

Original article

Serum levels of unique miR-551-5p and endothelial-specific miR-126a-5p allow discrimination of patients in the early phase of acute pancreatitis



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ABSTRACT

Objectives: Vascular dysfunction is a severe complication which can cause organ ischemia and damage during acute pancreatitis (AP). Laboratory assessment of AP is based on several routine parameters and does not reflect endothelial dysfunction or organ injury. Recently, small non-protein-coding RNAs (miRNAs) have been introduced to laboratory diagnostics as new biomarkers or predictive parameters. Candidate miRNAs (hsa-miR-16-5p, -103a-3p, -122-5p, -126-5p, -148a-5p, -216a-5p, -375, and -551b-5b) were selected to check their possible clinical application in stratification of patients with different AP severity. **Methods:** In this observational study, 62 patients with mild (MAP) and 26 with moderate and severe (SAP) acute pancreatitis were included. The control group consisted of 10 age and sex matched subjects. Circulating miRNAs were analyzed in serum using a quantitative real-time PCR method (q-RT-PCR) by means of 3'-locked-nucleic-acid primers.

Results: In SAP patients, a significant increase in most of the selected miRNAs (miR-126-5p, -148a-3p, -216a-5p and -551b-5p, and miR-375) was observed when compared to control subjects. In MAP patients, three miRNAs were significantly elevated: endothelial-specific miR-216a-5p, -551b-5p, as well as miR-375 that is highly abundant in pancreas. ROC analysis revealed that miR-126-p and miR-551b-5p can predict AP severity (AUC 0.748, sensitivity 60.0%, specificity 87.1%, $p < 0.001$) and (AUC 0.716; sensitivity 69.2%, specificity 72.6%, $p < 0.001$). miR-375 was not relevant (AUC 0.458; sensitivity 55%, specificity 44.4%).

Conclusions: A pancreatic miRNA signature can be useful for assessment of pancreatic injury in the acute phase of AP. Endothelial dysfunction during AP is reflected by levels of specific circulating miRNAs and may help in patient stratification.

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Introduction

The diagnosis of acute pancreatitis (AP) based on the revised Atlanta classification (2013) relies on typical signs and symptoms, an appropriate increase of pancreatic enzymes, and characteristic

findings on abdominal imaging (contrast-enhanced computed tomography, transabdominal ultrasonography, or magnetic resonance), and is clearly defined by the guidelines [1,2]. However, in some specific cases, including patients with infected necrosis (IN), these criteria should be considered carefully [3]. In contrast to diagnosing the disease, early differentiation between severe (SAP) and mild (MAP) form of AP remains challenging even for an experienced physician. Moreover, both forms of disease may be observed in patients with different etiology including heavy alcohol consumption or biliary etiology [4].

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From a clinical point of view, AP is a rapidly evolving patient condition, in which the severity can change very quickly during the time course of disease and diagnostic processes. Thus, early implementation (i.e., within 48 h from the onset of symptoms) of adequate therapy is often a key determinant of patient survival [5–7]. SAP often takes a clinical course with two phases, an early phase (which lasts about one week) and a late phase, which is characterized by common life threatening complications such as hypovolemia, hemoconcentration, drop in blood pressure, and finally acute renal injury (AKI), and acute lung injury (ALI), leading to shock [1,4]. In routine practice, multiple prognostic scoring systems – such as APACHE II (Acute Physiology and Chronic Health Evaluation), Ranson's, or, more recently, BISAP (Bedside Index For Severity in Acute Pancreatitis) scores may help to assess the prognosis [8]. Also, serum concentrations of various inflammatory markers and mediators, e.g., proinflammatory cytokines (tumor necrosis factor- α , interleukins 6 or 18), procalcitonin, or acute phase proteins (C-reactive protein, serum amyloid A, pentraxin 3) differ between SAP and MAP patients and may help in clinical practice [9–12]. However, none of them has so far been an ideal prognostic marker.

In the last decade, the complex nature of different pathologies was extended by the discovery of microRNAs (miRNAs), and clinical research on miRNAs has provided a new class of potential prognostic markers [13]. miRNAs are small (18–22-nucleotide) non-protein-coding RNAs that are generally conserved. In a cell, their function is to regulate gene expression through sequence-specific repression of their target mRNAs [14]. Recently, miRNAs have been found to be transferred outside of cells; they can circulate in many different body fluids including plasma, serum, and lymph [15,16]. Mechanisms which control the processes of extracellular miRNA transportation are varied. Most of circulating miRNAs are bound to argonaute 2 (Ago2) protein, which is a part of the RNA-silencing complex [17]. Nevertheless, a global circulation miRNA signature is carried within different populations of membrane-derived extracellular vesicles (EV) including apoptotic bodies, microparticles, or exosomes [16,18]. Their nomenclature and ontology has been recently characterized in a public database dedicated to EV research [19]. Similarly to soluble factors, circulating miRNAs participate in the cell-to-cell communication system, in both micro- and macroenvironments [18].

The main reason why we decided to investigate these master regulators as potential biomarkers, is that several miRNA signatures correlate with patient diagnosis, prognosis and response to treatment. To improve patient diagnostics and predict outcome in AP patients, new strategies based on miRNAs have been proposed recently [15,20,21]. However, results of some clinical studies are not concordant with animal (rat) models, where different microRNAs have been selected (e.g., miR-15b, miR-16, miR-216a) [22,23]. Similarly, some discrepancies between human and rat circulating microRNA profiles and dynamics have been observed in other acute diseases such as myocardial infarction (MI) [24,25] or AKI [26]. On the other hand, it may be assumed that some “organ-abundant” miRNAs should be increased in blood of AP patients due to a tissue or organ injury, e.g., liver- (hsa-miR-122-5p) or pancreatic-specific (hsa-miR-375), those related to endothelial function (hsa-miR-126-5p, -216a, -551b-5b), as well as apoptotic ones (hsa-miR-16-5p) [15,22,27–29].

In this study, we evaluated a hypothesis that some organ-abundant miRNAs can be considered biomarkers of AP severity. Hence, we selected candidate miRNAs to compare their levels in SAP and MAP patients with a control group: hsa-miR-16-5p, hsa-miR-103a-3p, hsa-miR-122-5p, hsa-miR-126-5p, hsa-miR-148a-5p, hsa-miR-216a-5p, hsa-miR-375, and hsa-miR-551b-5b, and we analyzed their profile in serum collected from AP patients in the early stage of disease. The second aim of the study was to

determine whether severe inflammatory state (SAP) is associated with the profile of miRNAs related to vascular (endothelial) dysfunction.

Materials and methods

Characteristics of the study group

The study included 88 consecutive patients admitted to the Surgery Department of the District Hospital in Sucha Beskidzka, Poland. Since AP is an acute inflammatory process with characteristic but nonspecific symptoms such as abdominal pain and vomiting, admission of patients to the clinic does not always correlate with the disease onset. In our study, we limited the group of patients to those who reported the onset of acute abdominal pain within 12–24 h before admission and in whom the preliminary diagnosis was confirmed by elevated serum amylase (mean 1248 [min–max 432–5548] IU/L) and lipase (mean 2450 [min–max 300–10,059] IU/L) activity, with our reference values of 25–125 IU/L for amylase and <160 IU/L for lipase. Additionally, transabdominal ultrasonography was applied after admission as the standard imaging modality. Contrast-enhanced computed tomography (CECT) was performed in patients in whom the diagnosis was unclear or who failed to improve clinically within the first 48–72 h after hospital admission. Severity of AP was determined as the actual stage of disease in a patient during admission, based on the revised Atlanta Classification (2013) [1]. In patients who declined clinically, the diagnosis needed revision in order to evaluate further complications, i.e., in moderate severe AP, transient organ failure and local peripancreatic or pancreatic complications were confirmed within 48–72 h after admission, according to the ACG recommendations [2]. Necrosis was evaluated in the later stage of disease.

Finally, the study group consisted of 52 women and 36 men with a mean age of 53 ± 16 years. Sixty two patients were diagnosed with mild acute pancreatitis (MAP) with the absence of both (peri)pancreatic necrosis and organ failure. In 26 patients, the course of disease was moderate and severe (SAP) with the presence of sterile (peri)pancreatic necrosis and/or transient organ failure. Patients were excluded when they had a diagnosis of chronic pancreatitis, post-ERCP (endoscopic retrograde cholangiopancreatography) pancreatitis, an intraoperative diagnosis of AP, liver or kidney failure, pregnancy, and malignancy, or when the onset of symptoms was more than 24 h before hospitalization. Patients who refused to give their informed consent were also excluded. The etiology of AP and other clinical characteristics are presented in Table 1.

The control group consisted of 10 healthy volunteers (53 ± 6.9 years) selected from the OKO-LASER Ophthalmology Outpatient Clinic, Krakow, Poland. Exclusion criteria for the control group were: age below 40 years, any systemic and chronic diseases including autoimmune diseases, cancer, diabetes, hepatic disorder proved by aspartate transaminase and alanine aminotransferases (AST and ALT, respectively) activity, diagnosed cardiovascular diseases including hypertension and coronary artery disease, any injury or acute state including infections proved by C-reactive protein levels, and hyperlipidemia.

The study protocol has been approved by the Bioethics Committee of Jagiellonian University Medical College (KBET/86/B/2012 and KBET/260/B/2013). All subjects signed their informed consent in accordance with the Declaration of Helsinki.

Laboratory methods

Study design

The specific criteria for the miRNA selection were their abundance in diseases affected organs (liver or pancreas) and

Table 1
Characteristics of study groups.

Parameters	Mild Acute Pancreatitis (n = 62)	Moderate and Severe Acute Pancreatitis (n = 26)	p Value
Gender			
Female	35 (56.5)	17 (65.4)	0.66 ^a
Male	27 (43.5)	9 (34.6)	
Etiology			
Biliary (in. gallbladder lithiasis)	30 (48.4)	7 (26.9)	0.27 ^a
Alcoholic	13 (20.9)	10 (38.5)	0.22 ^a
Hypertriglyceridemia	3 (4.8)	3 (11.5)	0.37 ^a
Idiopathic	12 (19.4)	2 (7.7)	0.34 ^a
Other	4 (6.5)	4 (15.4)	0.25 ^a
Age (years)	53 ± 17	53 ± 20	0.40 ^b
Duration of hospitalization (days)	6 [5–8]	50 [26–116]	<0.001 ^c
Glasgow score (mean ± SD)	0.75 ± 0.7	1.44 ± 1.38	0.04^b
Deaths	1 (1.6)	9 (34.6)	0.014^a

Values are given as mean ± standard deviation (SD), median with interquartile interval [Q1–Q3] or the number and percentage (%); bold values represent statistical significance.

^a Fisher 2-tailed exact test.

^b Student's *t* test.

^c Mann–Whitney *U*-test.

serum stability. The identification of candidate miRNA biomarkers was performed by means of database research, using non-commercially available tools: <http://atlas.dmi.unict.it/mirandola/>; <http://bioeng.swjtu.edu.cn/TSMiR/index.asp>. MiRandola is the first database that collects and classifies data about different kinds of extracellular miRNAs in human biological fluids [30]. For our study, we browsed miRandola using the “advanced search” tool, and we selected the “mature miRNA” category. After choosing our miRNAs of interest, we selected the “circulating miRNA” type and “serum sample” subcategory. Because miRandola provides only the data on extracellular miRNAs, we extended our search with a tissue-specific miRNA database – TSMiR for potential targets selection. Specific criteria for miRNA selection such as abundance in patient serum, organ specificity and significance for organ injury were established. Additionally, we included in our analysis those miRNAs which were prognostic biomarkers for acute pancreatitis, according to a recent paper by Liu et al. [21].

Sample collection

Samples were collected during the first 48 h after admission from the patient's cubital vein using Monovette Sarstedt tubes (Sarstedt, Nümbrecht, Germany). For miRNA analyses, samples were taken together with the blood used for routine laboratory tests (pancreatic enzymes) in order to avoid additional burden for patients. Because hemolysis can cause false increase either in common laboratory parameters or miRNA levels, every visually detected hemolysed serum samples were rejected.

miRNA extraction

Serum samples from patients and healthy donors were stored at - 80 °C until examinations. After thawing, samples were centrifuged at 3000 × *g* for 5 min to pellet any debris and insoluble components. Circulating miRNA was extracted using the miRCURY™ RNA Isolation Kit (Exiqon, cat. No. 300112), according to the manufacturer's instructions with minor modifications. The purified miRNA was eluted with 20 µl of RNase free water. The quantity and quality of the isolated miRNA were assessed using Agilent Bioanalyzer 2100 (Small RNA Analysis Chip, Agilent Technologies, Inc.).

miRNA quantification by qPCR

Reverse transcription (RT) was performed using the miRCURY LNA™ Universal RT kit (Exiqon, cat. No. 153002) with a synthetic RNA spike-in control template (UniSp6), which provides internal control for the quality of the cDNA synthesis reaction and PCR. The qPCR reaction was conducted using the Veriti Thermal Cycler (Applied Biosystems, USA). The reaction conditions were 42 °C for 60 min, followed by cooling to 4 °C. Then, the synthesized cDNA was amplified by quantitative PCR, using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPA BIOSYSTEMS, cat. No. KK4621). The reaction included a spike-in control primer set (UniSp6) and specific primers for candidate miRNAs. Target sequences specific to each miRNA primer set are presented in Table 2.

Primers were resuspended by adding 250 µl RNase free water. Master mix was prepared for each miRNA-specific assay. Each single reaction included 5 µl KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal, 1 µl LNA™ PCR primer set for an individual miRNA, 3 µl RNase free water. The RT products were diluted 1:20 in RNase free water, and 4 µl of the product were added to a single real-time PCR reaction tube as a template.

Because even very small changes in microRNA expression levels might be biologically significant, we applied three replicates to reveal any statistically significant differences. Real-time PCR was carried out in Rotor Gene Q (Qiagen, USA), using the following cycling conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 20 s, followed by a hold at 4 °C. Raw data were analyzed by Rotor-Gene Q Software series (1.7). The cycle threshold (Ct) was set manually at 0.40148. The level of Ct was set in the linear phase of the amplification plot, above the baseline but sufficiently low to be within the exponential growth region of the amplification curve in the case of all qPCR reactions. The correction for background fluorescence of a sample, which must ideally remain constant before amplification, was needed. Slope Correction option in Rotor-Gene Q uses a line-of-best-fit to determine the noise level instead of the average, and normalizes to that instead. Turning on this option can tighten replicates if sample baselines are noticeably sloped. miRNA data expression was quantified by the DeltaDeltaCt method using the DataAssist software (Life Sciences). Because the number of miRNAs analyzed in each sample was smaller than 50, global normalization was not recommended in our protocol. Relative abundance of each miRNA (fold change – RQ) was normalized by the

Table 2

Target sequences and nomenclature of selected miRNAs analyzed in the study. Accession sequences and names are presented according to miRBase 20.0.

miR name (human)	microRNA target sequence	Sequence reference	Corresponding LNA™ microRNA PCR primer set (cat. No)
hsa-miR-16-5p	UAGCAGCAGCUAAAUAUUGGCG	MIMAT0000069	EQ-205702
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	MIMAT0000101	EQ-204063
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	MIMAT0000421	EQ-205664
hsa-miR-126-5p	CAUUUUUUUUUUUGGUACGCG	MIMAT0000444	EQ-206010
hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU	MIMAT0000243	EQ-205867
hsa-miR-216a-5p	UAAUCUCAGCUGGCAACUGUGA	MIMAT0000273	EQ-204167
hsa-miR-375	UUUGUUUCGUUCGGCUCGGUGA	MIMAT0000728	EQ-204362
hsa-miR-551b-5p	GAAUCAAGCGUGGGUGAGACC	MIMAT0004794	EQ-204630

levels of hsa-miR-103a-3p according to the formula $\Delta\Delta Ct = (Ct_{\text{sample}} - Ct_{\text{ref}})_N - (Ct_{\text{sample}} - Ct_{\text{ref}})_T$, and the estimated expression ratio is equal to $2^{-\Delta\Delta Ct}$. Hierarchical clustering analysis was performed by the Altanalyze software, applying relative normalization to the median of the samples expression and the average clustering method [31].

Statistical analysis

Continuous variables were presented as the mean value \pm standard deviation (SD), or median with interquartile interval [Q1-Q3]. Categorical variables were given as absolute values, percentages, or both, and compared by means of the Fisher 2-tailed exact test. Differences between mean values were verified using the Student's *t* test when the distribution of variables was normal, in other cases applying the Mann–Whitney U test. The fold-change analysis of miRNA levels was performed by the non-parametric Kolmogorov–Smirnov test to compare data sets. A *p* value less than 0.05 was considered statistically significant. Clinical accuracy of significant miRNAs was assessed using receiver operator characteristic (ROC) analysis, and the area under the curve (AUC) was calculated for each of the miRNAs of interest separately, using DeLong's method. For those cut-offs, positive and negative predictive values (PPV, NPV), as well as sensitivity and specificity were calculated. Analyses were performed with Statistica Version 10 (StatSoft, Inc.) and Excel (Microsoft) software.

Results

Normalization of selected miRNA levels

To confirm a stable level of housekeeping miRNAs in serum the global normalization approach was performed. This global normalization strategy allowed us with some caution (number of analyzed miRNAs was below 50) to select hsa-miR-103a-3p as a housekeeping miRNA for further analyzes. Surprisingly, levels of the commonly recommended hsa-miR-16-5p were not stable using global normalization approach. We observed a significant fold

change (RQ: 6.4 ($p = 0.045$)) in MAP and SAP vs. controls (RQ: 28 ($p = 0.03$)). In the comparable study by *Blenkiron et al.* [15] the authors failed to achieve amplification reliability of plasma miR-16 and they reported raw Ct values, without normalization. In contrast to miR-16, if we used global normalization approach, we did not observe significant variability in hsa-miR-103a-3p levels in MAP and SAP patients vs. controls (RQ: 1.1 ($p = 0.454$) and 0.9 ($p = 0.099$), respectively) as well as in MAP vs. SAP patients (RQ: 0.8 ($p = 0.291$)). Raw Ct values for every analyzed miRNAs are presented in the supplementary file 2 (Supp 2), and the variability between samples is more significant for miR-16 than for miR-103.

Analysis of candidate miRNAs in an independent cohort of AP patients and controls

The differential profile of serum miRNA candidates by RT-qPCR in an independent cohort was finally performed on 88 AP patients (62 MAP cases and 26 SAP cases). Fold change (RQ) of analyzed miRNAs in patients with different severity of AP in compare to control subjects and the comparison between MAP and SAP patients is presented in Table 3. Interestingly, after endogenous control normalization, the fold change of hsa-miR-16-5p in SAP patients was even higher (RQ: 35 ($p = 0.016$)) than that after global normalization. In SAP patients, a significant increase in the level of most of the analyzed miRNAs was observed (miR-126-5p, -148a-3p, -216a-5p, and -551b-5p), including an increase in the level of specific miR-375, which is highly abundant in pancreas. Contrastingly, in MAP patients only 3 miRNAs were highly and significantly increased: miR-216a-5p, -375, and -551b-5p. Despite such a significant difference in miRNAs levels in AP patients in compare to the control group, the discrimination between MAP and SAP patients was not apparent.

Clustering of circulating miRNAs to enhance diagnostic discrimination of AP patients

For enhancing diagnostic discrimination of AP patients, a common pattern of hierarchical clustering was applied. The analysis of

Table 3

Fold change (RQ) of selected miRNAs in patients with different severity of AP. Analyses were performed by means of the DataAssist software (Life Sciences). Relative abundance of each miRNA was normalized by the expression level of hsa-miR-103-5p.

miR name (human)	SAP vs. MAP patients	MAP vs. control	SAP vs. control
hsa-miR-16-5p	2.8 ($p = 0.313$)	12 ($p = 0.098$)	35 ($p = 0.016$)
hsa-miR-122-5p	1.8 ($p = 0.465$)	190 ($p = 0.051$)	337 ($p = 0.055$)
hsa-miR-126-5p	2.1 ($p = 0.383$)	35 ($p = 0.098$)	74 ($p = 0.016$)
hsa-miR-148a-3p	0.4 ($p = 0.414$)	206 ($p = 0.088$)	91 ($p = 0.016$)
hsa-miR-216a-5p	0.9 ($p = 0.73$)	110 ($p = 0.005$)	95 ($p = 0.011$)
hsa-miR-375	0.5 ($p = 0.379$)	698 ($p = 0.003$)	381 ($p = 0.032$)
hsa-miR-551b-5p	2.5 ($p = 0.313$)	113 ($p = 0.005$)	279 ($p = 0.016$)

Abbreviations: AP – acute pancreatitis, MAP – mild acute pancreatitis, SAP – moderate and severe pancreatitis; *p*-values are presented in brackets, bold indicates statistical significance.

levels of the 7 selected miRNAs (Δ Ct plus) with Euclidean distance measurement was performed. As shown in Fig. 1, the algorithm clustered MAP and SAP patients with several outliers. No specific discrimination pattern was observed, but it was plainly demonstrated that significant miRNAs are up-regulated in most subjects with SAP and miR-551b-5p is down-regulated in most MAPs.

group, 6 of the 7 selected miRNAs were predictive of acute pancreatitis, apart from miR-16-5p (Table 4, Fig. 2A). The following predictors of AP severity were identified by ROC analysis: miR-126-p (AUC 0.748, sensitivity 60.0%, specificity 87.1%, $p < 0.001$) and miR-551b-5p (AUC 0.716; sensitivity 69.2%, specificity 72.6%, $p < 0.001$).

Receiver operating characteristic (ROC) analysis of significant miRNAs in ACS patients

To evaluate the predictive value of up-regulated miRNAs, ROC curves were calculated for each of them. In the case of the entire AP

Discussion

Our observational study clearly demonstrated that some organ-abundant miRNAs such as hsa-miR-122-5p (liver), hsa-miR-375, and has-mir-148a-3p (pancreas) can be considered biomarkers of

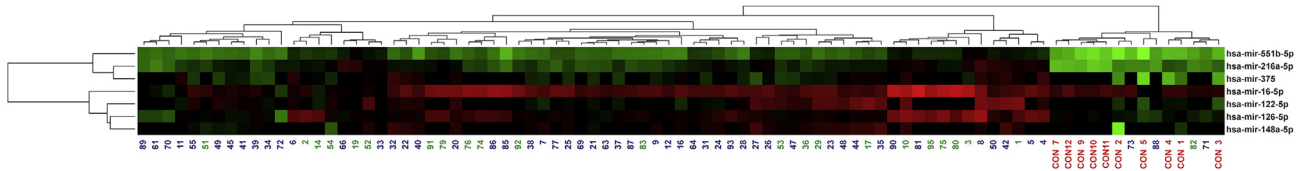


Fig. 1. Hierarchical clustering of median-normalized miRNA expression data in serum samples from the cohort of acute pancreatitis (AP) patients and control subjects. Mild acute pancreatitis (MAP) patients are depicted by blue numbers, moderate and severe acute pancreatitis (SAP) as green numbers and controls are in red.

Table 4
Results of ROC analysis for differentiation of acute pancreatitis patients (AP) from controls and stratification according severity of diseases (MAP vs. SAP).

miRNA	AUC	Standard error	(95% CI)	Sensitivity [%]	Specificity [%]	p-value
SAP vs. MAP patients						
hsa-miR-16-5p	0.613	0.074	0.468-0.758	48.4	51.6	NS
hsa-miR-122-5p	0.676	0.11	0.461-0.891	62.5	67.6	NS
hsa-miR-126-5p	0.748	0.059	0.633-0.863	60.0	87.1	<0.001
hsa-miR-148a-3p	0.481	0.12	0.246-0.716	62.5	54.5	NS
hsa-miR-216a-5p	0.671	0.059	0.555-0.787	58.1	41.9	NS
hsa-miR-375	0.458	0.088	0.286-0.631	55.6	44.4	NS
hsa-miR-551b-5p	0.716	0.056	0.605-0.827	69.2	72.6	<0.001
AP vs. control subjects						
hsa-miR-16-5p	0.559	0.071	0.421-0.697	65.9	50.0	NS
hsa-miR-122-5p	0.942	0.04	0.864-1	82.2	100	<0.0001
hsa-miR-126-5p	0.725	0.06	0.608-0.842	54.0	91.7	<0.001
hsa-miR-148a-3p	0.988	0.017	0.954-1	97.6	100	<0.0001
hsa-miR-216a-5p	0.958	0.021	0.918-0.998	83.0	100	<0.0001
hsa-miR-375	1	0	1	97.8	100	<0.0001
hsa-miR-551b-5p	0.977	0.014	0.949-1	89.0	98.7	<0.0001

Abbreviations: AUC – the area under the receiver-operator characteristic curve, NS – not significant, bold indicates statistical significance.

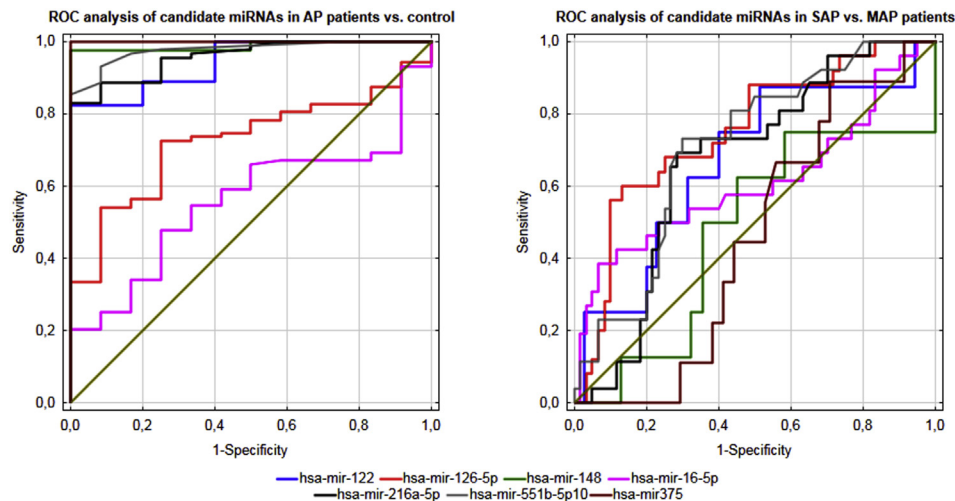


Fig. 2. ROC curves analysis in acute pancreatitis (AP) patients for selected miRNAs. (A) miRNAs discriminating value for AP vs. controls; (B) miRNAs discriminating value for mild (MAP) vs. moderate and severe (SAP) patients.

AP diagnosis, but only those related to vascular dysfunction (has-miR-126 and -551b-5p) are discriminative of disease severity.

Circulating miRNAs are derived from different cells, and their increased levels are usually associated with different pathological conditions. Extracellular miRNAs which are detectable in the bloodstream are usually highly stable; thus they may be used as blood-based biomarkers for cancer and other diseases [16,17]. However, the clearance and stability of circulating miRNAs depends not only on cellular exo- and endocytosis, but they are also regulated by individual profiles of miRNA-carriers (extracellular microvesicles and lipoproteins) in patients [18]. In contrast to serum, in plasma, there are a number of circulating phosphatidylserine-positive microvesicles, especially those from platelets, which can contribute to miRNA transfer. These microvesicles are scarce in serum (non-published observation) because they participate in coagulation processes. And *vice versa*, distinct profiles of miRNAs are usually consistent among individuals [32,33]. Thus, we should be aware that each biological fluid compartment contains distinct miRNA signatures [18]. In our study we decided to use serum as a most stable and reproducible fluidic sample, which may be a reason for some discrepancies between our results and other studies [15]. However, we confirmed the most important finding that miR-551b-5p and miR-216a-5b are increased in AP patients [15,21].

In the early phase of AP, hemoconcentration is often observed, which results in microcirculation impairment and plays a role in the development of pancreatic necrosis [34,35]. One of the most important components of AP therapy is early intravenous fluid resuscitation. It is without doubt that a decrease in mortality is due to prevention of pancreatic necrosis by maintaining microcirculation with more aggressive fluid resuscitation [4]. And *vice versa*, patients with pancreatic necrosis at admission were characterized by high (>47%) hematocrit (HCT) that was still elevated during the next 24–48 h [34]. In contrast, low HCT during the first 24 h of AP was associated with a low risk for pancreatic necrosis and with better outcomes [5,6]. Hemoconcentration generally does not correlate with common markers of AP (serum amylase and lipase). Nevertheless, our previous studies showed (non-published data) that HCT correlates with urine amylase ($R = 0.41$; $p < 0.01$) or plasma albumin ($R = 0.50$; $p < 0.01$). Additionally, we found a number of interesting correlations between red cell distribution width (RDW-CV) and the concentration of selected inflammatory mediators (e.g., interleukins 6 and 18, procalcitonin, or osteoprotegerin) [36]. In the current study, we did not analyze correlations between HCT and selected miRNAs; nevertheless, we found that mean HCT values in MAP patients were lower ($37.96 \pm 6.04\%$) than in SAP patients ($39.50 \pm 9.00\%$).

Intensive fluid resuscitation initiated during the first 12–24 h from admission is a recommended therapy in patients with AP, especially with SAP [2,7,35]. However, such therapy does not prevent hypoxia of distant organs, including pancreas, caused by higher oxygen demands. Moreover, in some patients, generalized anaerobic metabolism may lead to severe complications such as acute pulmonary edema, cerebral edema, abdominal compartment syndrome (ACS), and persistent organ failure [7,8,35]. According to Mentula *et al.* [7], ACS often results in multiple organ dysfunction and mortality in AP. This complicated net of relationships explains the mechanisms of multiorgan damage.

In the present study, the selected circulating miRNAs were analyzed depending on their specificity to organ dysfunction or damage. Specific criteria for miRNA selection were established: 1) abundance in patient serum [37], 2) organ specificity and 3) significance for organ injury. Taking these standards into consideration, we selected a set of miRNAs for our analyses: hsa-miR-16-5p, -103, -122-5p, -126-5p, -148a-5p, -216a-5p, -375.

Two selected miRNA: miR-16-5p and -103a-3p are commonly abundant in human plasma and are usually proposed to be in use as housekeeping or normalizing miRNAs [32]. We confirmed the usefulness of miR-103a-3p as a control for miRNA profiling; however, miR-16-5p appeared to be inappropriate for such purpose. miR-16-5b belongs to the "canonical" miR-16 family, which is proposed to be antiangiogenic and proapoptotic [23,38]. Increase of miR-16-5p in pancreatic stellate cells induces caspase-3, -8 and -9 activity *in vitro*. Moreover, inhibition of miR-16-5p as well as miR-15-b diminishes their proapoptotic effect [23]. This may suggest that circulating miR-16 may regulate apoptosis in even distant cells. Interestingly, inhibition of miR-16 enhances the proangiogenic potential of proangiogenic circulating cells; thus, miR-16-5p may serve as a prognostic biomarker in patients with vascular complications [39].

Other endothelial-specific miRNAs were increased in patients with AP: has-miR-126-5p and -551b-5p. While the role of miR-126 in endothelial dysfunction has been previously demonstrated, there is little information regarding the function of miR-551b-5p [40]. One of the most noteworthy relationships is down-regulation of miR-551b in hypoxia-stimulated HUVECs (human umbilical endothelial cells) [41]. Moreover, miR-551b has been uniquely detected in normal human plasma [37]. These observations, and the fact that miR-551b-5p has been significantly increased in SAP patients [21], encouraged us to select this miR for analysis. Our observations confirmed the data published by Liu *et al.* [21] that show that miR-551 could have possible diagnostic applications in discrimination between SAP and MAP patients. The role of miR-126-5p in vascular damage has been proposed by other authors [42]; nevertheless, we are the first who showed that miR-126-5p is elevated in AP. Previous investigations demonstrated that miR-126-5p is released from endothelial cells in diabetic patients and detectable in plasma samples [43]. A major source of miR-126-5p in plasma are platelets. In pathological state related to platelet activation and vascular injury, such as diabetes type 2, increased levels of circulating miR-126 can be used as a biomarker for progression of vascular damage [44]. In our study, we found that miR-126-5p is significantly increased in serum from SAP patients and can be used for discrimination between SAP and MAP patients.

Interestingly, increased levels of circulating liver-specific miR-122-5p were observed in our MAP and SAP patients, but they were not statistically significant. miR-122 is associated with cardiogenic shock in model systems, and a possible application in laboratory diagnostics of dysfunction in organs other than the liver could be proposed [45]. miR-375 has an unlimited potential for monitoring (in clinics) and understanding (in pathophysiology) the pancreas function. miR-375 is strongly related to diabetes and beta-cells function [46–48]. Increased levels of circulating miR-375 in patients with other acute diseases (myocardial infarction) is probably related to impaired glycogenesis, hyperglycemia, and hypoxia [27,49,50]. miR-375 is highly expressed in Langerhans' islets in both human and rat tissues, which was identified by microarray method [20]. It was demonstrated on a rat model that higher levels of circulating miR-375 correspond to time and size of pancreatic lesions [50].

Another miR related to pancreatic injury (miR-216a-5p) has been investigated in both pancreatic cancer and AP [20,51,52]. According to our search in the TSMiR database, miR-216a-5p regulates expression of the FOS transcription factor complex. The FOS proteins have been as regulators of cell proliferation, differentiation, and transformation; thus we may accept that miR-216a-5p may act as a regulatory factor in a generalized inflammatory process. Our observation that circulating miR-216a-5p is significantly increased in the acute phase of disease in both MAP and SAP patients are in concordance with studies by Endo *et al.*, Kong *et al.* and

Blenkiron *et al.* [15,20,22,53]. However, this pancreatic-abundant miR discriminates neither MAP vs. SAP patients nor mild vs. moderate AP patients [15]. An additional interesting finding is that animal models of AP are very complimentary to the time course of disease, giving us the opportunity to observe the dynamics of miRNA release. It was shown on rat and mice models that miR-216a (and other ones: -216b, -217) is actively released to the circulation in a time- and dose-dependent manner [20,48,52]. Concluding, a pancreatic miRNA signature displays several good characteristics that make it a valuable biomarker and can be useful for assessment of pancreas injury in the acute phase of pancreatitis; nevertheless, it is less specific in discrimination of the severity of disease.

In patients in shock related to sepsis and organ failure, significant correlations between miR-133a and disease severity have been revealed; patients with an APACHE-II score above 10 had significantly higher miR-133a levels than those with an APACHE-II below 10 [54]. The study by Tacke *et al.* illustrates that specific miRNAs can be used as predictors for an unfavorable prognosis, and for both short- and long-term mortality in critically ill patients. The same miR-133a has been described as a very early and sensitive biomarker for patients with acute MI, and its diagnostic relevance is comparable to or even better than common cardiac markers - troponins [25].

Some limitations of our study merit consideration. Firstly, the study design was based on the selection of candidate miRNAs rather than on microarray profiling. Systemic evaluation of specific miRNAs by means of the microarray method is a recommended approach for biomarkers selection; nevertheless, we must be aware that microarrays are designed for a wide application range, and do not cover every tissues optimally. Moreover, their sensitivity to plasma/serum and other fluidic samples is very limited due to a higher number of copies needed for hybridization. Additionally, in fluidic samples the yield of extracted miRNA is relatively lower than those from solid tissues. To increase the yield, some plasma/serum dedicated methods of miRNA extraction should be applied. Thus, in our study we decided to perform data-based research to select candidate miRNAs, rather than to perform microarray-based identification as that performed by Liu *et al.* recently [21]. Secondly, we did not apply kinetic analysis of circulating miRNA signatures. The time course of circulating miRNA levels may bring us some additional information about dynamic changes of the human miRNome during AP and may refine clinical diagnosis in such patients. Nevertheless, we focused our interest on patients with AP to find any significant differences in miRNA signatures corresponding to disease severity. Other limitations like sampling bias and data collection were avoided. In order to check if our population was sufficient to provide information about the impact of specific miRNAs levels on MAP and SAP patients discrimination, we performed the power analysis for normalized and non-normalized Ct. Accordingly, if we used the normalized or non-normalized Ct, to achieve the quantifiable standardized effect (Es) with a power equal to 0.4 and Alfa error on the level 0.05, we should have at least $n = 68$ subjects for analysis ($MO_{Ct} = 30$ and $Mu_{Ct} = 26$). If we normalized data ($MO_{Ct} = 0$) we will calculate the same number of subject for analysis.

In conclusion, a pancreatic miRNA signature can be useful for assessment of pancreas injury in the acute phase of AP. Endothelial dysfunction during AP is reflected by levels of specific circulating miRNAs and may help in patient stratification.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pan.2015.05.475>.

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