MINERVA CARDIOANGIOLOGICA

VOLUME 61 · N. 6 · DICEMBRE 2013

EDIZIONI · MINERVA · MEDICA

PUBBLICAZIONE PERIODICA BIMESTRALE - POSTE ITALIANE S.P.A. - SPED. IN A. P. D.L. 353/2003 (CONV. IN L. 27/02/2004 N° 46) ART. 1, COMMA 1, DCB/CN - ISSN 0026-4725 TAXE PERÇUE

Circulating microRNAs (miR-423-5p, miR-208a and miR-1) in acute myocardial infarction and stable coronary heart disease

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***Aim*. The microRNAs (miRs) are small non-coding RNAs which regulate expression of multiple genes involved in atherogenesis. MicroRNA are also present in circulation. The aims of this study were: 1) assessment of expression level of miR-1, miR-208a and miR-423-5p in plasma in patients with STEMI, stable CAD and healthy individuals; 2) evaluation of correlation between plasma miRs and left ventricle ejection fraction, end-systolic and end-diastolic diameters and troponin release in patients with STEMI.**

***Methods*. Study group consisted of 26 patients: 1) acute MI group (N.=17); 2) stable CAD group (N.=4); and 3) subjects with no history of CAD (control group, N.=5). Expression of miR-423-5p, miR-208 and miR-1 was measured in plasma before PCI, 6, 12 and 24 hours later. Expression level ofmiRs was measured using TaqMan® MicroRNA Assays. Expression was assessed by Pfaffl method, and miR-39 was used for normalization of the results.**

***Results*. In stable CAD in comparison to control group the expression level of miR-1, miR-208a and miR-423-5p did not show significant differences. Also there was no significant increase of number of miR copies at 6, 12 and 24 hours after PCI. There was a significantly higher number of miR-423-5p copies in patients with acute MI before the pPCI. After 6, 12 and 24 hours post-procedure the expression level was similar to the control group and significantly lower than the baseline level. Conversely, the expression level of miR-1 and miR-208a were not significantly different than in the control group. In patients with acute MI there were no significant correlations between the expression level of miRs and any of the echocardiographic parameters of LV as well as level of troponin I at any time-point of the follow-up.**

***Conclusion*. Early in acute myocardial infarction the expression of miR-423-5p in plasma is significantly increased with subsequent normalization within 6 hours. Potentially it is an early marker of myocardial necrosis.**

KEY WORDS: microRNAs - Myocardial infarction - Coronary disease.

The microRNAs (miRs) are non-coding RNAs (22 nucleotides long) which regulate translation and degradation of mRNAs and thus modulate expression of multiple genes, so can be regarded as pivotal post-translational regulators of cell function. Mi-

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cro RNAs are part of developmentally conservative mechanism of regulation of gene expression and were first discovered in cytoplasm of *C. elegans* cells.[1] So far about 1000 of miR particles have been identified in human genome. Genes coding miRs are located in several parts of chromosomes including introns. MiRs precursors (pri-miR) are synthesized as a few hundred base-pairs long particle by enzyme – RNA polymerase II. Then it is transformed to pre-miR by rybonuclease III – Drosha and extracted to cytoplasm by exportin-5. Pre-miR is transformed to about 22 nucleotide miRs in cytoplasm by enzyme called Dicer. Then it becomes a part of RNA-protein complex called RICS (RNA Silencing Complex) and performs its basic function. MiRs are involved in key metabolic pathways involved in the development of atherosclerosis, such as lipid metabolism, secretion of insulin as well as the regulation of endothelial and vascular smooth muscle cells function as well as platelet activity.[2] MiRs are also involved in the biology of stem and progenitor cells, cardiac lineage development and repair following ischemic injury.[3]

Several studies have also revealed the presence of miRs in the circulatory system. This sort of miRs are known as circulating micro RNAs. MicroRNA are also present in circulation in association with microparticles. There are a few theories about circulating miR function, *e.g.*, intercellular communication hypothesis or cellular destruction hypothesis. Various cardiovascular diseases such as acute coronary syndromes (ACS) and stable coronary artery disease (CAD) are characterized by specific signature of circulating miRs (Figure 1). Recently published studies confirmed the role of selected circulating miRs as biomarkers of myocardial injury in acute myocardial infarction (MI) and heart failure. However, the role of miRs in ACS still needs to be clarified. In this paper we assessed the role of several circulating miR particles (miR-423-5p, miR-208a and miR-1) in ST-segment elevation MI (STEMI).

Circulating miR-423-5p seems to serve as heart failure biomarker. However, target gens for that particle still are not identified. MiR-208 is widely known as cardiac specific particle. Function of miR-208a is connected with cardiac development and heavy myosin chain formation. It also seems to regulate insulin-induced smooth muscle cells proliferation.[4] Some animal model based studies also suggest that miR-208 can serve as potential target of heart failure prevention.[5] Furthermore miR-1 occurs as muscle tissue specific. It has been recognized as regulator of HSP-60 expression during MI and as pivotal regulator of skeletal muscle differentiation.[6, 7] Moreover, animal studies revealed that miR-1 can be involved in cardiac arrythmia *via* modulation of expression of KCNJ2, GJA1 and PP2A proteins.[8, 9] MiR-1 particle is also involved in regulation of Ca^{2+}-dependent signaling pathway regulating myocardial contractility.[10] Circulating miRs are potentially applicable as a novel biomarkers of many cardiovascular diseases. In this context, the assessment of post-infarction miRs release from cardiomyocytes may serve as an early diagnostic tool of myocardial injury.

The aims of this study were: 1) assessment of expression level of miR-1, miR-208a and miR-423-5p in plasma in patients with STEMI, stable CAD and healthy individuals; 2) evaluation of correlation between plasma miRs and left ventricle ejection fraction (LVEF), end- systolic (ESD) and end-diastolic (EDD) diameters and troponin release in patients with STEMI.

Materials and methods

Patients

Study group consisted of 27 patients: 1) acute MI group (N.=17); 2) stable CAD group (N.=5); and 3) subjects with no history of ischemic heart disease (control group, N.=5). The study protocol conforms to the Declaration of Helsinki and has been approved by the Ethics Committee of the Medical University of Silesia. All subjects signet written informed consent.

Inclusion criteria. 1) age 18-80 years; 2) written, informed consent; 3) MI group:

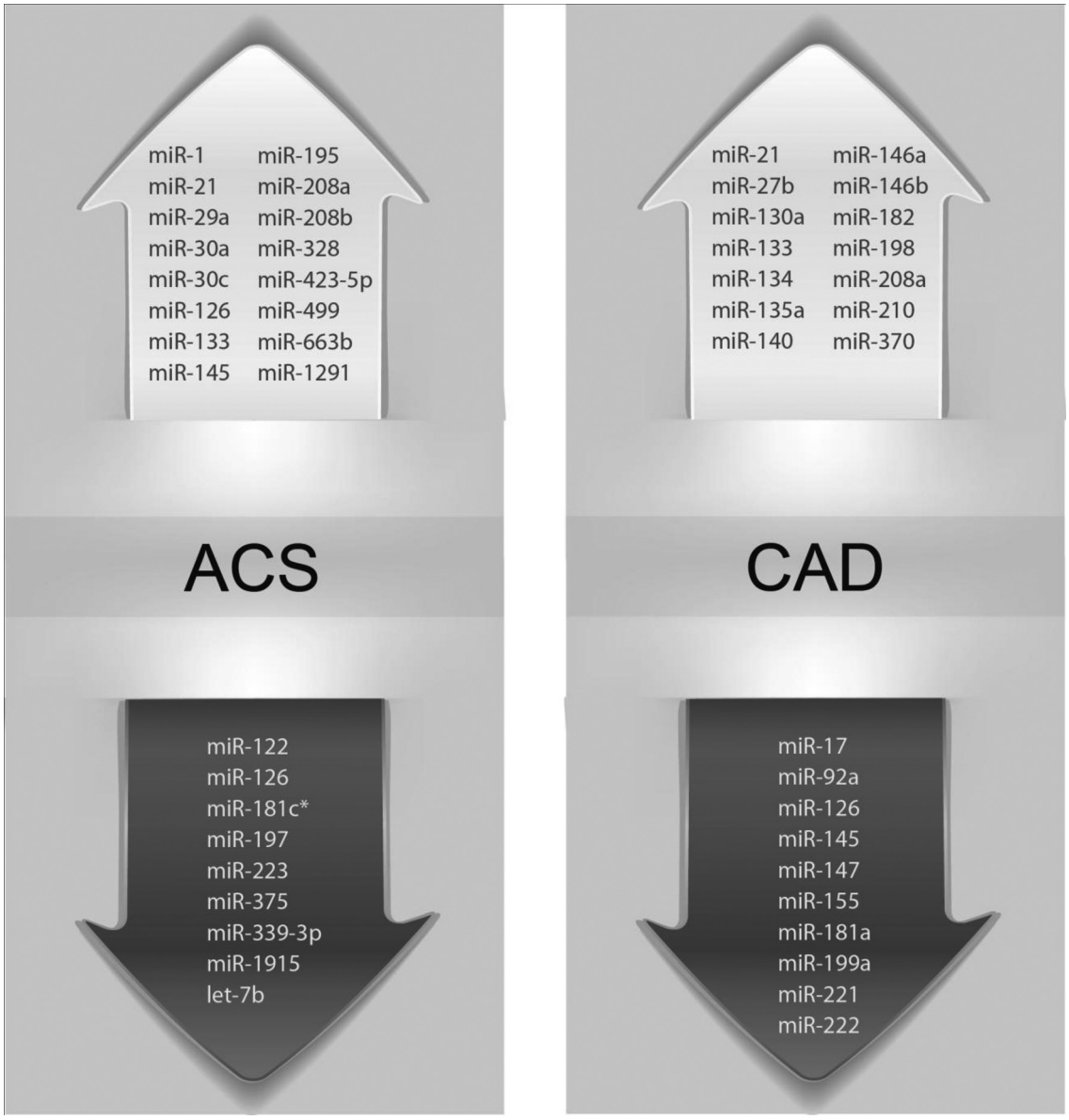

Figure 1.—The overview of circulating miRs signature in acute coronary syndrome (ACS) (A), and coronary artery disease (CAD) (B).

patients were diagnosed according to the current ST-elevation myocardial MI (STEMI) definition of the European Society of Cardiology, time interval between the onset of chest pain and primary PCI(pPCI) <12 hours. Abciximab was administered according to the decision of pPCI operator. All patients received unfractionated heparin (70 U/kg) to achieve ACT values >250s. In all patients TIMI3 flow in the infarct-related artery was achieved. Statins were administered starting from the first day of hospitalization; 4) stable CAD group diagnosed according to (a) typical clinical symptoms (angina reproducibly associated with physical exercise); (b) non-invasive test (positive treadmill stress test) and presence of ≥1 significant stenotic lesion (≥70%) in coronary artery amenable for elective PCI (ePCI); 5) control group individuals were free from

history of CAD. Patients treated with PCI received loading dose of clopidogrel (600 mg) with maintenance dose of 75 mg per day for 12 months.

Exclusion criteria: 1) acute myocardial infarction within 30 days prior to enrolment; 2) PCI or CABG in the last 30 days; 3) renal failure; 4) liver failure; 5) neoplastic disease; 6) autoimmune disease; 7) COPD; 8) infectious disease; 9) positive pregnancy test; 10) myopathies; 11) muscle injuries within 30 days prior to enrolment; 12) lack of informed consent.

Sample preparation and laboratory analysis

Blood samples

Blood samples were taken immediately before PCI, 6, 12 and 24 hours later in acute MI and stable CAD groups. Samples in the control group were taken once. The vacuum system (Vacutainer, Trisodium citrate) was used to take patient's blood sample from cubital vein (5 mL). Within 1 hour the sample was centrifuged at 4 °C for 10 min (1550 x g). Afterwards, the supernatant was transferred to RNase/DNase free tube and stored at -80 °C until miRs measurement.

Isolation and measurement of miR

Total RNA was isolated and purified with commercially available kit (mirVana PARIS, Ambion, CA, USA) according to the instruction. Synthetic cel-mir-39 RNA was added to the serum as a reference to control sample-to-sample variations of the RNA isolation efficiency.miR copy number was determined with TaqManTaqMan® MicroRNA Assays applying ABI 7900HT Fast Real-Time PCR System (ABI, CA, USA). The relative quantity of miR-1, miR-208a and miR-423-5p was determined from ΔΔCt. Selected miRs to cel-mi-39 RNA gene ratio was evaluated with the Pfaffl mathematical model.[11] Briefly, expression was calculated normalizing for expression of reference gene as a ratio of particular miR to miR-39 (reference gene).

Echocardiographic study

Echocardiography was performed after admission to hospital (<12 hours of chest pain symptoms) by experienced echocardiolographer. Transthoracic echocardiography (M-mode and typical 2D projections) was carried out in accordance to the American Society of Echocardiography guidelines. Evaluated echocardiography parameters were: left ventricle end-diastolic (EDD) and end-systolic (ESD) diameter and left ventricle ejection fraction (EF%) according to Simpson method.

Statistical analysis

Continuous variables are expressed as mean ± SD or median (interquartile range). Relative changes of the parameters with non-normal distribution were compared between groups using U Mann-Whitney test. Correlations between the number of miR copies and echocardiographic parameters were analyzed using the Spearman correlation test. Values of $P<0.05$ were considered statistically significant. Statistica 6.0 software was used for the analysis.

Table I.—*Baseline characteristics of the study groups.*

	Acute MI N.=17	Stable CAD N.=5	Control N.=5	P-value
Age (mean)	60	60.4	48.6*	P<0.05 *vs.* MI and CAD
Males, N. (%)	11 (65%)	4 (80%)	3 (60%)	P=NS
Hypertension, N. (%)	11 (65%)	4 (80%)	2 (40%)	P=NS
Diabetes, N. (%)	3 (18%)	1 (20%)	1(20%)	P=NS
Smoking, N. (%)	10 (59%)*	1 (20%)	2 (40%)	P<0.05 *vs.* MI and CAD
Hypercholesterolemia, N. (%)	8 (47%)	4 (80%)*	1 (20%)	P<0.05 *vs.* control and MI
Creatinine (μmol/L)	0.89	0.84	0.71	P=NS
Peaktroponin I (ng/mL), (mean±SD)	3,084±3,39	-	-	-

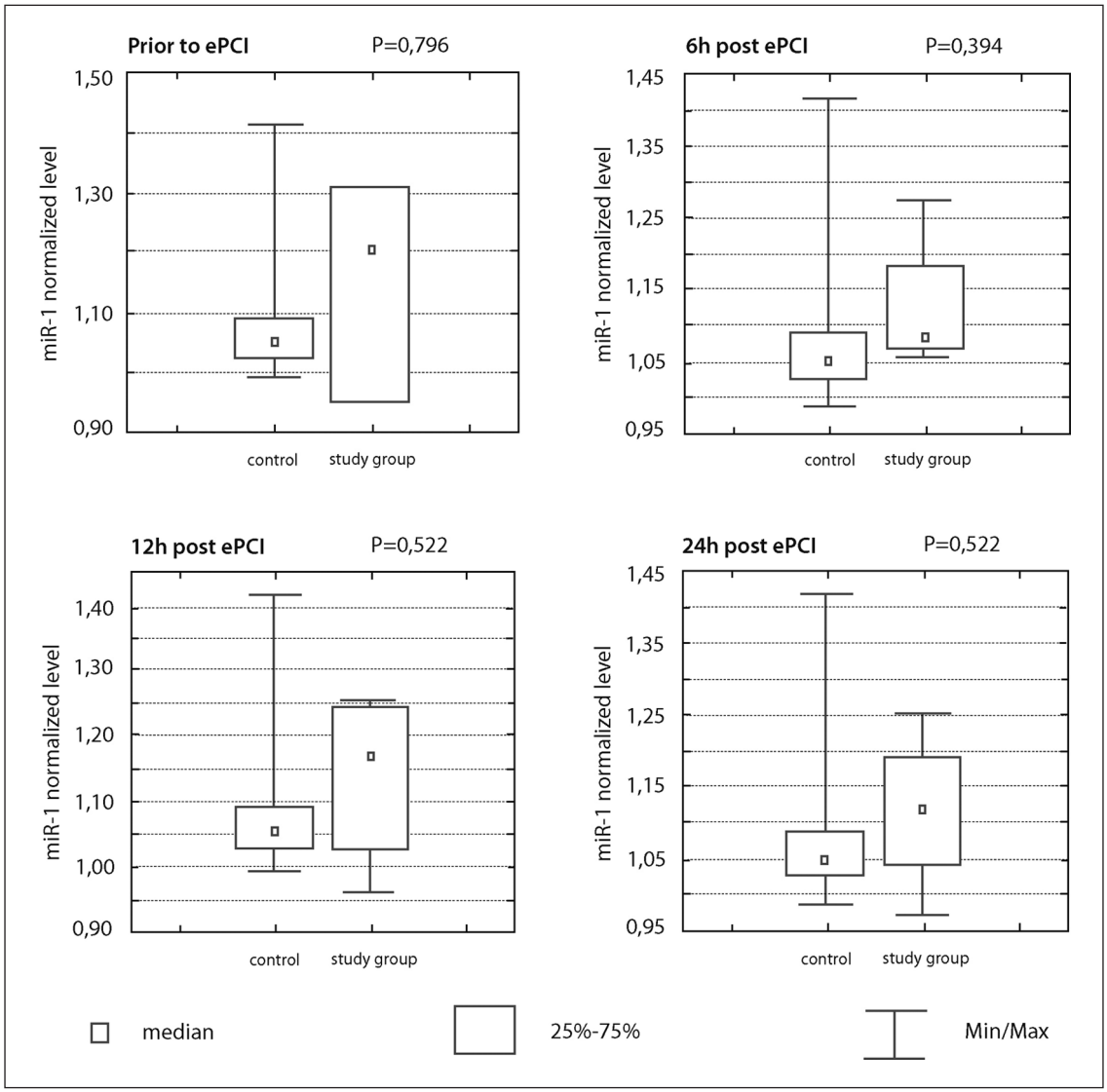

Figure 2.—miR-1 expression level in patients with stable CAD.

Results

The characteristics of the study group is shown in Table I. Control subjects were significantly younger than both groups with CAD. Also in stable CAD group the number of patients with hypercholesterolemia was significantly higher than in the acute MI and control groups. MI patients were significantly more often active smokers. There was numerically higher number of males in all groups. All PCI procedures were carried out with clinical and angiographic success. There were no deaths, repeat revascularizations or bleeding events during hospitalization.

Expression level of miR in stable CAD

Expression level of circulating miRs in stable CAD is shown in Figures 2-4. In comparison to control group the expression level of copies of miR-1 did not show statistically significant differences although normalized expression level was higher than in

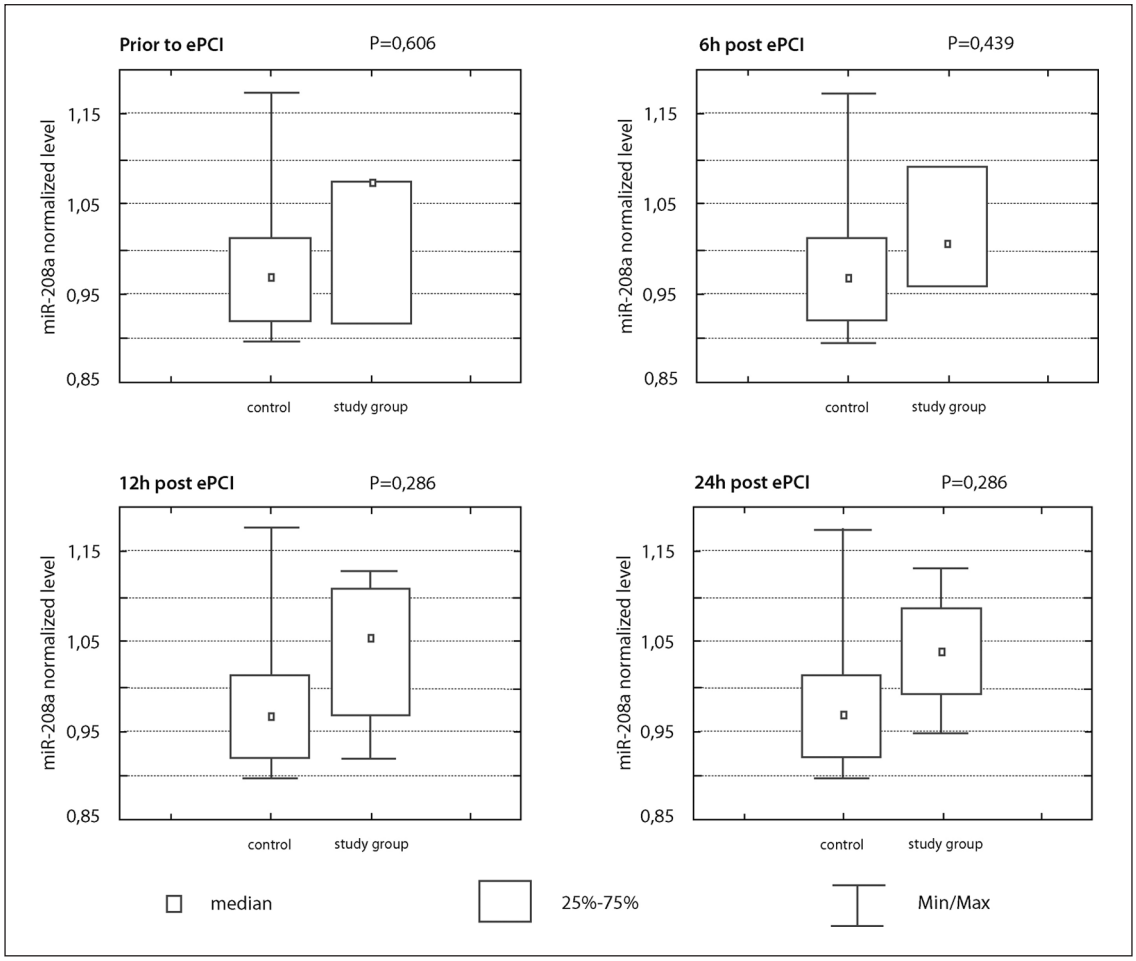

Figure 3.—miR-208a expression level in patients with stable CAD.

the control group. Also there was no significant changes in miR-1 copies during the 24-hour follow-up (Figure 2). There were no significant differences in miR-208a copies at baseline and 6-24 hours after elective PCI in comparison to control group (Figure 3). Number of miR-423-5p copies was numerically higher than in control group however the differences were not statistically significant and there was no significant increase of expression level at 6, 12 and 24 hours after PCI (Figure 4).

Circulating miRs in acute MI

We have observed a significantly higher expression level miR-423-5p in patients with acute MI before the pPCI. After 6, 12 and 24 hours post procedure the expression level was similar to the control group and significantly lower than the baseline level (Figure 5). Conversely, the expression level of miR-1 (Figure 6) and miR-208a (Figure 7) were not significantly different than in the control group. There was also no change in the level of these miRs after 6, 12 and 24 hours.

In patients with acute MI there were no significant correlations between the number of miR-423-5p, miR-1 and miR-208a and any of the echocardiographic parameters of LV ant any time-point of the follow-up. Also we observed no significant correlation between miRs and peak levels of troponin I (data not shown).

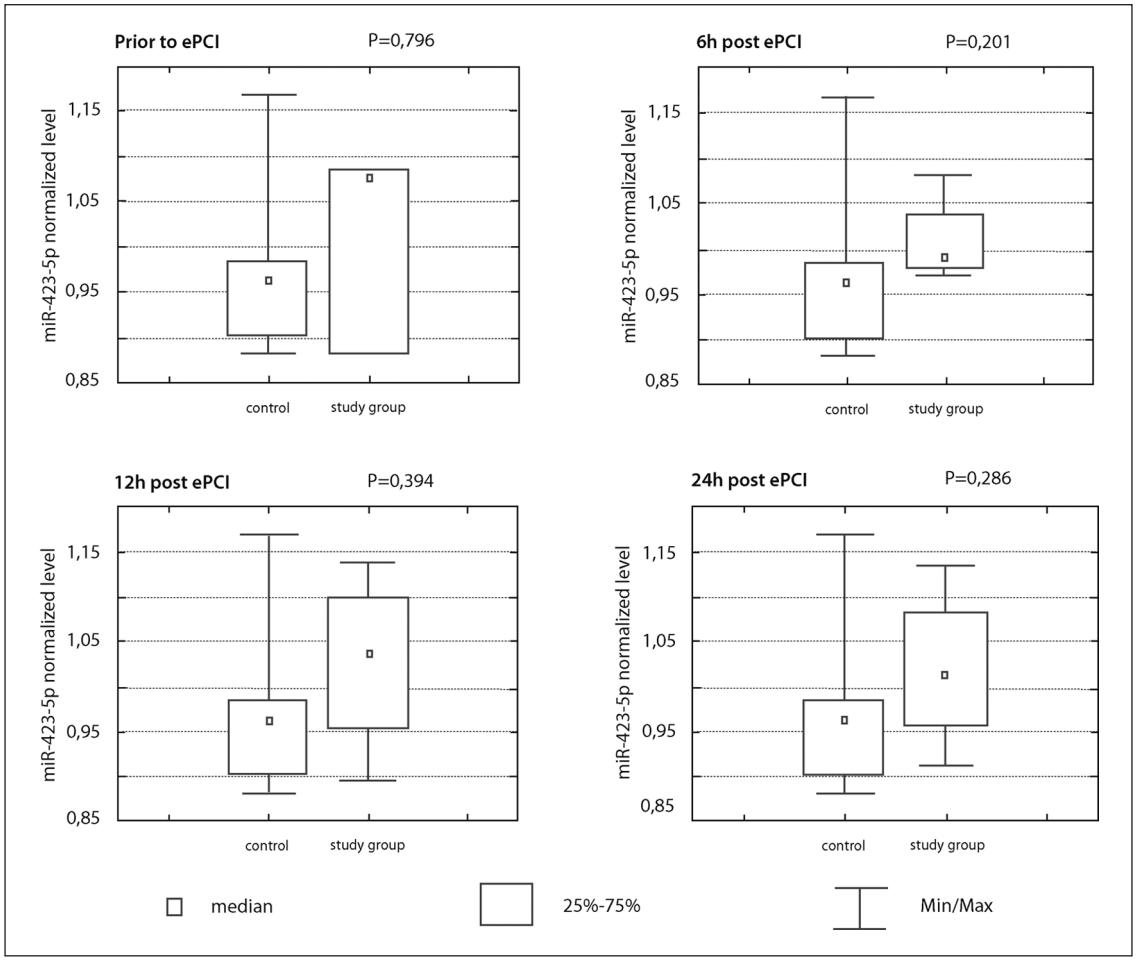

Figure 4.—miR-423-5p expression level in patients with stable CAD.

Discussion

We evaluated the changes of circulating miRs in patients with stable CAD undergoing elective PCI and patients with acute MI treated with primary PCI. We observed no significant differences between number of miR-423-5p, miR-1 and miR-208a copies before and 6-24 hours after PCI in patients with stable CAD. In acute MI there was a significantly higher level of miR-423-5p prior to revascularization. After pPCI there was no further increase of this miR copies in peripheral blood. Also we found no differences in miR-1 and miR-208a in acute MI. Furthermore there were no significant correlation between LVEF and troponin I release and studied miRs. The influence of myocardial necrosis in the setting of MIon miR-1 expression has already been investigated in several studies. However miR-1 is not specific for myocardium but it is a muscle-specific miR. First report focused on both animal and human models was presented by Cheng Y et al. Animal model revealed significantly increased level of miR-1 in MI-induced rats with peak 6 hours after MI. The results from clinical studyof 31 patients within 24 hours of STEMI confirmed the findings obtained in animal model. It proved that circulating miR-1 level is increased in acute MI. Moreover, it revealed significant correlation with CK-MB peak ($r=0.68$; $P<0.05$).[12] Similar results were ob-

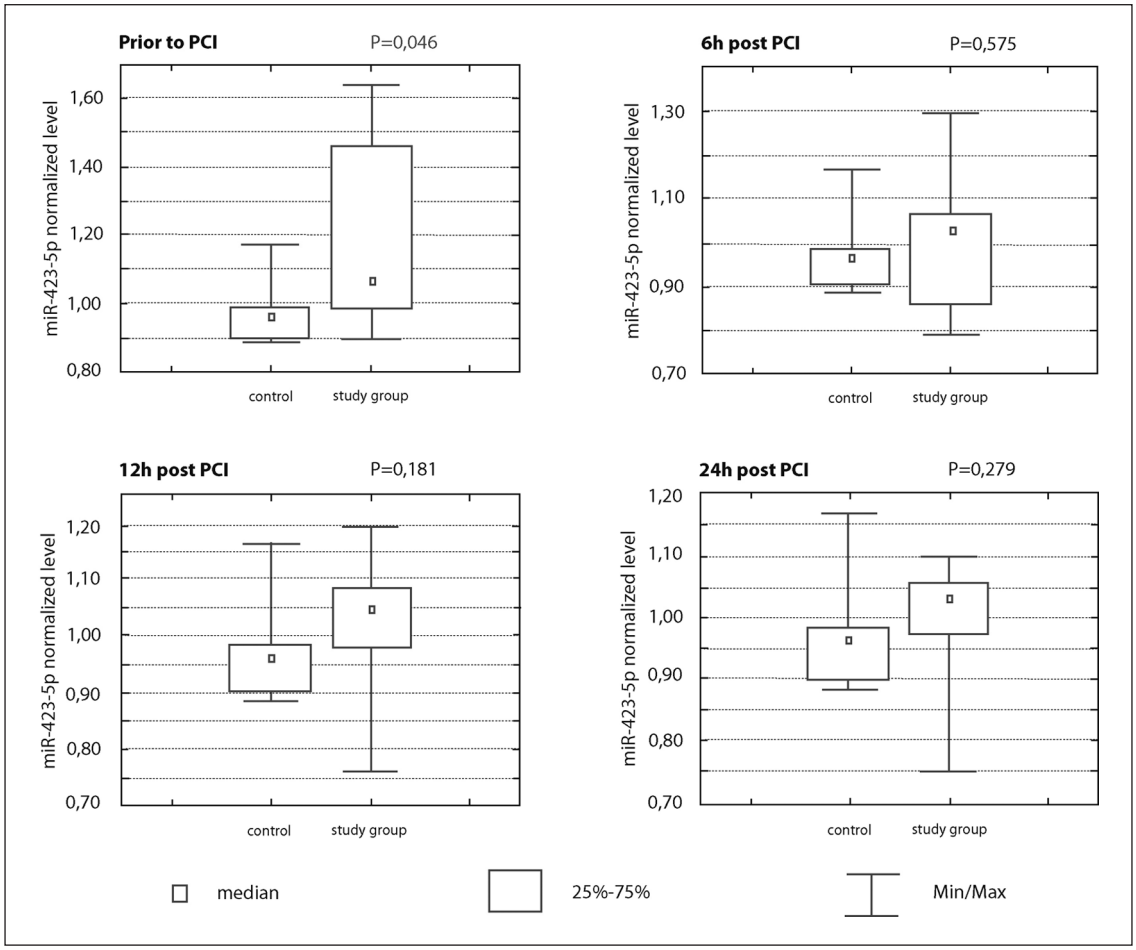

Figure 5.—miR-423-5p expression level in patients with acute MI.

tained by Long *et al*. They confirmed that miR-1 level is significantly elevated in 4, 8, 12, 24, 48, 72 hours after MI. They also confirmed correlation between troponins level and miR-1 level after MI (17).[13] Furthermore Kuwabara Y *et al*. carried out investigations in a group of 72 patients. The obtained results confirmed elevated circulating miR-1 level and decreased intracellular miR-1 level in patients after MI. The authors suggested to consider different mechanism of selected miR extraction.[14] The results obtained in our studies do not confirm the above one. We did not indicate statistically significantly elevated level of circulating miR-1 both in patients with STEMI and CAD. However there was a trend towards increased expression level. Our data concerning miR-1 were similar to the results obtained by Corsten *et al*. who did not observe significantly elevated level ofcirculating miR-1 in patients after AMI.[15]

It has been known that miR-208a is recognized as myocardial specific type of miR. Guo-Kun Wang *et al*. evaluated the potential role as ischemia biomarker of several cardiac specific and muscle tissue-specific microRNA. They confirmed myocardial origin of miR-208a. Moreover, they revealed that miR-208a can detect MI with 90.9% sensitivity at 100%, specificity. The most significant feature of miR-208a seems to be its peak at 1 hour after MI. That leads us to consider miR-208a as a valuable marker of

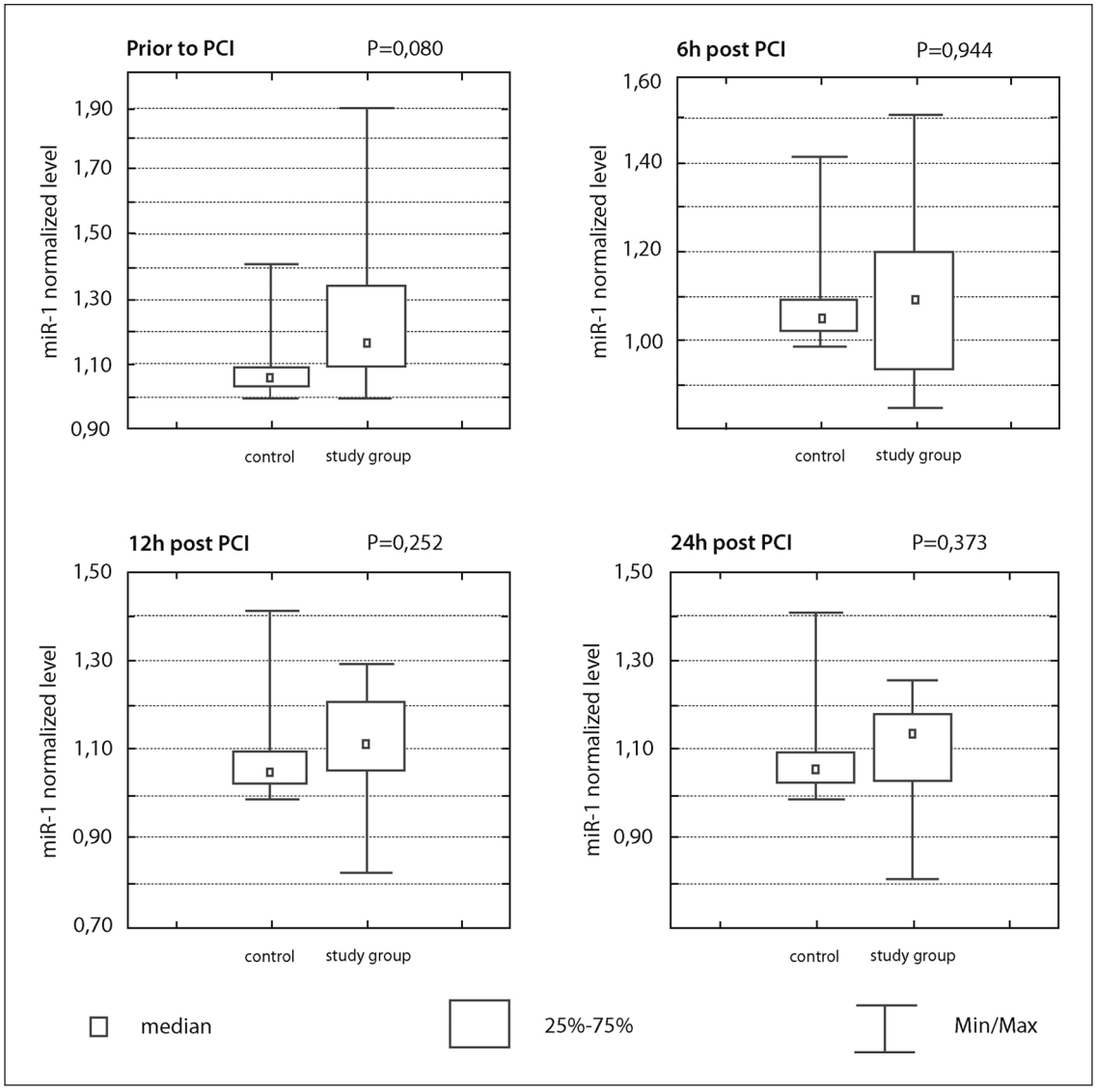

Figure 6.—miR-1 expression level in patients with acute MI.

cardiac ischemia and supplement for cardiac specific troponins.[16] The role of several micro RNAs was also investigated by another group of authors. This study was based on the similar group of patients. However, the authors focused on miR-208b which has common origin particle with miR-208a. It has been revealed that MI-induced significant increase of miR-208b and also correlation between miR-208b and troponin T level during AMI was demonstrated.[15] Our data are different from the above results. We did not observe elevated level of miR-208 after PCI and after 6, 12, 24 hours both in patients after AMI and with CAD.

The influence of MI on miR-423-5p level was investigated in our study. It has previously been confirmed on 12 patients group that miR-423-5p level is significantly increased in patients with HF.Correlations between miR-423-5p NT-proBNP and EF were also revealed.[17] Our data indicate significantly increased level of miR-423-5p in patients after AMI at time 0 (P=0.045).

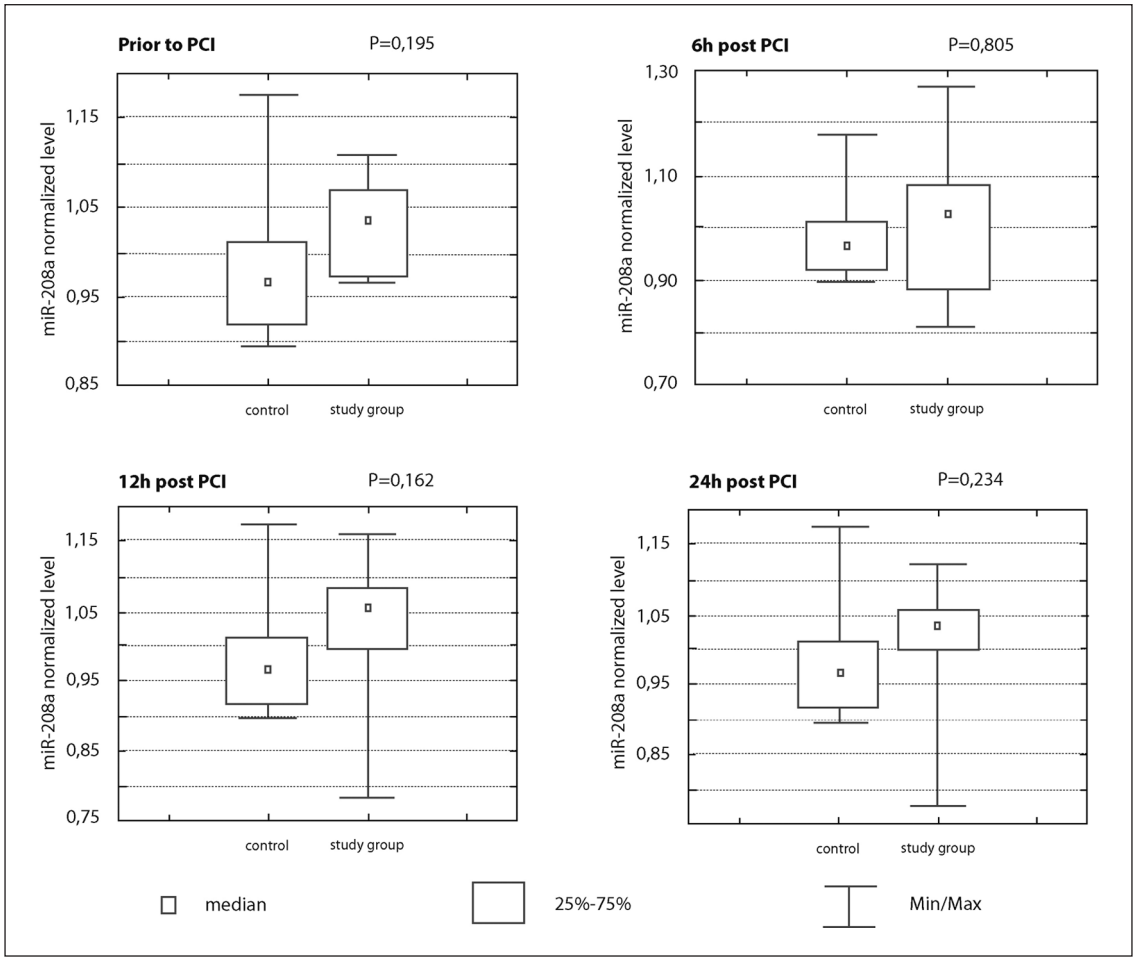

Figure 7.—miR-208a expression level in patients with acute MI.

We believe that the obtained results may be consequence of HF in our group of patients. We observed the expression of this parameter in the dynamic process, *i.e.*, in 6, 12 and 24 hours after AMI and the obtained results did not reach statistical significance. The interpretation of these facts is difficult. However we suggest that miR-423-5p might serve as a biomarker of AMI.

The differences in our results in comparison to the results obtained in other studies may be caused by relatively small group of patients and the differences in methodology of miR detection. Therefore further studies should be performed to confirm the role of miR-423-5p as a biomarker and clarify the role of miR-208 and miR-1 in AMI patients.

Conclusions

In the early phase of acute MI plasma miR-423-5p is significantly up-regulated and the expression level declines after revascularization of the infarct related artery.

Riassunto

MicroRNA circolanti (miR-423-5p, miR-208a e miR-1) nell'infarto miocardico acuto e nella cardiopatia coronarica stabile

Obiettivo. I microRNA (miR) sono piccole molecole di RNA non codificante che regolano l'espressione di molteplici geni coinvolti nell'aterogenesi. I microRNA sono anche presenti nel circolo. Obiettivo del presente studio è stato quello di: 1) valutare

il numero di copie di mir-1, miR-208a e miR-423-5p nel plasma di pazienti con STEMI, CAD stabile e individui sani; 2) valutare la correlazione tra i miR nel plasma e la frazione di eiezione del ventricolo sinistro, i diametri telesistolico e telediastolico e il rilascio di troponina in pazienti con STEMI.

Metodi. Il gruppo sperimentale era composto di 26 pazienti: 1) gruppo con IM acuto (N.=17); 2) gruppo con CAD stabile (N.=4) e 3) soggetti senza storia clinica di CAD (gruppo di controllo, N.=5). L'espressione di miR-423-5p, miR-208 e miR-1 è stata misurata nel plasma prima dell'intervento coronarico percutaneo, 6, 12 e 24 ore dopo. Il numero di copie di miR è stato misurato utilizzando il saggio TaqMan® per MicroRNA. L'espressione è stata valutata mediante il metodo Pfaffl e il miR-39 è stato utilizzato per la normalizzazione dei risultati.

Risultati. Rispetto al gruppo di controllo, nella CAD stabile il numero di copie di miR-423-5p, miR-208 e miR-1 non ha mostrato differenze significative. Non vi è stato neanche nessun aumento significativo nel numero di copie miR a 6, 12 e 24 ore dopo l'intervento coronarico percutaneo. Vi è stato un numero significativamente più elevato di copie miR-423-5p nei pazienti con IM acuto prima dell'intervento coronarico percutaneo primario. Sei, 12 e 24 ore dopo la procedura, il numero di copie era simile al gruppo di controllo e significativamente più basso rispetto al livello basale. Al contrario, il numero di copie di miR-1 e miR-208a non era significativamente diverso rispetto al gruppo di controllo. Nei pazienti con IM acuto non vi erano correlazioni significative tra il numero di miR e i parametri ecocardiografici del VS, oltre che il livello di troponina I in qualunque momento di osservazione del follow-up.

Conclusioni. Nella fase iniziale dell'infarto miocardico acuto, l'espressione di miR-423-5p nel plasma è significativamente aumentata con una successiva normalizzazione entro 6 ore. Questo è un potenziale marcatore precoce di necrosi del miocardio.

PAROLE CHIAVE: microRNAs - Miocardio, infarto - Coronaropatia.

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Funding.—The study was funded by the Polish Cardiac Society and Servier grant and by statutory funds of Medical University of Silesia.

Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.