4.5 vs. 1.1 copies/μL, P = 0.017 and 5.9 vs. 3.1 copies/μL, P = 0.015 respectively) yielding an AUC of 0.67 (0.55–0.78) and 0.67 (0.55–0.79) respectively. When adjusted for hs-cTnI concentrations using logistic regression analysis only miR-208a remained a significant predictor for functionally relevant CAD, with the combination of hs-cTnI and miR208a having an AUC of 0.80 (0.71–0.90) as compared to the AUC 0.74 (0.63–0.85) for hs-cTnI alone. However, none of the assessed miRNAs was associated with death or MACE in this subgroup.

3.5. Subgroup analysis: patients undergoing exercise stress only

Overall, 103 (52%) patients underwent exercise stress only and in 36 (35%) of these functionally relevant CAD was detected. In contrast to hs-cTnI, which had a high AUC in this subgroup, none of the examined miRNA was associated with functionally relevant CAD (Table 2B).

4. Discussion

In this large diagnostic pilot study, we evaluated whether the cardiovascular miRNAs miR-21, miR-208a, miR-499 serum concentrations before, after stress and exercise-induced changes provide clinical utility for the detection of functionally relevant CAD.

The hypothesis that miR-21, miR-208a, miR-499 serum concentrations might be associated with functionally relevant CAD was based on recent following observations. miR-21 is up-regulated in cardiomyocytes shortly after initiation of myocardial ischaemia [25]. It promotes cardiac muscle function and is highly expressed in the cardiovascular system [26–28]. Notably, it regulates several functions relevant to exercise and has been found to be upregulated by exercise [29]. In AMI it contributes to post-AMI fibrogenesis, suggesting a role in remodelling [30,31]. miR-21 has been demonstrated to significantly increase the diagnostic value of AMI when added to hs-cTn [32]. miR-208a is cardiac specific and miR-499 is found highly expressed in myocardial but also found in skeletal muscle [33]. Both have been shown to be increased in AMI

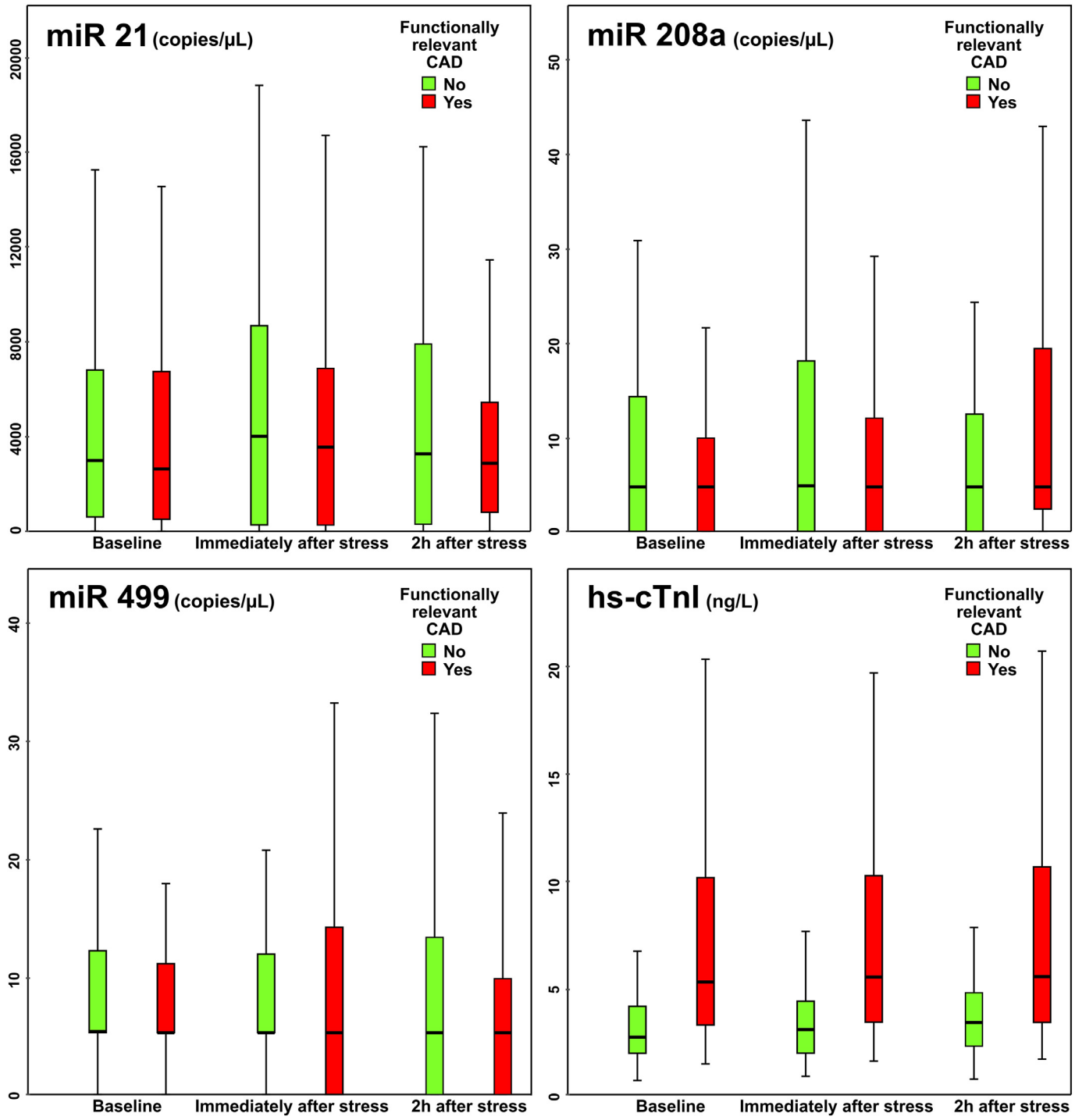

Fig. 1. Boxplots of miR-21, miR-208a, miR-499 and hs-cTnI concentrations at rest, stress and 2 h after stress.

[32,34,35]. miR-208a is highly detectable in AMI patients, but not in healthy controls or in non-AMI patients [35]. miR-208a may even have advantages over cTn in the early phase of AMI. miR-499 is detected at a higher level, but released slower. The combination of the two miRNAs might therefore detect recent cardiac injury (miRNA-208), whereas miR-499 may detect myocardial injury that occurred longer ago [33]. In a small pilot study, miR-499 and miR-21 have been shown to significantly increase the diagnostic value when added to hs-cTn, while a combination of microRNAs had a higher diagnostic value than hs-cTn [32]. In contrast, another study found that none of the tested miRNAs outperformed cTn [36].

Digital polymerase chain reaction has been shown to exhibit superior technical qualities (decreased variability, increased day-to-day reproducibility and superior sensitivity) for quantifying miRNA concentrations in the circulation [37–39]. Droplet digital polymerase chain reaction (ddPCR) is an end-point analysis, allowing direct absolute quantification of microRNAs by using a Poisson statistical analysis of fluorescent signals from positive and negative droplets [37]. We therefore used ddPCR to quantify serum miRNA.

We report six major findings. First, of the analyzed miRNAs miR-208a, miR-21 and miR-499 neither were found to be significantly different in patients with or without functionally relevant CAD when

Table 2A
Diagnostic performance of miRNAs and hs-cTnI.

	AUC	P-value
miR-21, copies/μL		
Rest (n = 200)	0.53 (0.45–0.61)	0.447
Stress (n = 189)	0.53 (0.45–0.61)	0.470
2 h after stress (n = 186)	0.51 (0.43–0.60)	0.765
miR-208a, copies/μL		
Rest (n = 200)	0.55 (0.46–0.63)	0.269
Stress (n = 189)	0.55 (0.46–0.63)	0.283
2 h after stress (n = 186)	0.55 (0.47–0.64)	0.226
miR-499, copies/μL		
Rest (n = 200)	0.55 (0.47–0.63)	0.209
Stress (n = 189)	0.53 (0.45–0.61)	0.458
2 h after stress (n = 186)	0.53 (0.45–0.62)	0.417
hs-cTnI, ng/L		
Rest (n = 198)	0.76 (0.70–0.83)	<0.001
Stress (n = 193)	0.74 (0.66–0.81)	<0.001
2 h after stress (n = 190)	0.73 (0.66–0.81)	<0.001

AUC – area under the receiver operating characteristics curve.

Table 2B
Diagnostic performance of miRNAs and hs-cTnI in the subgroup of patients undergoing exercise stress only.

	AUC	P-value
miR-21, copies/μL		
Rest (n = 103)	0.51 (0.39–0.63)	0.885
Stress (n = 95)	0.51 (0.39–0.64)	0.868
2 h after stress (n = 93)	0.54 (0.42–0.66)	0.540
miR-208a, copies/μL		
Rest (n = 103)	0.52 (0.41–0.64)	0.688
Stress (n = 95)	0.51 (0.39–0.64)	0.830
2 h after stress (n = 93)	0.51 (0.39–0.63)	0.862
miR-499, copies/μL		
Rest (n = 103)	0.51 (0.39–0.63)	0.912
Stress (n = 95)	0.52 (0.37–0.61)	0.793
2 h after stress (n = 93)	0.58 (0.45–0.70)	0.219
hs-cTnI, ng/L		
Rest (n = 103)	0.82 (0.73–0.91)	<0.001
Stress (n = 98)	0.80 (0.70–0.90)	<0.001
2 h after stress (n = 99)	0.78 (0.69–0.88)	<0.001

AUC – area under the receiver operating characteristics curve.

assessing all patients. Second, stress-induced changes of miRNA concentrations did not differ between patients with or without functionally relevant CAD. Third, neither miR-208a, miR-21 nor miR-499 concentrations predicted all-cause mortality or the occurrence of MACE during long-term follow-up. Fourth, in the subgroup analysis of patients without known CAD, lower miR-208a concentrations were associated with functionally relevant CAD, independent of hs-cTnI. Fifth, in the subgroup of patients undergoing exercise stress only, none of the examined miRNA was associated with functionally relevant CAD. Sixth, hs-cTnI concentrations were significantly higher in patients with functionally relevant CAD as compared to patients without functionally relevant CAD and were predictors for death and the occurrence of MACE.

In our study, and in agreement with previous studies, miR-21 expression was very stable and showed the highest serum expression [29]. miR-21 was detectable in all patients. In our experimental setting miR-21 significantly increased after stress in the entire population, and decreased again after 2 h. It has been reported that circulating miR-21 increases in healthy athletes immediately after acute exhaustive exercise and then decreases again after 1 h of rest [29]. miR-21 has also been shown to be upregulated in response to acute exhaustive exercise in chronic heart failure patients, however no clinical correlation was identified [40].

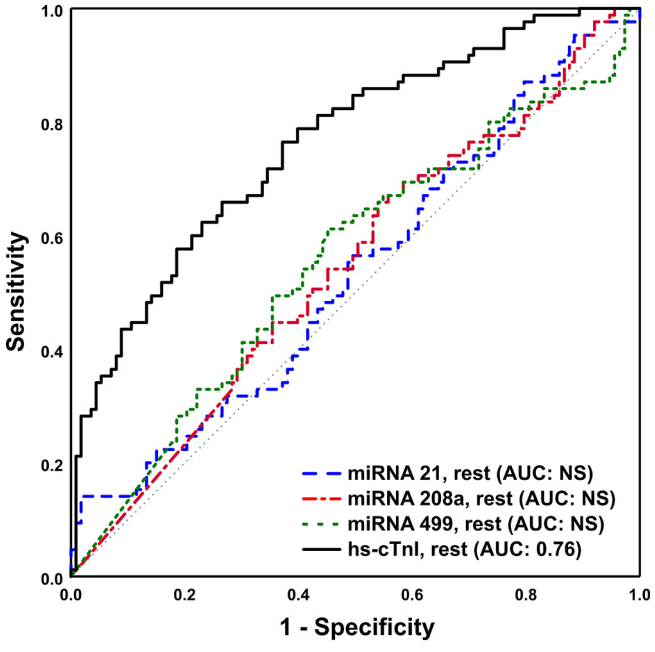

Fig. 2. Receiver operating characteristic curves miR-21, miR-208a, miR-499 and hs-cTnI concentrations at rest.

miR-499 and miR-208a demonstrated a much lower level of serum expression. miR-499 was detectable in 79% of all patients and miR-208a in 70% of all patients. Again, this is in agreement with previous studies showing that miR-208a was undetectable in non-AMI patients (coronary heart and cardiovascular disease) and healthy controls, whereas miR-499 is expressed at a higher level but with low abundance [35,41]. It can be hypothesized that the higher expression of miR-208a in our non-AMI population is a result of increased sensitivity of ddPCR.

In our setting miR-499 and miR-208a did not change with stress. In healthy male runners miR-499 and miR-208a were shown to be upregulated after running a marathon [42]. The authors also reported an elevation of creatine kinase and hs-cTn after a marathon. In contrast miR-208a was at the same level after acute exhaustive exercise for a period shorter than 30 min. This possibly reflects changes only seen in miR-499 and miR-208a expression after excessive exercise with a cardiac injury. Differences in the findings compared to our study might be attributed to the different types of exercise. In our study we found a significant increase of hs-cTn after exercise compared to the cardiac-specific miR-208a and cardiac-enriched miR-499. This might be a matter of sensitivity. We used a novel high-sensitivity assay able to adequately detect even minimal cardiac injury.

The findings of our predefined subgroup analysis in patients without pre-existing CAD are provocative. This subgroup was selected to avoid any interference of biochemical signaling related to chronic pre-existing CAD with the acute ischemic response during functional testing. In this subgroup, lower miR-208a and lower miR-499 concentrations were associated with functionally relevant CAD. This result must be interpreted with caution for two reasons. First, the concentration of the reported miRNAs was per se very low. Demonstrating a significant decrease in low abundant targets with minor copy number changes may be affected by analytical imprecision [43]. Second, this contradicts prior studies suggesting higher levels of miR-208a in patients with CAD [44,45]. However, miR-499 has been shown to be downregulated in ischemic hearts and hypoxic cardiomyocytes [46]. These findings therefore warrant validation in future studies.

5. Limitations

Several limitations should be considered when interpreting our results. First, our findings are specific to the three validated cardiovascular microRNAs and cannot be generalized to other miRNAs that could be assessed e.g. in a global miRNA profiling. Second, this study included

only a moderate number of patients. However, as the sample size was comparable to that used in prior studies evaluating hs-cTnI in this indication as well as miRNAs in other indications, it is very unlikely that we have missed a clinically relevant finding. Third, the unexpected finding derived from the predefined subgroup analysis in patients without pre-existing CAD can neither be verified nor negated within this diagnostic study. Fourth, patients were enrolled in a single institution. Nevertheless, the fact that MPI-SPECT/CT was the standard non-invasive imaging modality and when applied to patients with a wide range of pre-test probability for CAD should have counterbalanced the inherent possibility of referral bias in a single-center study.

6. Conclusions

In conclusion, we have demonstrated that miR-208a, miR-21 and miR-499 concentrations at rest, after exercise and exercise-induced changes do not provide clinical value for the detection of functionally relevant CAD or the prediction of MACE during long-term follow-up.

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Appendix A

Table A.1
Correlation of miRNAs and hs-cTnI.

	Hs-cTnI	miR-208a	miR-499
miR-21	Spearman's rho 0.106 P = 0.138	Spearman's rho 0.380 P < 0.001	Spearman's rho 0.391 P < 0.001
miR-208a	Spearman's rho 0.022 P = 0.761	–	Spearman's rho 0.538 P < 0.001
miR-499	Spearman's rho 0.128 P = 0.071	–	–

Table A.2
Univariate Cox regression of hs-cTnI and miRNAs with all-cause mortality (42 events) and MACE (25 events) as outcome.

	Hazard ratio, 95% CI P-value
All-cause mortality	
miR-21	0.90, 0.78–1.03 0.117
miR-208a	1.06, 0.84–1.21 0.945
miR-499	1.04, 0.85–1.27 0.724
hs-cTnI	2.21, 1.66–2.96 <0.001
MACE	
miR-21	0.90, 0.75–1.07 0.224
miR-208a	0.96, 0.76–1.22 0.732
miR-499	0.86, 0.64–1.16 0.318
hs-cTnI	2.66, 1.90–3.72 <0.001

Variables were log transformed.

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