Original article

The relationships among monocyte subsets, miRNAs and inflammatory cytokines in patients with acute myocardial infarction

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ABSTRACT

Background: Acute myocardial infarction (AMI) causes irreversible myocardial damage and release of inflammatory mediators, including cytokines, chemokines and miRNAs. We aimed to investigate changes in the levels of cytokines (IL-6, TNF-α and IL-10), miRNAs profiles (miR-146 and miR-155) and distribution of different monocyte subsets (CD14++CD16-, CD14++CD16+, CD14+CD16++) in the acute and post-healing phases of AMI.

Methods: In eighteen consecutive AMI patients (mean age 56.78 ± 12.4 years, mean left ventricle ejection fraction – LVEF: $41.9 \pm 9.8\%$), treated invasively, monocyte subsets frequencies were evaluated (flow cytometry), cytokine concentrations were analyzed (ELISA) as well as plasma miRNAs were isolated twice – on admission and after 19.2 ± 5.9 weeks of follow-up. Measurements were also performed among healthy volunteers.

Results: AMI patients presented significantly decreased frequencies of classical cells in comparison to healthy controls (median 71.22% [IQR: 64.4–79.04] vs. 84.35% [IQR: 81.2–86.7], $p = 0.001$) and higher percent of both intermediate and non-classical cells, yet without statistical significance (median 6.54% [IQR: 5.14–16.64] vs. 5.87% [IQR: 4.48–8.6], $p = 0.37$ and median 5.99% [IQR: 3.39–11.5] vs. 5.26% [IQR: 3.62–6.2], $p = 0.42$, respectively). In AMI patients both, analyzed plasma miRNA concentrations were higher than in healthy subjects (miR-146: median 5.48 [IQR: 2.4–11.27] vs. 1.84 [IQR: 0.87–2.53], $p = 0.003$; miR-155: median 25.35 [IQR: 8.17–43.15] vs. 8.4 [IQR: 0.08–16.9], $p = 0.027$, respectively), and returned back to the values found in the control group in follow-up. miR-155/miR-146 ratio correlated with the frequencies of classical monocytes (r=0.6, $p = 0.01$) and miR-155 correlated positively with the concentration of inflammatory cytokines − IL-6 and TNF-α.

Conclusions: These results may suggest cooperation of both pro-inflammatory and anti-inflammatory signals in AMI in order to promote appropriate healing of the infarcted myocardium.

Introduction

In the course of acute myocardial infarction (AMI), irreversible myocardial injury occurs due to sustained ischemia. In consequence, necrotic cardiomyocytes release ligands and inflammatory mediators, including cytokines and micro RNA (miRNAs), which activate the components of innate immune system [1]. Therefore, previous findings revealed significantly increased concentration of either pro-inflammatory and anti-inflammatory cytokines in AMI patients [2,3].

In the early phase of AMI, adrenergic signaling alerts bone marrow niche cells, which in turn leads to hematopoietic cell (HSC) mobilization from bone marrow to spleen and induce extra-medullary hematopoiesis [4,5]. Importantly, recent reports have indicated that acute inflammation observed in the acute phase of AMI leads to innate immune cells mobilization and recruitment to the infarcted myocardium zone [6]. Notably, monocytes represent an important cellular component of innate immune response and in the early phase of AMI. Although circulating monocytes that once leave bloodstream and enter infarcted myocardium are referred to as macrophages, their peripheral activation affects directly the biological activity of the latter cells. More importantly, due to pleiotropic biological activities, macrophages may play a crucial role in both myocardial injury and repair [7,8].

Monocytes are heterogeneous cell population [9]. Due to the differences in CD14 (lipopolysaccharide receptor) and CD16 (FcγRIII) expression levels, three functionally distinct subsets can be distinguished – classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16++) monocytes. Classical monocytes are referred to as unstimulated cells that are characterized by high phagocytic activity [10–12]. In contrast to classical cells, remaining two subsets represent activated CD16+ cells. Interestingly, intermediate monocytes present low or no reactive oxygen species (ROS), myeloperoxidase and lysozyme production, and upon lipopolysaccharide (LPS) stimulation they produce high amounts of anti-inflammatory interleukin 10 (IL-10) [13,14]. Furthermore, they were shown to support angiogenesis, tissue regeneration and repair [15]. Therefore, intermediate monocytes are believed to differentiate into anti-inflammatory/reparatory M2 macrophages and thus may support post-AMI myocardial regeneration. Conversely, non-classical monocytes possess high cytotoxic and antigen presenting abilities. Furthermore, in response to pro-inflammatory signaling they produce high amounts of pro-inflammatory tumour necrosis factor α (TNF-α) and IL-1β [16,17]. Therefore, non-classical monocytes are recognized as pro-inflammatory cells (M1 macrophage-like cells), involved in necrotic cell clearance, antigen presentation and immune activation, among others.

The involvement of distinct monocyte subsets to the myocardial ischemic injury area for the first time on the animal model of mice. Nahrendorf et al. have found that MI repair is characterized by a biphasic response [18]. Ly-6Chi monocytes, which were thought to be equivalent of CD14+CD16- monocytes in humans and accumulate via CCR2, predominate at the site of injury during the first 3 days, whereas the second phase, between 4 and 7 days after infarction, is dominated by Ly-6Clo monocytes, which may correspond to CD14+CD16+ in humans and accumulate via CX3CR1. These two monocyte subsets present different roles. Ly-6Chi monocytes promote inflammation and scavenge necrotic debris by a combination of inflammatory mediator expression, proteolysis and phagocytosis, whereas anti-inflammatory Ly-6Clo monocytes attenuate inflammation and promote healing via collagen accumulation and promotion of angiogenesis. It has been suggested that monocytes response after AMI is also biphasic in humans [19], however, according to the current knowledge, we distinguish three monocyte subsets and none of them is unequivocally anti- or proinflammatory. Tsujoka et al. demonstrated that patients with ST-segment elevation AMI, treated with PCI, show a sequential mobilization of CD14+CD16- and CD14+CD16+ monocytes, which peak on day 2.6 ± 0.8 and 4.8 ± 2.9 after onset of AMI, respectively [19]. They described the ability of classical CD14+CD16- monocytes to escalate inflammation, which was substantiated by their negative correlation with the extent of myocardial salvage after 7 days, and left ventricular ejection fraction after 6 months since AMI. Tapp et al. examined dynamic changes and relation to left ventricular ejection fraction (LVEF) of the three human monocyte subsets following STEMI [20]. The comparison to the study group revealed significantly higher counts of classical and intermediate cells, with a peak on day 1 after STEMI and a return to proportions similar to stable coronary artery disease (CAD) within a month. Furthermore, intermediate monocytes showed a significant correlation with plasma cytokines and troponin level as well as independently predicted 6-week LVEF [20]. Moreover, the assessment of intermediate monocytes trajectories from day 1 to day 7 of symptom onset in STEMI patients turned out a novel risk factor for post-STEMI adverse outcomes [21].

Previous studies, which was undoubtedly substantial, usually assessed two monocyte subsets or was limited to the acute phase of AMI without the assessment of immunological changes in following months. Therefore, to resolve the mechanism of monocytes activity in AMI, further continuous trials in humans are needed. Furthermore, as far as we know, none of the studies included the assessment of monocytes with altogether: cytokines, circulating miRNAs and echocardiographic parameters.

Interestingly, monocyte recruitment and activation is controlled by a number of proteins, including cytokines, chemokines and growth factors. However, recent evidence indicated that circulating short non-coding RNA (miRNA) may play an important role in controlling both monocyte activation and differentiation towards macrophages and macrophage activity within inflamed or injured tissue [22,23].

Recently, miRNA dysregulation has been reported in cardiovascular diseases, including AMI [24]. Observed changes in circulating miRNA levels in AMI are probably not only associated with their release from injured myocardium, but they might be also influenced by other cells associated with local or systemic responses, including activated and apoptotic leukocytes, endothelial cells and platelets. Therefore, circulating miRNAs associated with myocardial infarction could be divided into myocardium and muscle-delivered miRNAs, vascular wall-delivered miRNAs, and leukocyte-delivered miRNAs. Circulating miR-499, miR-1, miR-133a/b and miR-208 belong to myocardium delivered miRNAs and are passively released from cardiomyocytes after ischemic injury. Muscle-specific miRNAs positively correlate with other, well-known markers of myocardial infarction, such as troponins or CK-MB and may, therefore, may serve as novel markers of myocardial infarction [25,26]. In our study, however, we focused on immunological reactions in the course of AMI and miRNAs associated with circulating leukocytes, namely: miR-146 and miR-155.

miRNAs released from these cells are transported to the recipient ones in order to regulate post-transcriptional gene expression [27]. Previous findings concerning mechanisms of monocytes accumulation in the infarct zone as well as their immune activity and differentiation into macrophages indicated the contribution of miR-155 and miR-146. The first one promotes monocyte recruitment into the ischemic tissue by increased production of chemokine (C-C motif) ligand 2 (CCL2) in macrophages, aggravates inflammatory response by increasing cytokine (e.g. TNF-α, IL-6) and ROS expression, and eventually promotes polarization into pro-inflammatory M1 macrophages [22,28]. Meanwhile, simultaneously induced expression of miR-146 may

serve to dampen excessive inflammation in a negative feedback manner, in order to prevent the cardiac damage [29]. miR-146 has been shown to suppress the exertion of CCL2, leading to inhibition of migratory signals for leukocytes, and downregulate pro-inflammatory cytokines production in human monocyte/macrophage cells by targeting interleukin-1 receptor-associated kinase 1 (IRAK1)/TNF receptor associated factor 6 (TRAF6) [30,31]. Unfortunately, the exact effect of circulating miRNA on the phenotype and function of peripheral blood monocytes in the course of AMI has not been fully elucidated yet.

The aim of the study was to determine immunological reactions occurring in the AMI, as well as during the following months of heart muscle healing, with a special concern for the role of monocytes. Therefore, we aimed to investigate the changes in monocyte subset composition, monocyte/macrophage delivered cytokine levels and selected circulating miRNA levels (miR-146 and miR-155) in the course of AMI [24] and assess mutual relationships between all this immune components. The other purpose was to determine their correlations with other inflammatory mediators, as well as clinical parameters, including those related to the range of destroyed myocardial tissue.

Material and methods

Analyzed population and blood sampling

Eighteen patients with diagnosed AMI (Table 1) and eighteen healthy volunteers (mean age 56.78 ± 12.4 years old, without any acute or chronic diseases) were enrolled in the study. AMI was diagnosed according to the European Society of Cardiology (ESC) guidelines and treated with primary percutaneous coronary intervention (pPCI) within the first 12 h [32]. Exclusion criteria were as follows: 1) a history of severe pre-existing cardiac disease; 2) AMI for > 24 h before onset; 3) unstable angina pectoris/ myocardial infarction in a period of 6 months; 4) chronic auto-inflammatory disease; 5) active inflammatory process in a period of a month; 6) a history of proliferative disease; 7) serious renal disease requiring dialysis; 8) age below 18 years old. Clinical parameters including age, gender, traditional coronary risk factors (smoking, hypertension, diabetes mellitus, hyperlipidemia and obesity) were assessed.

In this study, patients have received similar pharmacotherapy on admission (100% received acetylsalicylic acid, clopidogrel, unfractionated heparin, statin and proton-pump inhibitors), however, in the course of time, they required different doses of hypotensive drugs or diuretics. On discharge, patients received coronary artery disease pharmacotherapy according to the ESC guidelines: 100% - dual antiplatelet therapy, statin, beta-blocker; 88.2% - angiotensin-converting-enzyme inhibitors, 38.9% - loop diuretics, 38.9% – aldosterone inhibitors, and 11.1% - calcium-blockers.

Infarct size based on the impairment of left ventricular function was assessed on the basis of echocardiographic measurements performed during the first 24 h of the hospital stay. Analyzed parameters included left ventricle ejection fraction (LVEF) and wall-motion score index (WMSI) assessed in a 16-segment model called Bull's-eye [33]. Segmental WMSI was graded as 1, 2, 3, and 4 (normal, hypokinetic, akinetic, and dyskinetic, respectively). WMSI was calculated by averaging the scored segments when 16 segments were accepted for the analysis. The mean follow-up period was 19.2 ± 5.9 weeks.

All subjects signed written informed consent for participating in the study, including taking and storing blood samples. The study was approved by the institutional Medical Ethics Committee.

Venous blood samples were obtained first time on admission (before pPCI procedure, at a median time of 4 h [IQR: 3–7] after the onset of symptoms) in the amount of 15 ml and were used for the

Table 1
Patients' baseline characteristics.

Demographic and clinical parameters	
Age, years	64.7 ± 13.5
Female, % (n)	22.2 (4)
Weight, kg	76.7 ± 12.6
BMI, kg/m^2	25.8 ± 3.4
NYHA class, % (n)	
I	44.4 (8)
II	50 (9)
III	5.6 (1)
Systolic blood pressure, mmHg	153.7 ± 15
Heart rate, beats/min.	79.4 ± 13.5
Previously diagnosed CAD (ACS/PCI > 6 months ago), % (n)	16.7 (3)
Previously diagnosed heart failure, % (n)	11.1 (2)
Atrial fibrillation, % (n)	5.6 (1)
Arterial hypertension, % (n)	38.9 (7)
Hyperlipidemia, % (n)	27.8 (5)
Diabetes, % (n)	5.6 (1)
Laboratory results	
CRP, IU/L	6.8 (4.9–12.1)
Troponin max, ng/L	9.9 (4.7–42.6)
Creatinine, mg/dL	0.9 ± 0.25
Total cholesterol, mg/dL	189.7 ± 28.6
LDL, mg/dL	115.7 ± 30.9
Haemoglobin, g%	13.76 ± 1.32
Fasting glucose, mg/dL	107.6 ± 17.1
Glucose on admission, mg/dL	138.5 ± 36.2
Echocardiography	
LVEF, %	41.9 ± 9.8
WMSI	1.79 ± 0.53
LVIDd, cm	4.92 ± 0.4
LA, cm	3.97 ± 0.43
Left ventricular mass index	114.6 ± 17.58
E/A ratio	1.06 ± 0.34

Data are presented as mean ± standard deviation or median and interquartile range (IQR).
BMI - body mass index; NYHA - New York Heart Association; CAD – coronary artery disease; ACS – acute coronary syndrome, PCI – percutaneous coronary intervention; CRP – C-reactive protein; LDL – low-density cholesterol; LVEF – left ventricular ejection fraction; WMSI– wall motion score index; LVIDd -left ventricular end-diastolic dimension; LA – left atrium; E/A - ratio of the early (E) to late (A) ventricular filling velocities.

analysis of monocytes, cytokines and miRNA. After follow-up period, blood samples were drawn again and used to repeat the same analysis.

Flow cytometry

Fresh EDTA-anticoagulated whole-blood samples (9 mL), obtained from patients, were used for flow cytometry. Monocytes were stained from whole blood according to stain-lyse-and-then-wash protocol. Briefly, 200 μL of whole blood was incubated for 30 min at room temperature with 10 μl of the following murine anti human monoclonal antibodies: CD14 PE (clone: M5E2), CD16 FITC (3G8), HLA-DR APC (TU36) (all from Becton Dickinson Biosciences, Belgium). Next, erythrocytes were lysed with FACS Lysing Solution (BD), followed by 15 min incubation in the dark. Cells were washed twice with PBS (Biomed Lublin, Poland) and fixed with CytoFix (BD). Appropriate FMO (fluorescence-minus-one) controls were used for setting compensation and gating. Samples were acquired with FACSCalibur flow cytometer (BD, CA, USA) and analyzed by using FlowJo software (Tree Star Inc., Ashland, OR, USA), Fig. 1.

Plasma and serum collection

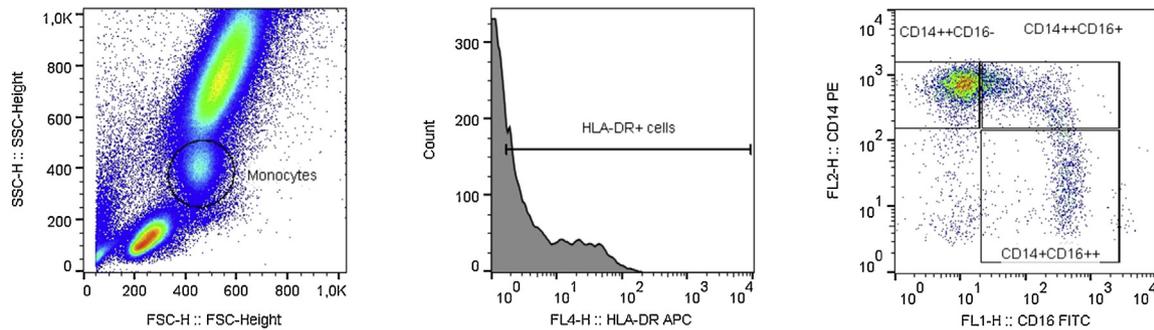To obtain plasma, venous blood was collected into EDTA containing vials and centrifuged for 15 min at 1000×g within

Fig. 1. Gating strategy for monocytes. First, events were gated based on a forward and side scatter (FSC/SSC) dot plot. Next, FSChighSSCint events were displayed on histogram plot and HLA-DR + cells were gated and referred to as monocytes. Finally, classical, intermediate and non-classical subsets were defined as CD14++CD16−, CD14++CD16+ and CD14+CD16++ cells, respectively.

30 min of collection. To obtain serum, whole blood samples were collected to the serum separator tubes (SST), left for 30 min at room temperature and centrifuged for 15 min at 1000xg. All plasma and serum samples were stored at −80 °C until cytokine analysis.

Cytokine assay

IL-10 (from plasma samples) and IL-6 levels (from serum samples) were analyzed with commercially available ELISA kits (R&D System). Samples were directly assayed according to manufacturer's instructions. The detection range of used ELISA tests was between 7.85–500 pg/mL for IL-10 and 3.1–300 pg/mL for IL-6. The samples were analyzed with automated light absorbance reader Ledetect 96 system (Dynamica) for IL-10 and Eti-max (DiaSorin) for IL-6. The results were calculated by using MicroWin 2000 software.

miRNA isolation and qPCR

RNA from plasma samples was isolated using miRCURY RNA Isolation Kit Biofluids (Exiqon) according to manufacturer's instructions. The RNA spike-in controls (Exiqon) were used according to manufacturer's instruction for validation of RNA isolation (UNiSp2, UNiSp4 and UNiSp5), cDNA synthesis and PCR amplification (UniSp6, cel-miR-39-3p). Moreover, an additional template-free control was purified by the samples and profiled. Briefly, EDTA-plasma samples were thawed on melting ice and centrifuged to avoid the presence of cell debris. Next, debris-free samples were lysed by using lysing solution and RNA isolation was performed. Isolated RNA was reveres-transcribed by using miRCURY LNA Universal RT cDNA Synthesis kit (Exiquon). The cDNA was diluted 50x and assayed according to the protocol for miRCURY LNA Universal RT microRNA PCR system (Exiqon). All LNA primers were obtained from Exiqon. Step-One Plus system was used for amplification (Applied Biosystems). All data were normalized to cel-miR-39-3p control.

Statistical analysis

Data are presented as medians with inter-quartile ranges (IQR) for non-normally distributed variables and means ± standard deviation (SD) for normally distributed continuous variables. Categorical variables were displayed as numbers and percentages. The statistical analysis was performed using *t*-student test or Mann-Whitney U test for continuous data and χ2 test for categorical variables. Pearson's or Spearman's correlation coefficient was used to examine the relationship between two continuous variables; *p* < 0.05 was deemed statistically significant.

A statistical software package Statistica 10 (USA) was used for the analysis.

Results

The assessments of monocyte subsets in the acute phase of AMI

As mentioned above, the early phase of AMI is characterized by increased production and release of monocytes leading to monocytosis [14]. Therefore, our first aim was to analyze, whether systemic increase of monocyte numbers entails changes in their activation status. Interestingly, we found decreased frequencies of CD14++CD16- monocytes in the acute phase of AMI when compared to normal donors – median 71.22% [IQR: 64.42–79.04] *vs.* 84.35% [IQR: 81.2–86.7], *p* = 0.001, Fig. 2. Consequently, the frequencies of activated CD16+ monocytes were elevated – median 18.04% [IQR:9.26–25.41] *vs.* 11.81 [IQR:10.17–15.04], *p* = 0.037, Fig. 2. No differences in the composition of CD16+ monocyte subsets, namely intermediate and non-classical monocytes (6.54% [5.14–16.6] *vs.* 5.87% [4.48–8.6], *p* = 0.37 and 5.99% [3.39–11.5] *vs.* 5.26% [3.62–6.2], *p* = 0.42 respectively) between patients and controls were found, Fig. 2.

The assessments of monocyte subsets in the post-healing phase of AMI

Similar to previous observations in the acute phase of AMI, patients in the post-healing AMI phase showed decreased frequencies of classical monocytes as compared to the healthy controls (median 64.45% [IQR: 55.89–77.68] *vs.* 84.35% [IQR: 81.2–86.7], *p* < 0.001). In some contrast to previous observations, however, patients revealed significantly higher frequencies of non-classical monocytes (median 6.81% [IQR: 5.71–9.47] *vs.* 5.26% [IQR: 3.62–6.2], *p* = 0.009), while frequencies of intermediate monocytes tended to increase (median 11.55% [IQR: 5.09–20.35] *vs.* 5.87% [4.48–8.6], *p* = 0.07), Fig. 2.

The changes in the composition of monocyte subsets and monocyte delivered cytokines between acute and post-healing phases of AMI

No significant changes among the analyzed monocyte subsets during the study were found, Fig. 3. We observed, however, substantial decrease in IL-10 concentrations (median 24.02 pg/ml [IQR: 8.35–53.48] to 8.35 pg/ml [IQR: 5.30–12.39], *p* = 0.003), but not TNF levels (median 6.28 pg/mL [IQR: 4.35–8.21] to 5.74 pg/mL [IQR: 4.64–8.76], *p* = 0.16). Moreover, we found significantly reduced IL-6 levels in the post-healing phase (median 2.92 pg/mL [IQR: 1.48–5.59] as compared to the acute phase of AMI (median 7.21 pg/mL [IQR: 4.41–33.45], *p* = 0.001), Fig. 3.
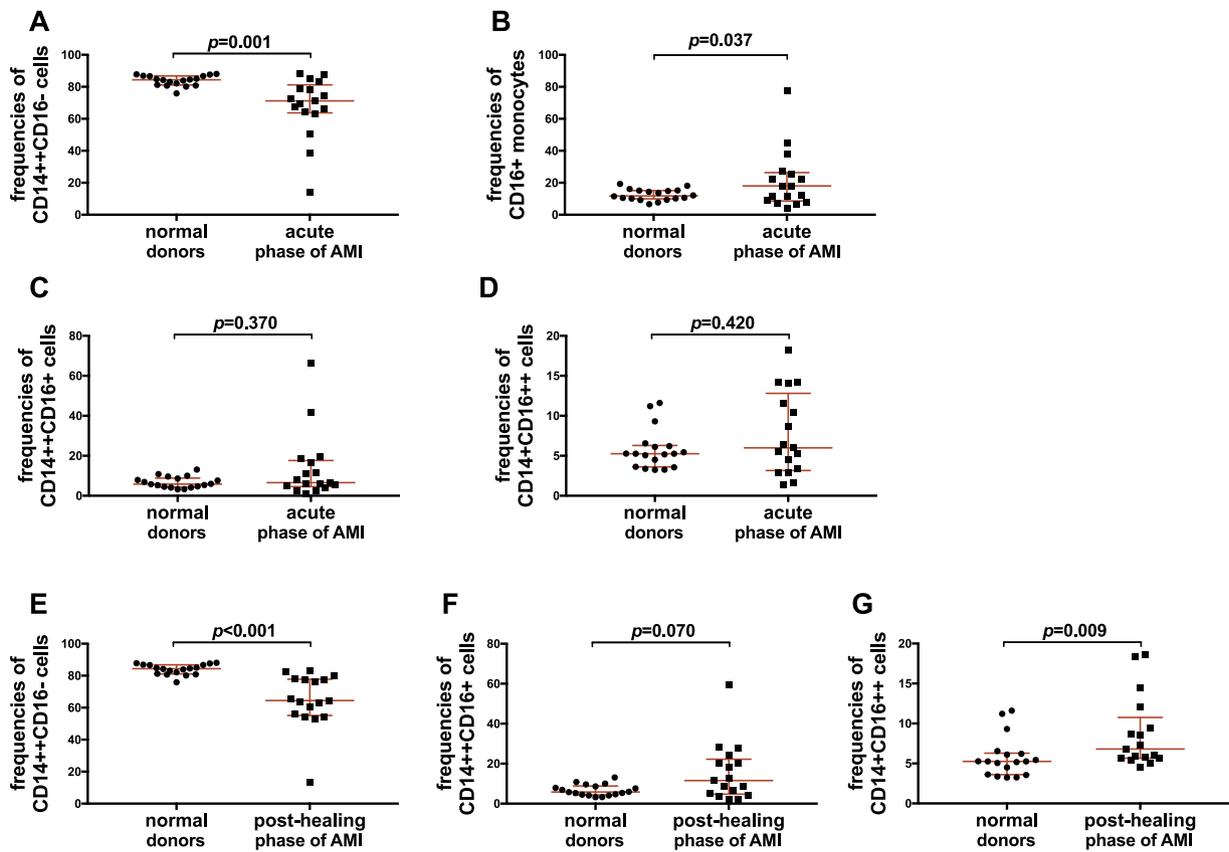
Fig. 2. Differences in classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16++) monocytes: A–D) between AMI patients and controls; E–G) between AMI patients in the post-healing phase and controls. Data presented as median frequencies [interquartile range] of total monocytes in peripheral blood. AMI – acute myocardial infarction.

The balance between pro-inflammatory and anti-inflammatory cytokines described as IL-6/IL-10 and TNFα/IL-10 ratio did not reveal any statistically significant changes in the acute phase of AMI and post-healing phase – median 0.69 (IQR:0.1–2.33) *vs.* median 0.4 (IQR:0.15–1.42), *p* = 0.4 and 0.29 (IQR:0.11–0.85) *vs.* 0.65 (IQR:0.37–1.05), *p* = 0.1, respectively.

The extension of myocardial injury could be reflected by increased markers of myocardial necrosis, such as troponin, and impaired cardiac function parameters – reduced LVEF and increased WMSI. The more extensive ischemia, the worse LV wall contraction with higher WMSI and lower LVEF. Therefore, we searched for any association between inflammatory reaction and the range of myocardial damage. In the acute phase of AMI, a significant correlation between IL-6 levels and maximal troponin I concentrations (r = 0.55, *p* = 0.02) was observed. In the post-healing phase IL-6 concentration correlated with both classical (r=0.63,
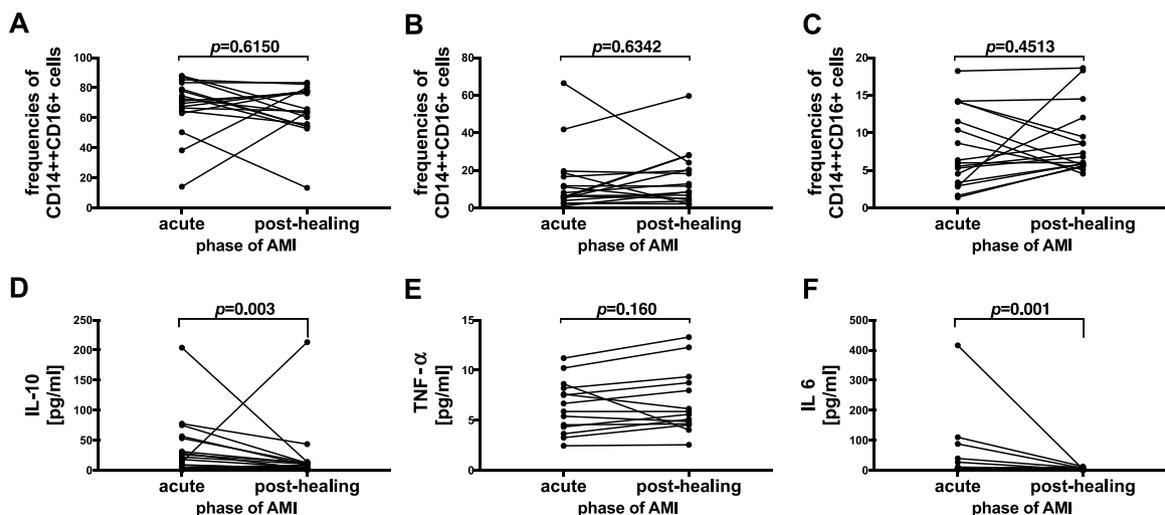

Fig. 3. The changes in the composition of monocyte subsets and monocyte delivered cytokines between acute and post-healing phases of AMI. Data presented as median frequencies and concentrations [interquartile range]. AMI – acute myocardial infarction; IL-6 – interleukin 6; IL-10 – interleukin 10; TNF-α – tumor necrosis factor α.

$p = 0.01$) and intermediate cells (r=0.55, $p = 0.03$). Correlations between WMSI and IL-10 (r=−0.7, $p = 0.002$) as well as IL-6/IL-10 ratio (r=0.6, $p = 0.03$) were found, Fig. 4. In the post-healing phase IL-10 levels correlated positively with LVEF assessed on admission (r = 0.51, $p = 0.04$).

miRNA assessments

As mentioned above, monocyte activation and phenotype might be influenced by circulating miRNAs. Therefore, we aimed to determine whether observed differences in the composition of monocyte subsets and observed time course variability in monocyte delivered cytokine levels in AMI are associated with changes in circulating miRNA profile. First, in the early phase of AMI, we found significantly increased levels of plasma miR-146 and miR-155 as compared to the healthy volunteers (median 5.48 [IQR: 2.4–11.27] *vs.* 1.84 [IQR: 0.87–2.53], $p = 0.003$) and median 25.35 [IQR: 8.17–43.15] *vs.* 8.4 [IQR: 0.08–16.9], $p = 0.027$, respectively, Fig. 5. Next, both analyzed miRNAs significantly decreased in the post-healing phase– miR-146 median 5.48 [IQR: 2.4–11.27] *vs.* 1.69 [IQR: 1.06–3.59], $p = 0.002$ and miR-155 median 25.35 [IQR: 8.17–43.15] *vs.* 6.36 [IQR: 0.21–19.88], $p = 0.02$, respectively, Fig. 5. More importantly, all analyzed circulating miRNA reached normal levels since in the post-healing phase no differences between healthy volunteers and AMI patients were discovered, Fig. 5. Positive correlations between miR-155 and IL-6 (r = 0.6, $p = 0.01$) as well as TNF-α (r = 0.78, $p = 0.0009$) were revealed, Fig. 6.

We have not discovered any significant relationship between our results and patients' pharmacotherapy.

Discussion

In the course of AMI ischemia-induced necrosis of cardiomyocytes activates innate immune responses and, consequently, leads to the accumulation of macrophages in the infarcted myocardium. In general, post-ischemic immune response may be divided into three subsequent and overlapping phases: acute (pro-inflammatory), healing and post-healing (maturation) phases. Notably, in all of them, monocytes and macrophages orchestrate systemic and local immune response and cardiac repair [34]. In this study, we analyzed changes in the composition of circulating monocyte subsets between acute and late phase of AMI, as well as differences in circulating miRNAs and monocyte/macrophage delivered cytokines.

The extension of myocardial damage is reflected by increased markers of myocardial necrosis, such as troponin, and impaired cardiac function parameters, namely reduced LVEF and increased WMSI. We inquired whether observed elevated inflammatory response may be associated with the range of myocardial damage.

In the acute phase of AMI, M1 (classically activated) macrophages are involved in the local inflammatory reaction leading to the removal of dead cells and debris, while M2 (alternatively activated) macrophages play a regulatory role by controlling M1-dependent activities. Therefore, appropriately balanced local pro-inflammatory (M1 macrophage-dependent) and anti-inflammatory (M2 macrophage-dependent) reactions seem to be crucial for myocardial salvage and proper healing [35–37]. As mentioned, macrophage polarization and function depend on monocyte peripheral activation. According to our results, we may probably expect, that increased accumulation of macrophages in infarcted myocardium is reflected by systemic activation and subsequent peripheral maturation of blood monocytes, which acquire CD16 in the process of maturation. As a result, we observed increased frequencies of CD16+ monocytes and simultaneously decreased classical monocyte frequencies compare to healthy individuals. Moreover, increased release of cytokines and miRNAs was observed as a consequence of systemic inflammation. Somewhat surprisingly, however, this does not change the composition of circulating monocyte subsets, namely frequencies of intermediate and non-classical monocytes (M2 and M1 precursors, respectively). Contradictory to our results, some other studies showed significantly higher counts of classical and intermediate monocytes in STEMI patients compared with stable coronary artery disease and healthy individuals [20,38]. However, authors assessed absolute count of monocyte subsets. Tapp et al., who additionally presented changes in monocyte subsets frequencies, interestingly revealed significantly increased percent of intermediate cells, whereas decreased classical and non-classical monocytes percent. On the other hand, considering only CD14 and CD16 experssion, Costantini et al. didn't find any differences in monocytes proportion between AMI patients and healthy, elderly subjects [39].

Previous observations indicated that monocyte accumulation in the acute phase of AMI is directly associated with CCR2, which is expressed predominantly on classical and intermediate monocytes. The production of chemokines such as CCR2 ligand −CCL2, is regulated by different miRNAs, including miR-155 and miR-146. Interestingly, in our study we observed significantly elevated levels of all above mentioned miRNAs in the serum of patients in the acute phase of AMI. miR-155 was previously shown to promote monocyte recruitment into inflamed myocardium by the increased production of CCL2 and favored monocyte differentiation towards M1 macrophages [22]. Furthermore, miR-155 by direct targeting
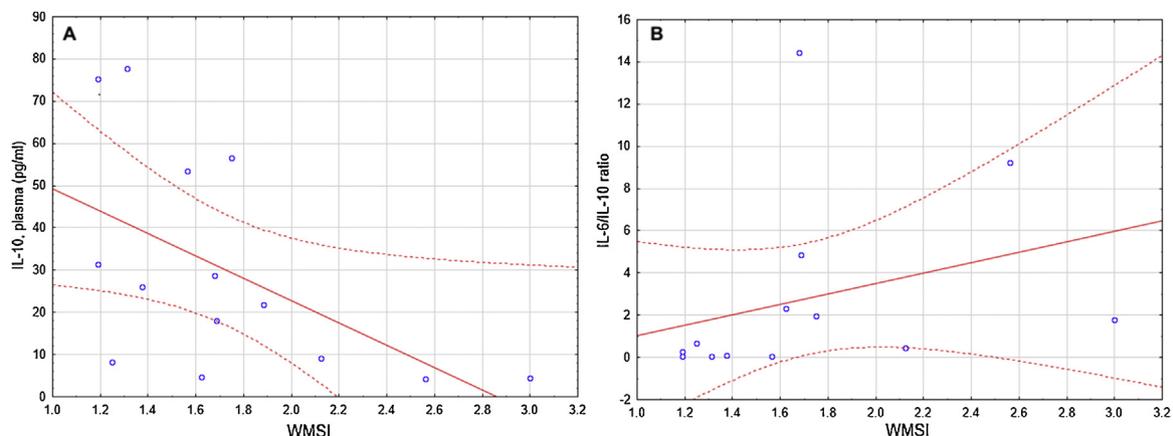

Fig. 4. Spearman correlations between WMSI and: A) IL-10 ($r = −0.7$, $p = 0.002$), B) IL-6/IL-10 ratio ($r = 0.6$, $p = 0.03$). IL – interleukin; WMSI – wall motion score index.
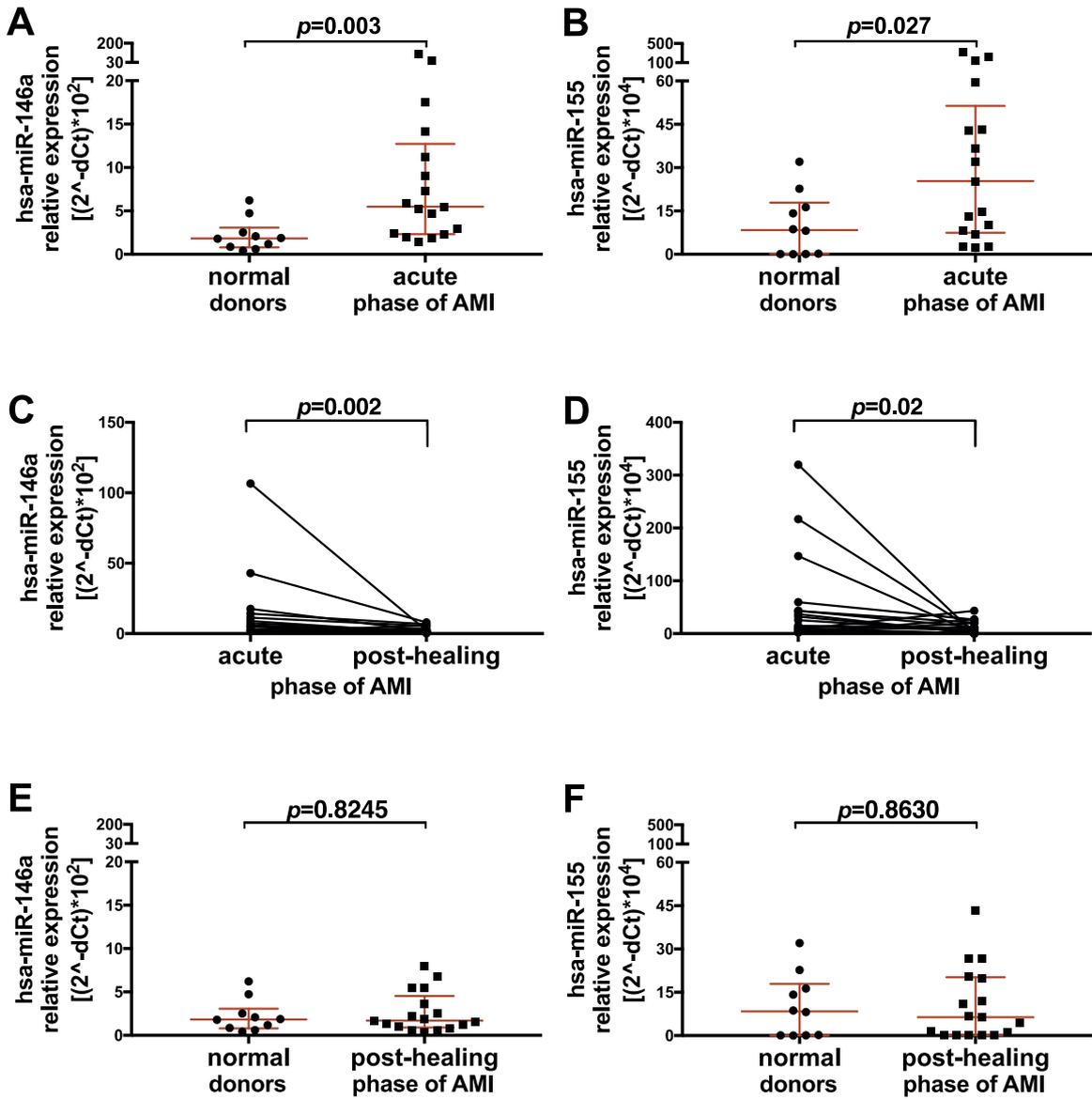
Fig. 5. miRNAs concentration changes in the acute phase of AMI and in the follow-up. Results derived by the $2 - \Delta CT \times 10^4$ (miR-155) or $2 - \Delta CT \times 10^2$ (miR-146) normalized to the volume and to the expression of cel-miR-39-p3. AMI – acute myocardial infarction; miR – microRNA.
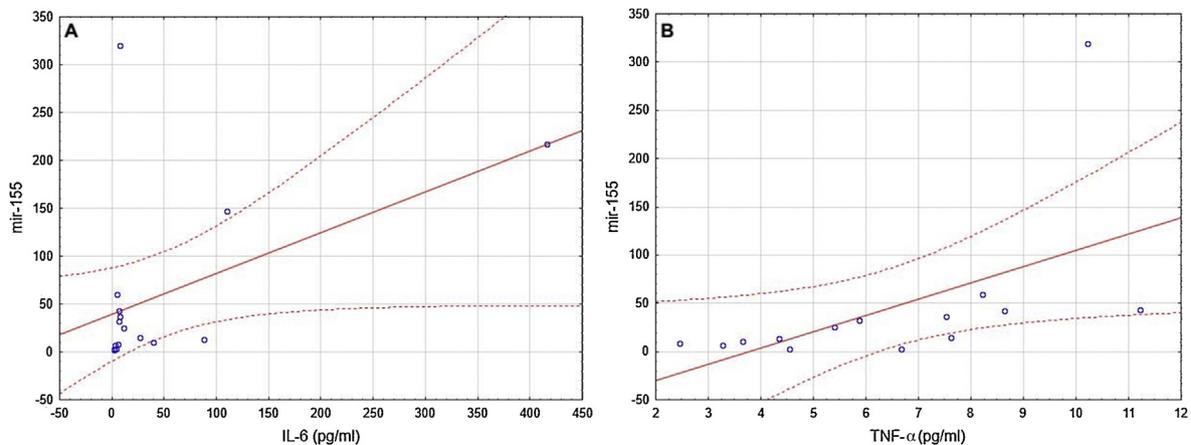

Fig. 6. Spearman correlations between mir-155 and A) IL-6 (in 17 patients) $r = 0.6$, $p = 0.01$, B) TNF-α (in 14 patients) $r = 0.78$, $p = 0.0009$. miR – microRNA; IL – interleukin; TNF-α – tumor necrosis factor α.

Socs1 and Inpp5d may increase TNF-α and IL-6 production by monocytes and macrophage [22,40]. In contrast to miR-155, miR-146 was shown to promote anti-inflammatory responses by downregulation of CCL2 and pro-inflammatory cytokine production in monocytes and macrophages [29].

Interestingly, we observed here a positive correlation between IL-6 and TNF-α plasma levels and circulating miR-155, but not miR-146. Therefore, we hypothesized that the observed increase of pro-inflammatory cytokines in patients in the acute phase of AMI may be additionally supported by miR-155 modulatory function on monocytes and macrophages. Furthermore, miR-155/miR-146 balance, through the regulation of chemokines exertion, may play an important role in the regulation of monocyte recruitment to infarcted myocardium since miR-155/miR-146 ratio correlated positively with the frequencies of classical monocytes. However, further studies are needed to explain this phenomenon.

Classical monocytes are known as a main subset able to secrete IL-6 after ischemic injury. IL-6, in turn, also induces the secretion of CCL2 by peripheral blood mononuclear cells and injured cardiomyocytes enhancing further recruitment of CD14++CD16- monocytes to the infracted area [41]. In this study we observed significantly higher concentration of IL-6 in the onset of AMI and a decrease in the following months. Baseline IL-6 concentration correlated with a concentration of well-known marker of myocardial necrosis - troponins. Therefore, IL-6 level probably reflect the extent of myocardial infarction in AMI patients [42]. In response to local and systemic inflammation we observed an elevation of anti-inflammatory IL-10 concentration, which probably constitutes a sort of counteraction in order to suppress synthesis of pro-inflammatory cytokines. Furthermore, increased levels of anti-inflammatory IL-10 may represent early manifestation of M2 macrophage dependent healing process. Notably, IL-10 signaling induces monocyte polarization towards M2 macrophages [43]. Additionally, IL-10 contributes to the restoration of the infarct area by extracellular matrix remodeling and thus prevents ischemic myocardium from excessive inflammatory destruction and heart failure development [44–46]. More importantly, increased IL-10 levels reduce risk of re-infarction and death [23,47]. We revealed a negative correlation between baseline IL-10 and WMSI as well as a positive correlation between follow-up concentration of IL-10 and LVEF. These results may suggest that the positive effect of IL-10 activity on LV function is not only associated with the acute phase of AMI, but it is also maintained after resolution of potent stimulus such as ischemia and reperfusion in follow-up period. Karpiński et al. [2] observed that correlation between LVEF and IL-10 measured on the first days after AMI was negative and became positive after 6 months. According to the authors, it implicates major enhancement of damaging factors on the first days after AMI stimulating protective action of IL-10 and beneficial effect of increased IL-10 concentration in the following days.

Directly after the acute phase of AMI, M1-associated pro-inflammatory reactions are dominated by M2-dependent reparatory, proangiogenic and fibrotic activities [48–50]. However, anti-inflammatory switch in the local immune response, observed between the acute and maturation phase of AMI, does not change peripheral monocyte activation. This observation confirms significant involvement of monocytes/macrophages in the late phase of myocardial healing. Interestingly, however, the maturation phase is manifested by increased frequencies of M2 macrophage precursors, namely intermediate monocytes [51]. Notably, optimal healing and maturation mechanisms require inhibition of pro-inflammatory response and significant reduction of inflammatory mediators. In fact, all analyzed miRNA levels as well as IL-6 and IL-10 levels decreased significantly. Somewhat surprisingly, TNF-α serum levels did not change between the acute and maturation

phase. Notably, TNF-α regulates extracellular matrix metabolism by reducing collagen synthesis and enhancing matrix metalloprotease activity in cardiac fibroblasts [52]. Furthermore, TNF-α was shown to support cardiac fibroblast migration [53], as well as wound epithelization and neovascularization. These may suggest its important role in wound maturation phase [54] and explain stable concentration in AMI patients during following months. However, further studies are needed to explain the role of TNF-α in the maturation phase of AMI.

Acute myocardial infarction is followed by a balanced cooperation of different monocyte subsets, miRNAs and cytokines in order to provide effective dead cells clearance and healing process. Innate immune response is activated locally and systemically, which is reflected by monocytes' increased expression of CD16 and overproduction of inflammatory mediators, namely cytokines and miRNAs. Subsequent increase of both miR-146 and miR-155 in the acute phase of AMI may regulate cytokine production and monocyte migration to the infarcted area and their differentiation towards macrophages. However, further studies are needed to explain the regulatory role of circulating miRNAs on monocytes in the course of AMI.

Study limitations

The main limitation of the study is a small sample size of the studied patients. However, the patients were studied consecutively and prospectively by using state-of-the-art techniques and the results were compared to an age and gender matched controlled group. In this study, the authors focused on the changes among monocyte subsets and presented them as percent values without an absolute number of cells per mL blood. The assessment of circulating miRNAs was limited to leukocyte-specific miRNAs that can affect monocyte maturation, polarization and function that are poorly described in AMI patients, namely mir-146 and mir-155, whereas muscle-specific miRNAs, such as mir-1 or mir-499 remained unmentioned, mainly because of different mechanisms of release and function. However, this is worth considering and evaluating in another, novel paper. Hypothetical relationship between analyzed miRNAs, cytokine expression and monocyte migration was based only on clinical premises and no doubt it requires further research.

Conflict of interest

The authors have no conflict of interest.

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