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ARTYKUŁ ORYGINALNY / *ORIGINAL ARTICLE*

Release kinetics of circulating miRNA-208a in the early phase of myocardial infarction

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Abstract

Background: The biochemical confirmation of myocardial infarction is based on cardiac troponin (cTnI or cTnT) determination. Recent scientific results suggested that microRNAs (miRNAs) might become a new biomarker of tissue injury.

Aim: To evaluate the release kinetics of circulating heart-specific miRNA-208a and also to test the hypothesis that miRNA-208a can serve as an accessible, diagnostically sensitive plasma biomarker of ST-elevation acute myocardial infarction (STEMI).

Methods: Nineteen STEMI patients (four women and 15 men, aged 44–85 years), 12 patients with stable coronary artery disease (CAD), and eight patients with a negative observation of CAD as a control group were studied. Blood samples were collected on admission and at three, six, 12, 24, and 48 h afterwards; in the CAD and control group blood samples were taken only once. Plasma levels of miRNA-208a determined by real-time polymerase chain reaction and their relative fold changes were calculated. cTnI and creatinine kinase (CK)-MB mass were also measured in the patients' serum samples.

Results: miRNA-208a was increased in STEMI patients at the time of admission and nearly undetectable in CAD patients and controls. The peak of miRNA-208a was observed at 3 h after reperfusion ($p < 0.001$). The traditional biomarkers (cTnI and CK-MBmass), which increase later in comparison to miRNA-208a reaching the maximum concentrations 6 h after reperfusion, were observed. Circulating miRNA-208a levels strongly correlated with cTnI and CK-MBmass released from the infarcted area.

Conclusions: These results demonstrate that plasma miRNA-208a is an interesting and promising candidate for a new biomarker released early after onset of myocardial infarction.

Key words: miRNA-208a, myocardial infarction, cardiac markers

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INTRODUCTION

Early identification of myocardial infarction (MI) in chest pain patients is crucial for early treatment initiation. Diagnosis of acute myocardial infarction (AMI) relies, besides clinical symptoms and electrocardiographic (ECG) findings, primarily on biomarker level [1]. High-sensitivity cardiac troponin (hs-cTnI) assays add a new dimension to the assessment of chest pain patients [2, 3]. These assays detect lower concentrations of cardiac troponin (cTnI), which made it possible to shorten the time to AMI. However, since they also detect a variety of acute and chronic coronary conditions that are not associated with an AMI, the positive predictive value of an elevated cTnI for an AMI has decreased, causing confusion about how best to use hs-cTnI in the clinical setting [4]. Therefore, the ideal biomarker for rapid and reliable diagnosis of AMI is still lacking. Scientific approaches should involve the analysis of multiple biomarkers that cover different aspects of the pathophysiological processes associated with disease progression. The dynamic development of analytical techniques in molecular biology has defined new directions for research on an ideal biomarker in myocardial necrosis.

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MicroRNAs (miRNAs) are short, single-stranded, non-coding RNA molecules consisting of 16–29 nucleotides, whose primary function is the negative regulation of gene expression (so-called "sleep genes"). The mechanism of action involves binding of miRNAs to specific non-translated 3′ regions of the mRNA, which results in the destabilisation and blocking translation of the transcripts [5, 6]. It is estimated that about 60% of the transcriptome of mammals is regulated by miRNA [7]. Therefore, since its discovery, miRNA is an object of interest as a molecule controlling many physiological processes, such as differentiation and proliferation of stem cells, neurogenesis, angiogenesis, and pathological conditions such as carcinogenesis or endothelial dysfunction and myocardial hypertrophy. In particular, molecules miRNA-1, -133, -195, and -208a affect proliferating cardiomyocytes and myocardial hypertrophy [8]. It seems that the small molecule miRNA-208a released from cardiomyocytes to plasma may be an almost perfect biochemical marker of ischaemia prior to cell necrosis.

Therefore, the immediate goal of the our study was a quantitative assessment of circulating miRNA-208a in patients with ST elevation MI (STEMI) both at initial presentation and during the course of AMI up to 48 h. We also compared the changes of the miRNA-208a expression in plasma with cTnI and creatinine kinase (CK)-MB mass concentration in serum.

METHODS

This was a prospective, observational, open-label study. Participation in the study had no effect on therapeutic decisions, invasive procedures, and the course of hospitalisation. The study received approval from the bioethical committee of the Medical University of Warsaw. Patients were enrolled during the years 2010–2012.

Study population

All 39 patients signed an informed consent form to participate in the research. Based on the initial diagnosis they were allocated to one of three groups:

- STEMI group (n = 19) included patients with acute STEMI, who were admitted to the hospital within the first 3 h after the onset of symptoms. The initial diagnosis was based on the presence of chest pain or its equivalent and persistent ECG ST-segment elevations (at least 2 mm in two adjacent leads), all patients underwent coronary angiography according to medical indications;
- stable CAD group (n = 12) included patients with stable symptomatic coronary artery disease (CAD) who underwent elective coronary angiography according to medical indications;
- control group (n = 8) included patients who had a negative observation of CAD.

The blood sample in each case was taken before coronary revascularisation. All patients had a coronary angiography, and then according to the indications, percutaneous coronary intervention (PCI) of infarct-related coronary artery was performed.

The exclusion criteria were: lack of written consent, not meeting the criteria for inclusion, paraneoplastic syndromes, severe anaemia (with a concentration of a blood haemoglobin less than 7 g/dL), history of chronic heart failure in New York Heart Association class III or IV, or the presence of acute severe heart failure (pulmonary oedema and/or cardiogenic shock).

Plasma miRNA-208a determination

Whole blood was collected in K_2EDTA tubes and subjected to two-step centrifugation. Plasma samples were stored at –80°C. Total RNA containing fraction of miRNA was isolated with mirVana PARIS kit (Ambion) according to the protocol of the manufacturer. MicroRNA-208a expression was analysed using TaqMan-based real-time polymerase chain reaction (PCR) (Applied Biosystems). Synthesis of cDNA and qPCR reactions were prepared with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan MicroRNA assay (Applied Biosystems) according to the manufacturer's protocols. qRT-PCR reactions were carried out on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression level of miRNA-208a was calculated using the ddCt method. The HY3 molecule was used as an endogenous control.

Biochemical analyses

We measured hs-cTnI and CK-MBmass by using the Dimension Xpand Plus Integrated Chemistry System. The analytical sensitivity of cTnI assays were determined to be less than 0.015 ng/mL. Functional sensitivity of cTnI is defined as the lowest concentration of cTnI with a total precision (%CV) of 10%, based on a two replicate per run, one run per day, 20-day reproducibility study. Five serum samples were tested, and the troponin I concentration with a 10% total CV was read from the curve. The functional sensitivity was determined to be approximately 0.04 ng/mL.

Statistical analyses

Statistical analysis was performed using Statistica 8.0. In the process of hypothesis testing the level of significance was set at $p < 0.05$, where the choice is up to the researcher sided critical region. Results with non-normally distribution are presented as the median value and interquartile range. Between group comparisons of distributions were used the Wilcoxon rank test. Correlations among continuous variables were used the Spearman's rank correlation coefficient. Categorical variables are expressed as numbers (percentages) and analysed using Fisher's exact test.

RESULTS

Clinical characteristics of the study groups are listed in Table 1. The STEMI population were mostly male (78.9%), and

Table 1. Clinical characteristics of the study groups. Results are expressed as medians (Q1–Q3)

Characteristics	Control (n = 8)	Stable CAD (n = 12)	STEMI (n = 19)	P		
				Control vs. CAD	Control vs. STEMI	CAD vs. STEMI
Age [years] (Q1–Q3)	60 (58–67)	66 (62–74)	58 (55–65)	0.068	0.141	0.022
Male	3 (37.5%)	5 (41.7%)	15 (78.9%)	0.612	0.037	0.035
Previous MI	0 (0%)	0 (0%)	3 (15.7%)	0	0.233	0.148
Hypertension	7 (87.5%)	10 (83.3%)	7 (36.8%)	0.656	0.016	0.011
Diabetes	1 (12.5%)	1 (8.3%)	3 (15.8%)	0.653	0.663	0.546
Glucose [mg/dL] (Q1–Q3)	100 (89–110)	93 (79–131)	105 (97–115)	0.329	0.241	0.134
Hyperlipidaemia	6 (75%)	1 (8.3%)	13 (68.4%)	0.002	0.558	0.001
TC [mg/dL] (Q1–Q3)	172 (141–214)	153 (139–198)	207 (188–219)	0.231	0.069	0.021
TG [mg/dL] (Q1–Q3)	90 (53–150)	140 (107–169)	114 01 (107–173)	0.061	0.046	0.262
HDL [mg/dL] (Q1–Q3)	63 (44–87)	43 (37–55)	42 (39–48)	0.039	0.005	0.210
LDL [mg/dL] (Q1–Q3)	85 (68–114)	86 (71–118)	135 (116–150)	0.481	0.003	0.004
Creatinine [mg/dL] (Q1–Q3)	0.93 (0.74–1.22)	1.04 (0.85–1.21)	0.92 (0.83–0.98)	0.221	0.407	0.073
eGFR > 60 [mL/min]	2 (25%)	5 (41.7%)	2 (10.5%)	0.392	0.334	0.043
eGFR > 30 [mL/min]	6 (75%)	7 (58.3%)	17 (89.5%)	0.392	0.334	0.043

MI — myocardial infarction; TC — total cholesterol; TG — triglycerides; HDL — high density lipoprotein; LDL — low density lipoprotein; eGFR — estimated glomerular filtration rate; CAD — coronary artery disease; STEMI — ST segment elevation myocardial infarction

the median age was 58.0 years (Q1–Q3: 54.5–65.5). Three (15.7%) patients had a history of previous MI. No patients had any history of advanced chronic kidney disease, and median concentration of creatinine was 0.9 mg/dL (Q1–Q3: 0.81––1.01). The median duration of acute symptoms to the collection of the first blood sample for isolation of miRNA-208 was 130 min (Q1–Q3: 100–140). In 63.1% of STEMI patients the infarct-related artery was completely occluded, in the rest of the patients (36.9%) it was critically narrowed (at least 90% of the diameter). The left main artery was not the culprit vessel in any of the cases. In all STEMI patients, markers of myocardial necrosis (cTnI, CK-MBmass) were significantly elevated, exceeding the 99th percentile standard for the laboratory. The initial diagnosis of a STEMI was confirmed in all patients enrolled to the STEMI group. One patient had a ventricular fibrillation during PCI. None of the patients had acute heart failure manifesting as a pulmonary oedema or cardiogenic shock. Echocardiography was performed in the subsequent days of hospitalisation, and the median left ventricular ejection fraction was 50% (Q1–Q3: 46–54.5%).

In the stable CAD group 41.7% were male, and the median age was 66 years (Q1–Q3: 61.75–74.25). None of the patients had a history of previous MI or chronic advanced kidney disease; median concentration of creatinine was 0.96 mg/dL (Q1–Q3: 0.885–1.123). All patients had a coronary angiography, and in 66% cases there was at least 50% stenosis in at least one coronary artery.

In the control group 37.5% were men, the median age was 60.5 years (Q1–Q3: 58–67.5). None of the patients had a history of previous MI or chronic advanced kidney disease, and the median concentration of creatinine was 0.89 mg/dL (Q1–Q3: 0.775–1.005). Furthermore, 87.5% of patients underwent coronary angiography, with no findings of significant (> 50% stenosis) changes in the coronary arteries.

Concentrations of miRNA-208a were measured in the plasma of 12 healthy controls, in eight patients with stable CAD, and in 19 patients with STEMI. Detectable concentrations of miRNA-208a were seen in only one of the controls and in two patients from the CAD group. However, miRNA expression in these groups was weak and very close to the limit of detection of the assay. The mean level of expression of miRNA-208a in these subjects was used to calculate the increase of miRNA-208a (fold change) in patients with STEMI (Fig. 1). A significant increase of the level of plasma miRNA-208a on admission (time 0) in patients with STEMI was observed. Plasma concentration of miRNA-208a increased within the first 3 h after presentation and reminded increased until 12 h. In the time points 6 h and 12 h the level of miRNA-208a was still significantly higher than those at 0 time. After 24 h and 48 h the concentration of miRNA-208a return to the baseline level.

We next determined the serum concentrations of traditional cardiac markers: cTnI and CK-MBmass (Fig. 2). It is noteworthy that the concentrations of both cTnI and CK-MBmass were below the cut off for MI at the time of admission. The maximum concentrations of both cardiac biomarkers 6 h after admission were stated. The levels of cTnI as well as CK-MBmass were increased during observation up to 48 h.

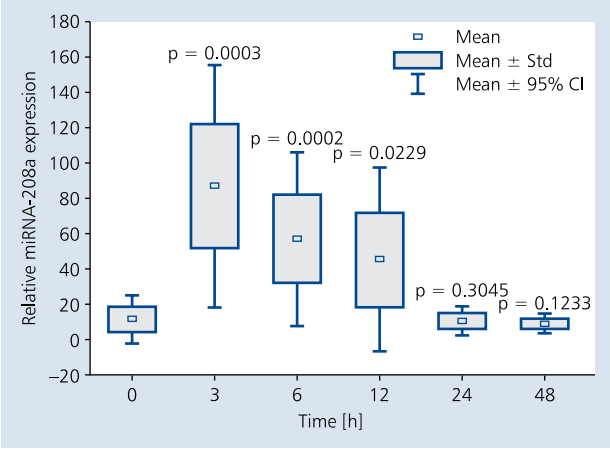

Figure 1. Time course of miRNA-208a plasma levels in patients with ST-segment elevation myocardial infarction

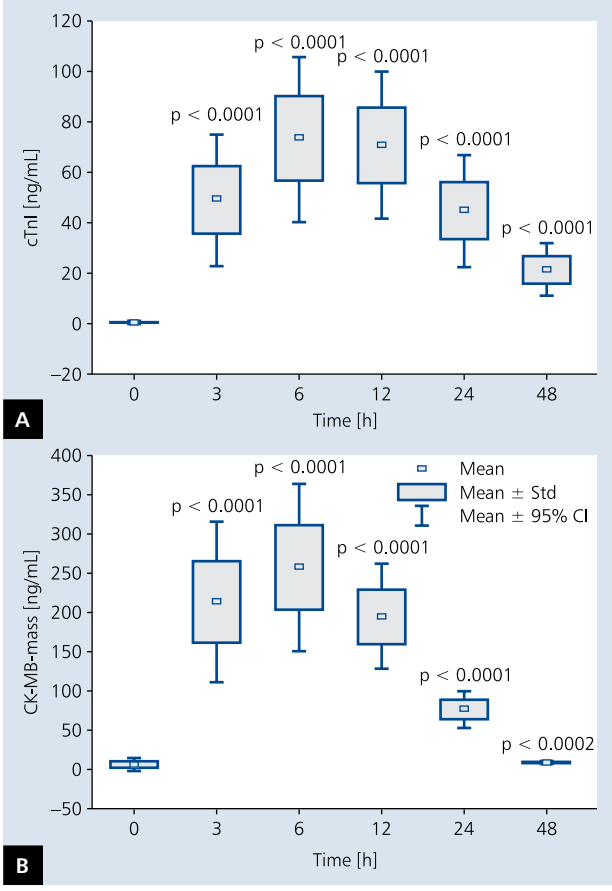

Figure 2. Time course of cardiac troponin I (cTnI) and creatinine kinase (CK)-MBmass plasma levels in patients with ST-segment elevation myocardial infarction

To eliminate the possibility that the observed increase in plasma miRNA-208a was caused by nonspecific insult we also determined whether plasma miRNA-208a expression measured at

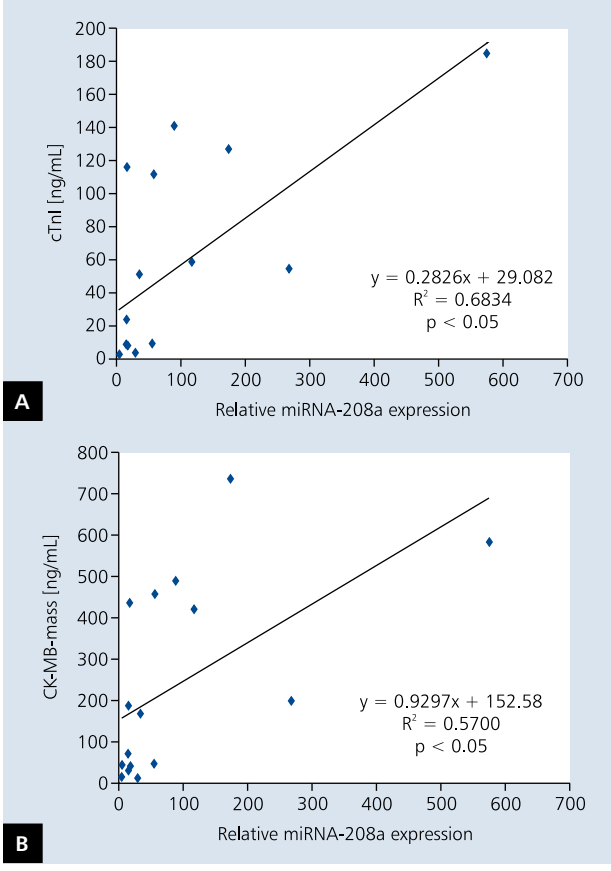

Figure 3. Correlation between miRNA-208a and cardiac troponin I (cTnI) (**A**) and miRNA-208a and creatinine kinase (CK)-MBmass (**B**)

peak (3 h) correlated with the level of both cardiac biomarkers, also measured at the peak their concentrations (6 h). As presented in Figure 3, we observed significant correlations between changes of miRNA-208a and cTnI and CK-MBmass released from the infarct area.

DISCUSSION

The first biomarkers of tissue injury were enzymes that are released into circulation after injury. The subsequent development of immunoassays that quantified proteins expanded the repertoire of specific cardiac proteins of potential clinical utility in the diagnosis of AMI and heart failure. Many of these markers have shortcomings such as reduced sensitivity, insufficient specificity or extended time to diagnosis [3, 4]. Cardiac troponins are currently the best validated biomarkers for the diagnosis of AMI. However, measurable amounts of troponin proteins are usually not released from damaged myocardium within 4–8 h of onset of symptoms [2, 3].

Recent advances in molecular biology and technology have initiated considerable interest in nucleotide-based biomarkers [9]. Accumulating evidence suggest the usefulness of circulating miRNAs as a stable blood-based biomarker for

the detection of tissue injury [10–14]. Various studies have demonstrated that miRNAs are released into the peripheral blood during AMI [15–18]. Increased circulating levels of miRNA-1, miRNA-133a, miRNA-133b, and miRNA-499, which are highly expressed in the skeletal muscle as well as in the heart, were observed in several studies investigating AMI patients and animal models [19, 20].

In terms of specificity, cardiac-restricted miRNA-208 represents a very attractive candidate. MicroRNA-208 is encoded by an intron of the alpha-myosin heavy chain gene. The miRNA-208 family includes two subfamilies: miRNA-208a and miRNA-208b. miRNA-208 is involved in cardiomyocyte hypertrophy, fibrosis, and regulation of other cardiac muscle gene expression and function [21]. Two studies found that miRNA-208 was very low or absent in control subjects and that its plasma levels were sharply increased upon AMI [18, 22]. However, other human studies measured detectable levels of miRNA-208 only in a sub-set of AMI patients, questioning the sensitivity of miRNA-208 as an AMI biomarker [20, 23]. Interesting results were reported by Fichtlscherer et al. [24], who found that in patients with stable CAD, plasma miRNA-126, miRNA-17, miRNA-92a, and miRNA-155 were decreased, while miRNA-133a and miRNA-208a were increased.

Our data show that the plasma concentration of miRNA-208a, which is produced exclusively in the heart, increases in STEMI and/or reperfusion-induced myocardial injury. Circulating miRNA-208a was hardly detectable, very close to the detection limit of the assay in healthy humans and in patients with stable CAD. A rise in miRNA-208a in plasma was evident (10-fold increase) at the time of admission (less than 3 h after onset of chest pain) in patients with STEMI when cTnI was not yet affected. This indicates that miRNA-208a may leak into the bloodstream at an earlier stage of myocardial injury. In addition, miRNA-208a achieved its peak before both cTnI and CK-MBmass (3 h vs. 6 h, respectively). The plasma expression of miRNA-208a shows a good correlation with the classic markers of myocardial damage at the time of their maximal concentrations. More recently, unexpected results were published by Nabialek et al. [25]. It was demonstrated in this study that circulating serum miRNA-208a levels are unchanged in AMI patients at six, 12, and 24 h after PCI. These differences may be due to the measuring time. It is possible that these time-points represent the times in which the release of miRNA-208a into the circulation is decreasing. In a very interesting study, Zile, et al. [26] demonstrated late release of miRNA-208 in patients after MI. The miRNA-208 increased five days post AMI and remained elevated for up to 90 days. These time-dependent changes were accompanied by a progressive increase of left ventricular and diastolic volume. From our and other results it may be suggested that the release of miRNA-208 after onset of AMI is biphasic. Early release is probably due to myocardial ischaemia and, in contrast, late release to metabolic changes leading to left ventricular structural remodelling.

However, the release mechanism of miRNAs is unclear, but they may be freed to the bloodstream as a consequence of passive release of the cell contents as apoptotic bodies, exosomes, and microparticles [27, 28]. Microparticles containing miRNAs may be formed during myocardial ischaemia as well as during necrosis. The findings of our study do not enable us to establish whether circulating miRNA-208a is an indicator of cardiac necrosis and/or myocardial ischaemia. Early release (before cTnI) and decrease after successful reperfusion seem to suggest that miRNA-208a is marker of myocardial ischaemia rather than necrosis.

Limitations of the study

It should be noted that the consideration of circulating miRNA-208a as a biomarker for AMI is at present based on the results from a relatively small sample. Larger clinical studies are definitely required to establish the clinical usefulness of miRNAs in the early diagnostics of MI. Nonetheless, the present study lays the groundwork for future efforts to establish precise mechanisms leading to expression of miRNA-208a in the circulation after AMI and also to identify of miRNA-208a as a novel class of cardiac biomarker and its diagnostic and prognostic significance.

CONCLUSIONS

There is an increased level of miRNA-208a plasma concentration in STEMI patients, not detected in stable CAD undergoing PCI and individuals without coronary disease. In STEMI patients the miRNA-208a increases within first 3 h after onset of chest pain, before any rise of cTnI and CK-MBmass is detected. MicroRNA-208a exhibited a very short life in the circulation during AMI and may be promising new marker for early AMI diagnosis.

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Conflict of interest: *none declared*

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Kinetyka uwalniania krążącego miRNA-208a we wczesnej fazie zawału serca

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Streszczenie

Wstęp: Biochemiczne potwierdzenie zawału serca bazuje na oznaczaniu stężenia troponin sercowych: cTnI lub cTnT. Wiele badań wskazuje, że nowym obiecującym biomarkerem uszkodzeń narządowych mogą być cząsteczki mikroRNA (miRNA).

Cel: Celem badania było określenie kinetyki ekspresji krążącego miRNA-208a oraz weryfikacja hipotezy, że krążące cząsteczki sercowo swoistego miRNA-208a mogą być użytecznym markerem diagnostycznym zawału serca z przetrwałym uniesieniem odcinka ST (STEMI).

Metody: Przebadano 19 pacjentów z STEMI (4 kobiety i 15 mężczyzn w wieku 44–85 lat), 12 osób ze stabilną chorobą wieńcową (CAD) oraz 8 osób z negatywną obserwacją CAD jako grupę kontrolną. Krew pobierano w momencie zgłoszenia (czas 0) oraz po 3, 6, 12, 24 i 48 godzinach; w grupie CAD i kontrolnej krew do badań pobierano jednorazowo. Osoczowy miRNA-208a oznaczano w rekcji RT-PCR i obliczano jego względny przyrost. W próbkach surowicy krwi oznaczano stężenie cTnI i CK-MBmass.

Wyniki: Oznaczalne, podwyższone stężenie miRNA-208a stwierdzono u pacjentów z STEMI w momencie przyjęcia, podczas gdy w grupie CAD i kontrolnej było bardzo niskie, w wielu przypadkach nieoznaczalne. Najwyższe stężenia miRNA-208a zaobserwowano w trzeciej godzinie po reperfuzji ($p < 0{,}001$). Tradycyjne biomarkery cTnI i CK-MBmass wzrastały później, osiągając maksimum w 6. godzinie po reperfuzji. Stężenie krążącego miRNA-208a silnie korelowało ze stężeniami uwolnionych z obszaru zawału cTnI i CK-MBmass.

Wnioski: Uzyskane wyniki wskazują, że miRNA-208a jest obiecującym markerem uwalnianym do krążenia we wczesnej fazie zawału, który może być przydatny w jego diagnostyce.

Słowa kluczowe: miRNA-208a, zawał serca, markery sercowe

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