Serum miRNA Profile in Diabetic Patients with Ischemic Heart Disease as a Promising non-invasive Biomarker

Agnieszka Bielska (✉ agnieszka.bielska@umb.edu.pl)

Uniwersytet Medyczny w Białymstoku

Witold Bauer

Uniwersytet Medyczny w Białymstoku

Anna Szalkowska

Uniwersytet Medyczny w Białymstoku

Iwona Sidorkiewicz

Uniwersytet Medyczny w Białymstoku

Anna Skwarska

Uniwersytet Medyczny w Białymstoku

Justyna Raczkowska

Uniwersytet Medyczny w Białymstoku

Damian Ostrowski

Uniwersytet Medyczny w Białymstoku

Kamil Gugala

Uniwersytet Medyczny w Białymstoku

Sławomir Dobrzycki

Uniwersytet Medyczny w Białymstoku

Magdalena Niemira

Uniwersytet Medyczny w Białymstoku

Adam Kretowski

Uniwersytet Medyczny w Białymstoku

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Abstract

Background

The increasing morbidity and mortality of type 2 diabetic mellitus (T2DM) patients with ischemic heart disease (IHD) highlights an urgent need to identify early biomarkers, which would help to predict individual risk of development of IHD. Here, we postulate that circulating serum-derived miRNAs may serve as potential biomarkers for early IHD diagnosis and help to identify diabetic individuals with a predisposition to undergo IHD.

Methods

We obtained serum samples from T2DM patients either with IHD or IHD-free and analysed the expression levels of 798 miRNAs using the NanoString nCounter Technology Platform. The prediction of the putative miRNAs targets was performed using the Ingenuity Pathway Analysis (IPA) software. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic value of identified miRNAs.

Results

Our data showed that 9 miRNAs (miR-1224-5p, miR-1303, miR-3147, miR-4455, miR-498, miR-548b-3p, miR-548d-3p, miR-615-3p, miR-651-5p) were significantly upregulated in T2DM IHD patients compared to T2DM patients without IHD. In patients with upregulated miRNA, functional enrichment analysis of target genes by IPA indicated networks and canonical pathways involved in the pathology of the cardiovascular system. All tested miRNAs showed high diagnostic value (AUC > 0.8).

Conclusions

Taken together, our findings suggest that circulating miRNAs might have a crucial role in the development of IHD in diabetic patients and may be used as a potential biomarker for early diagnosis.

Background

Diabetes mellitus is a group of endocrine and metabolic disorders characterised by insulin secretion that is insufficient to maintain the right blood glucose level. According to the International Diabetes Federation, 463 million people suffered from diabetes in 2019 and this number is expected to rise up in the next years [1]. Several forms of diabetes are distinguished, however, type 2 diabetes mellitus is the most common and accounts for over 90% of cases [2]. T2DM is characterized by cell resistance to normal concentration of insulin circulating in the blood. With the progression of the disease, pancreatic β -cell may also become dysfunctional and inevitably stop to produce insulin [3]. As a result, prolonged hypoglycaemia develops, which may cause serious cardiovascular problems [4]. The most common long-

term complications of diabetes are nephropathy, retinopathy, neuropathy, stroke, atherosclerosis or ischemic heart disease [5]. Those complications can become serious and may ultimately lead to the death of the patient. It is well known that cardiovascular disease, and especially ischemic heart disease (IHD), are the leading causes of morbidity and mortality in diabetes [6, 7]. IHD results from imbalance between blood supply and oxygen demand in myocardial cells caused by different degree of a coronary artery obstruction. Typically, IHD occurs when atherosclerosis develops in the coronary artery. This process is characterised by remodelling of arteries by an accumulation of deposits of calcium and fatty lipids. Ischemia is not only related with inadequate oxygen supply but also with reduced availability of nutrients and insufficient removal of metabolic products [8]. This pathological state is often the main reason of damage in the heart muscle and myocardial infarction (MI) [9]. Patients with T2DM have a higher risk of developing IHD than non-diabetic patients. Additionally, they also have a higher risk of mortality following IHD compared with healthy ones [10].

Diabetes complications, such as heart diseases, develop much earlier before they are clinically diagnosed [11]. To prevent the progression of the disease, special attention should be paid to its early detection. Despite the rapid progress in cardiovascular research, there is no reliable tool for prompt diagnosis and identification of people at risk of developing IHD. The coronary angiography remains the gold standard in the diagnosis of IHD. Unfortunately, it is an invasive medical procedure that uses contrast dye to detect blockages in coronary arteries at x-ray pictures [12]. Additionally, this method can only diagnose the disease at a later stage. Therefore, quick and safe detection of this condition at the initial state or at diagnosis of diabetic patients with a predisposition to IHD is crucial. Currently, scientists focus efforts to find the most suitable method for early IHD diagnosis. Understanding the exact mechanisms underlying diabetes complications could be helpful to develop better therapeutic approaches, provide more effective monitoring of the disease progression and allow early diagnosis of heart dysfunction.

Recent research shows that inflammatory mediators like chemokines CXCL12 and macrophage migration-inhibitory factor (MIF) play an important role in the pathology of IHD. In humans, MIF is abundantly produced by various cells in different stages of plaque development, indicating MIF playing an important role in the development of atherosclerotic plaque [13, 14]. Elevated levels of MIF in plasma can serve as an early biomarker for acute myocardial ischemia [15]. Also, CXCL12 was described as a potential marker for IHD diagnosis. It has been shown that CXCL12 levels in plasma are better predictors of coronary artery disease outcomes than traditional risk factors such as serum creatinine levels, hypertension or left ventricular ejection fraction [16]. Unfortunately, the concentration of MIF and CXCL12 in the serum seems to be characteristic not only for the ischemic heart disease but also for different inflammatory states [17], raising the need for more selective markers for IHD diagnosis.

A promising tool for understanding the pathogenesis of IHD are miRNAs [18]. MiRNAs are small (17–25 nucleotides) noncoding RNAs that play an essential role in the regulation of gene expression. MiRNAs control the expression of target genes by base pairing to the 3' untranslated regions (3' UTRs) of mRNA and inducing repression of the target mRNA. This effect can occur by transcript destabilization or inhibition of translation [19]. Bioinformatics predictions indicate that one miRNA could target more than

hundred mRNAs [20]. MiRNAs participate in critical biological processes such as proliferation, differentiation, and apoptosis of cells [21]. Abnormal expression of miRNAs is associated with the development of various diseases, such as cancer, cardiovascular diseases or metabolic disorders. Some miRNAs can be upregulated or downregulated in cardiac or cardiovascular cells under pathophysiological conditions [6]. It is commonly known that levels of certain circulating miRNAs might be predictive for long-term diabetes complications [22]. MiRNAs may be a useful biomarker because of its stability in biofluids even after prolonged time since collection and several freezing-thaw cycles [23]. Moreover, those small particles can be easily collected and measured with specific, sensitive assays [22].

Recent studies have shown that miRNAs play an important role in the development of heart diseases. It has been proven that miRNAs (miR-33, miR-144, miR-30c, miR-378, miR-96, miR-185) are involved in maintaining of lipoprotein homeostasis by regulation of cholesterol transport and its uptake [23]. MiRNAs such as miR-92a, miR-503, miR-126 can also control and regulate angiogenesis, crucial for the repair of myocardial cells after ischemic injury [24, 25]. MiR-155, miR-342-5p are involved in vascular inflammation by modulation of inflammatory response and atherosclerosis progression [26]. MiRNAs like miR-125b, miR-205 are able to regulate process of vascular calcification that contributes to cardiovascular conditions such as atherosclerosis [27, 28]. Additionally, decreased level of miR-126 is known as a predictor of diabetes and it also occurs in patients with IHD [21]. Many studies describe cardiac muscle-specific circulating miRNAs, such as miR-208, miR-133a/b, miR-499-5p as an useful tool in the prognosis of the risk of MI or major adverse cardiac events [29]. MiR-130, miR-134, miR-204 play important roles in endothelial dysfunction. Endothelial cells are important in the pathological progression of vascular problems [21]. In diabetes, there is an established connection between miR-223 and activation of platelets, the latter which significantly contributes to the development of cardiac disease [30]. However, despite evident progress, our understanding of the regulation and function of specific miRNAs in IHD is still limited.

In the present study, we have investigated the differential expression of IHD-associated miRNAs in the serum samples from T2DM patients with (+) and without (-) IHD using the NanoString platform, a novel technique offering a high level of precision and sensitivity without amplification reaction [31]. Furthermore, we assessed the levels of MIF and CXCL12 in serum and correlated those results with obtained miRNAs profile in patients with T2DM and IHD. Our results indicate that the panel of nine miRNA has the potential to improve the early IHD diagnosis in patients with T2DM.

Methods

Baseline characteristics of the patients

To diagnose IHD in a group of over 600 patients with T2DM which participated in the cohort study, coronary angiography was performed. This procedure has distinguished two subsets of T2DM patients - with and without IHD. Exclusion criteria for this study included: other T2DM complications, other inflammatory states (rheumatoid arthritis, systemic sclerosis), cancers, human immunodeficiency virus

(HIV) or hepatitis C virus (HCV) infection, alcohol consumptions and smoking. A group of 43 individuals was qualified for further analysis. Serum samples were collected, centrifuged and stored at -80°C .

All study participants provided written informed consent and received information on the study and associated risks prior to enrolment. The methods were carried out in accordance with the approved guidelines and were conducted in accordance with the ethical standards of the institutional research committee, with the 1964 Helsinki declaration and were approved by the local ethics committee of the Medical University of Bialystok, Poland (approval number: R-I-002/583/2019).

MiRNAs isolation

The miRNeasy Serum/Plasma Kit (Qiagen, Germany) was used for RNA extraction (smaller than 1000 nucleotides) using 200 μL of serum aliquots from one patient according to the manufacturer's instructions. The miRNA concentration was measured by The Qubit microRNA Assay Kit with the Qubit® 2.0 Fluorometer.

Detection of miRNAs profile

A total of 43 samples were prepared for nCounter miRNA expression profiling according to the manufacturer's recommendations (NanoString Technologies, USA). A 3 ng of isolated microRNA were used as input material. Unique DNA tags were ligated onto the 3' end of each mature miRNA, providing an identifier for each miRNA in the sample. Tagging was performed in the ligation reaction followed by an overnight hybridization (65°C) to nCounter Reporter and Capture probes that allowed to complex sequence specific probes with targets. After hybridization, samples were placed into the nCounter Prep Station for automated sample purification and target/probe complexes immobilization on the cartridge for data collection.

Each sample was scanned for 555 FOV (fields of view) on the nCounter Digital Analyzer (NanoString Technologies, USA) to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. NanoString raw data were analysed with nSolver™ software (NanoString Technologies, USA). The NanoString data were deposited in the Gene Expression Omnibus (GEO) database (accession number: GSE153593).

Measurement of MIF and CXCL12 in serum

The concentration of MIF and CXCL12 in serum was determined in duplicate samples by enzyme-linked immunosorbent assay (ELISA) (Quantikine Human M-CSF Immunoassay; R&D systems, Abingdon, United Kingdom), according to the manufacturer's recommendations.

Statistical analysis

Statistical analysis was performed using STATISTICA version 13.1 (StatSoft, Tulsa, Oklahoma). To examine the statistical difference in clinical parameters between the groups the U-Mann Whitney test was performed. miRNAs were tested for differential expression using nSolver 4.0 Analysis software (NanoString) including normalization using the average geometric mean of the top 100 miRNAs detected.

The *p*-values were adjusted using the False Discovery Rate (FDR) correction for multiple comparisons. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic value of miRNAs and for each miRNA and the area under the curve (AUC) was calculated. The Spearman rank-order correlation coefficient (*r*) was determined to estimate the correlation between the identified miRNAs and clinical parameters. It was assumed that *r* > 0.8 indicates strong correlation and *r* > 0.3 indicates moderate correlation. The statistical significance level was set at *p* < 0.05. Ingenuity Pathway Analysis (QIAGEN Inc) was performed to generate a list of predicted targeted genes for studied miRNAs, identify canonical pathways, diseases and functions and gene networks in which target genes were involved.

Results

Patient baseline characteristic

The study and control groups consisted of 43 patients with T2DM, 24 of which were also diagnosed with IHD. IHD (-) and IHD (+) groups did not show significant differences in clinical parameters such as duration of diabetes, platelets, fibrinogen, body mass index (BMI), glucose level, glycated haemoglobin (HbA1c) level, cholesterol, triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The clinical characteristics of patients enrolled in this study were summarized in Table 1.

Table 1
Patient baseline characteristics.

Characteristics	Patients (n = 19) (+) T2DM / (-) IHD	Patients (n = 24) (+) T2DM / (+) IHD	<i>p</i> -value
Mean age	60.35 (CI 56.31–64.39)	58.55 (CI 55.41–61.68)	0.91
Duration of T2DM	7.36 (CI 4.66–10.07)	5.89 (CI 4.06–7.73)	0.77
Platelets	231 (CI 205.53–256.47)	249.42 (CI 211.39–287.45)	0.98
Fibrinogen	357.38 (CI 327.18–387.57)	422.45 (CI 375.8–469.11)	0.39
BMI	31.65 (CI 29.45–33.86)	30.5 (CI 28.69–32.32)	0.56
Glucose level	147.88 (CI 114.87–180.9)	145.24 (CI 122.87–167.6)	0.90
HbA1c level	6.85 (CI 6.09–7.61)	7.52 (CI 6.92–8.12)	0.55
Cholesterol	199.07 (CI 181.3–216.83)	181.94 (CI 160.63–203.26)	0.55
TG	163 (CI 111.43–214.57)	165.29 (CI 138.74–191.85)	0.77
LDL	120.14 (CI 103.99–136.3)	101.65 (CI 84.78–118.52)	0.55
HDL	48.73 (CI 43.29–54.18)	42.12 (CI 37.11–47.13)	0.46
CI, confidence interval; the <i>p</i> -value describes significance of difference between patients with T2DM without IHD in comparison to patients with T2DM and IHD using Mann-Whitney U-test.			

Baseline levels of circulating miRNAs

The NanoString Technology Platform has been used as a tool for the identification of miRNAs with potential diagnostic value. miRNA profiling from the serum of diabetic patients with IHD (n = 24) and without IHD (n = 19) that constituted the control group was performed. nSolver 4.0 analysis indicated that expression of 9 miRNAs was statistically different between two compared groups ($FC > 1.5$, $FDR \leq 0.05$). Importantly, these 9 miRNAs were upregulated in serum samples from IHD diabetic patients (Table 2.)

Table 2
miRNAs upregulated in T2DM with IHD patients.

miRNA	FC	FDR
hsa-miR-498	2.71	< 0.01
hsa-miR-3147	2.28	< 0.01
hsa-miR-615-3p	2.25	< 0.01
hsa-miR-548b-3p	2.15	< 0.01
hsa-miR-1224-5p	1.92	< 0.01
hsa-miR-1303	1.77	< 0.01
hsa-miR-548d-3p	1.67	< 0.01
hsa-miR-4455	1.59	0.05
hsa-miR-651-5p	1.54	0.04
FC, fold change; FDR, false discovery rate, adjusted <i>p</i> -value; $FC \geq 1.5$; $FDR \leq 0.05$		
Functional enrichment analysis in T2DM IHD (+) patient's samples		
IPA analysis indicated 858 putative target genes for 9 studied miRNAs. Most potential targeted genes were regulated by miR-4455 (323 potential target genes) (Supplementary Table 1.).		
Through further analysis of target genes, 70 top diseases or function connected with the cardiovascular system were found (Supplementary Table 2.). Among them, 4 most significant were connected with an acute coronary syndrome, a disorder of coronary artery, abnormal morphology of coronary artery and ischemic heart disease (Table 3).		

Table 3

Top 4 diseases and functions associated with the cardiovascular system indicated by analysis of target genes of the 9 tested miRNA using IPA.

Categories	Diseases or Functions Annotation	p-value	genes	number of genes
Cardiovascular Disease, Organismal Injury and Abnormalities	Acute coronary syndrome	0.000232	<i>ADORA1, APOB, AR, ATP4A, CACNG1, CAMLG, GABRA1, GABRG2, IL6, NOS3, PDE3A, PLA2G5, PPARD, PTGS1, TNNC1, TNNT2, TOR2A, TUBB2A, VEGFA</i>	19
Cardiovascular Disease, Organismal Injury and Abnormalities	Disorder of coronary artery	0.012	<i>AADAT, ADORA1, AMY2A, APOB, AR, ATP4A, CACNG1, GABRA1, GABRG2, IL6, LTBP1, MEF2A, NOS3, PDE3A, PTGS1, RASL12, SAMD12, SGCG, TEX36, TNNT2, TUBB2A, VASP, VEGFA</i>	23
Cardiovascular Disease, Cardiovascular System Development and Function, Organ Morphology	Abnormal morphology of coronary artery	0.0143	<i>LTBP1, NOS3, VEGFA</i>	3
Cardiovascular Disease, Organismal Injury and Abnormalities	Ischemic heart disease	0.0216	<i>AADAT, ADORA1, APOB, AR, GABRA1, IL6, MEF2A, NOS3, PDE3A, PTGS1, RASL12, SAMD12, TEX36, TNNT2, VASP, VEGFA</i>	16

The most relevant four gene networks, based on Fisher's exact test, were associated with cardiovascular system development and function, endocrine system disorders, cardiac arrhythmia, and skeletal and muscular system development pathways (Fig. 1).

The pathway analysis identified top 24 canonical pathways including apelin cardiomyocyte signalling pathway and atherosclerosis signalling pathway (Supplementary Table 3.). Top 10 of deregulated canonical pathways are shown in Fig. 2.

Levels of MIF and CXCL12 in serum

No statistical differences were observed between the serum concentration of MIF and CXCL12 in IHD (+) diabetes and IHD (-) diabetes groups. The medians values of MIF were 0.65 ng/ml in the IHD (+) group compared to 0.85 ng/ml in the HD (-) group. The median values of CXCL12 in IHD (+) and IHD (-) group were 2001.54 pg/ml and 2158.37 pg/ml, respectively ($p > 0.05$). These results indicate that neither MIF nor CXCL12 can serve as good IHD prognostic markers in T2DM patients.

Table 4
The levels of MIF and CXCL12 in serum from T2DM IHD (+) and T2DM IHD (-) patients.
Median and range results are shown.

	Patients (+) T2DM / (-) IHD	Patients (+) T2DM / (+) IHD	<i>p</i>-value
MIF ng/ml	0.85 (0.17–6.60)	0.652 (0.23–2.38)	> 0.05
CXCL12 pg/ml	2158.37 (508.38-2598.33)	2001.537 (807.11-3024.30)	> 0.05

Correlation of circulating miRNAs with clinical data

A Spearman's rank-order regression analysis was performed to study the relationship between miRNAs levels and clinical parameters of patients (Fig. 4). MiR-1224-5p and miR-1303 negatively correlated with HDL level ($r = -0.43$ and $r = -0.38$ respectively; $p < 0.05$). MiR-3147, miR-498, miR-548d-3p, miR-615-3p and miR-4455 were negatively correlated with LDL levels ($r = -0.34$; $r = -0.37$; $r = -0.39$; $r = -0.36$; $r = -0.34$ respectively; $p < 0.05$). Additionally, miR-615-3p, miR-498 and miR-651-3p were negatively correlated with HDL level ($r = -0.43$; $r = -0.39$ and $r = -0.34$, respectively $p < 0.05$). A moderate positive correlation between fibrinogen level and miR-1303, miR-3147, miR-498, miR-548d-3p and miR-615-3p was observed ($r = 0.32$; $r = 0.35$; $r = 0.41$; $r = 0.38$; $r = 0.3$, respectively; $p < 0.05$). None of the tested miRNAs levels were significantly correlated with clinical parameters such as BMI, platelet levels, fasting glucose, HbA1c or triglycerides, consistent with lack of differences in these parameters between IHD (+) and IHD (-) patients at diagnosis. Statistical analysis showed strong positive correlation between serum levels of all studied miRNAs. MiR-498 showed the strongest positive correlation with miR-1224-5p, miR-1303, miR-3147, miR-548d-3p, miR-615-3p and miR-4455 ($r > 0.8$; $p < 0.05$). No significant correlations between MIF and CXCL12 levels and upregulated miRNAs were found.

Figure 3. Spearman's rank – matrix correlation between tested miRNAs, MIF, CLXC12 and clinical parameters. Blue colour indicates positive correlation and red indicate opposite.

Evaluation of diagnostic values of tested miRNAs

To estimate the possible roles of identified miRNAs as biomarkers for IHD in diabetic patients, ROC curve analysis was performed (Fig. 5.). The ROC curve illustrates the relationship between diagnostic sensitivity and specificity and describes the diagnostic value of the tested parameters [32]. AUC (area under the ROC curve) for all 9 miRNAs reached statistical significance comparing to AUC = 0.5 ($p < 0.001$ in all cases). The AUC values > 0.9 were found for miR-1224-5p, miR-1303, miR-3147, miR-498. Importantly, AUC scores for the other upregulated miRNAs (miR-548d-3p, miR-615-3p, miR-548b-3p, miR-651-5p, miR-4455) were also high (0.875; 0.877; 0.86 0.857; 0.836 respectively). The AUC values for MIF and CXCL12 were less than 0.5 (AUC = 0.5 borderline of the diagnostic usefulness of the test).

Discussion

The latest research show that miRNA can play an important role as a biomarker for diabetes and its complications [3]. The potential for biofluids–derived miRNA to serve as a diagnostic tool has stimulated a wide range of study regarding the disease-specific expression of miRNA and its stability [33]. In order to find the unique miRNA profile in T2DM patients with IHD, we used a state-of-the-art NanoString nCounter platform that provides the opportunity to profile a large number of miRNAs that have not been previously investigated in relation to IHD in diabetes. In comparison to other methods of miRNA detection, the NanoString offers greater sensitivity and specificity with high quality of data due to the elimination of amplification [34]. It has been shown that this platform detects miRNAs in biofluids with sensitivity and specificity greater than other miRNA detection methods like Real-Time PCR or microarrays. [35]. This makes NanoString as a best strategy to identify novel biomarker candidates for IHD prognosis. To our best knowledge, this study is the first miRNA profiling in the serum of IHD patients using NanoString platform.

Our research revealed 9 miRNAs differentially expressed in patients with T2DM and IHD in comparison to diabetic individuals without IHD. Among the differently expressed miRNAs, several were previously described as associated with the IHD or T2DM. For instance, it has been shown that platelet miR-615-5p was upregulated in patients with IHD as compared to healthy patients. The activation of platelets and the formation of a thrombus on a ruptured atherosclerotic plaque is an important mechanism in the pathogenesis of IHD [36]. Another research showed that the level of miR-1303 in serum was significantly increased in T2DM patients. Importantly, the level of miR-1303 was higher in serum of patient with microvascular complications (neuropathy, nephropathy, and retinopathy) than those without complications [37]. Fittingly, our results indicated upregulation of miR-615-3p and miR-1303 in the serum of T2DM IHD (+) patients. No significant expression of miR-615-3p and miR-1303 was observed in T2DM without IHD. These results suggest that miR-615-3p and miR-1303 are common for T2DM IHD (+) phenotype and could play a pivotal role in the IHD diagnosis in diabetic patients. Chen et al. has shown that miR-548d-3p regulates the expression of the *ERBB2* gene. This gene encodes an oncogenic HER2 tyrosine kinase receptor, highly expressed in various types of human tumours [38]. Moreover, *ERBB2* has an essential role in cardiac function and development [39, 40]. Clinical studies have indicated that downregulation of *ERBB2* leads to heart dysfunctions [41]. Correspondingly, our results showed significant overexpression of miR-548d-3p in T2DM IHD (+) patients. Given the involvement of miR-548d-3p in regulation of *ERBB2*, required for the proper functioning of the heart, it is plausible to expect miR-548d-3p to be a potential biomarker for IHD. We expect that the rest identified miRNAs candidates (miR-498, miR-1224-5p, miR-3147, miR-548b-3p, miR-651-5p and miR-4455) may also have a high probability of being specific for diabetic patients with IHD.

The area under the ROC curve remains a major criterion for diagnostic biomarkers [32]. To the best of our knowledge, there is no evidence in the literature describing the usefulness of miR-1224-5p, miR-1303, miR-3147, miR-498, miR-548d-3p, miR-615-3p, miR-548b-3p, miR-651-5p and miR-4455 as diagnostic tool for IHD in T2DM. Interestingly, miR-1303 in plasma was tested as a biomarker for acute myocardial infarction [42]. The AUC for miR-1303 was 0.884, but it was lower than AUC for high-sensitive cardiac troponin I (hs-cTnI) which seems to be a more specific biomarker in MI. In this study, the highest AUC

values were observed for miR-1224-5p, miR-1303, miR-3147 and miR-498. Furthermore, other tested miRNA (miR-548d-3p, miR-615-3p, miR-548b-3p, miR-651-5p) also showed high AUC results. High AUC scores for these miRNAs provide the groundwork for future confirmatory studies with a comprehensive validation in a larger cohort of patients. Additionally, AUC values for MIF and CXCL12, which play an important role in IHD, were below 0.5, what disqualifies these two proteins as a good diagnostic tool for IHD (+) diabetic patients. MIF as a proinflammatory factor is involved in processes and disorders such as atherosclerosis and myocardial infarction [43, 13]. It is also considered as a potential biomarker for heart diseases in patients with T2DM [44]. Similarly, CXCL12 has an important role in cardiovascular dysfunctions. Research shows that levels of this chemokine are prognostic tools to the prediction of future cardiovascular risk in patients with myocardial infarction, IHD or heart failure [45–47]. However, one has to consider that diabetes is also an inflammation state and non-specific inflammatory parameters like MIF or CXCL12 can be elevated in patients either with IHD or without it. Low AUC values for those proteins support our observations that indeed the changes in miRNA levels could be better prognostic IHD biomarkers than the level of MIF or CXCL12 in T2DM patients.

We could not find any statistically significant correlation between the level of miRNAs and clinical parameters such as BMI, platelet levels, fasting glucose, HbA1c or triglycerides. However, those parameters are not specific only for ischemic heart disease. Interestingly, we indicated a positive moderate correlation between fibrinogen and miR-1303, miR-3147, miR-498, miR-548d-3p. Fibrinogen is not only an indicator of hypercoagulability but, as an acute phase protein, is also an indicator of inflammation. In epidemiological and clinical studies, elevated blood fibrinogen levels have been shown to be an independent risk factor for cardiovascular diseases [48–50]. It has been proven that miRNAs are able to regulate fibrinogen production [51]. In our study, we indicated that the relationship between levels of fibrinogen and four miRNAs (miR-1303, miR-3147, miR-498, miR-548d-3p) that have not been previously described as being related to this protein. A strong or moderate positive correlation between all miRNAs level suggested they may belong to the miRNA group which participates in the dysregulation of mechanisms responsible for diabetic complications.

IPA analysis indicated 858 molecules regulated by identified miRNAs. Further analysis of these genes showed their connection with cardiovascular diseases, including IHD. Among genes regulated by miRNA were apolipoprotein B and *MEF2A*. The apolipoprotein B encoded by *APOB*, potentially regulated by miR-615-3p, is showing association with risk of ischemic heart disease [52]. The same miRNA regulates the *MEF2A* gene. *MEF2A* encodes transcription factor, that has an important role in the differentiation of cardiomyocytes. Also, it has been demonstrated that *MEF2A* is involved in the homeostasis of cardiac cells [53]. Studies show that *NAMPT*, possibly regulated by miR-548b-3p, is one of the major genes involved in hypertrophy of the heart [54]. Hypertrophy is known as a disease that can lead to IHD and other cardiovascular dysfunctions [55]. On the other hand, *TBX5* (T-box transcription factor) has also a crucial role in the development of cardiac disorders. *TBX5* is potentially regulated by miR-4455 and it is considered as a key regulator of heart development [56]. Similarly, it was indicated that Scm Polycomb Group Protein Like 4, encoded by *SCML4*, participates in atherosclerosis mechanism that can lead to ischemic heart disease [57]. In our study, IPA analysis has shown, that miR-1224-5p is involved in the

regulation of *SCML4* gene. This analysis pointed out considerable relationship between tested miRNAs and genes involved in cardiovascular dysfunctions.

Ingenuity core analysis allowed us to identify which canonical pathways are dysregulated by tested upregulated miRNAs. Our results showed that one of the most important dysregulated canonical pathways in the IHD (+) T2DM patients was apelin cardiomyocyte signalling pathway. Apelin is an endogenous peptide widely expressed in cardiomyocytes, brain, pancreatic islets and adipose tissue [58]. Under normal conditions, it is involved in lowering of blood pressure [59]. Recent studies have found that this apelin-mediated signalling is connected to heart failure and ischemic heart disease. Furthermore, apelin participates in the pathology of diabetes by playing a key role in increasing glucose uptake and insulin sensitivity [60]. Another important dysregulated signalling pathway was atherosclerosis signalling. Atherosclerosis is the process, when plaque builds up inside arteries underlying pathology of cardiovascular diseases and can lead to myocardial infarction [61]. Next altered pathway presented in our study was connected with granzyme B. Granzyme B is an enzyme of the serine protease family and is able to induce cell apoptosis by activating intracellular caspases. Apoptosis induced by granzyme B plays an essential role in cardiovascular diseases such as atherosclerosis, IHD and myocardial infarction [62]. Moreover, it was shown that in patients with chronic kidney disease, granzyme B can be a predictive factor of ischemic heart disease [63]. Additionally, plasma levels of granzyme B are increased in patients with acute coronary syndrome [64]. Endothelin-1 signalling was another canonical pathway highlighted by IPA analysis. Endothelin-1 is a potent vasoconstrictor and pro-inflammatory protein and is an important contributor to the pathogenesis of hypertension, atherosclerosis, hypertrophy and diabetes [65]. In normal conditions, endothelin-1 is involved in increase arterial blood pressure and reduction of heart rate. Influenced by risk factors for cardiovascular disorders its expression is altered, what plays an important role in the pathology of the cardiovascular system [66]. Furthermore, patients with T2DM have increased vasoconstrictor activity induced by endothelin-1 [67]. Certainly, these identified canonical pathways play a significant role in the mechanisms leading to the development of cardiovascular diseases. It allows to suppose that determined by us miRNAs play a relevant role in the regulation of genes associated with cardiovascular diseases and top dysregulated canonical pathways.

Conclusion

Presented data indicate that there is a specific serum miRNA profile of patients with T2DM and IHD and the different levels of selective miRNA expression might have a crucial role in further IHD diagnosis. We believe that identified circulating miRNAs might serve as new, noninvasive biomarkers for early detection of IHD in T2DM patients. However, this data should be considered as preliminary and additional studies on larger cohort of patients are required to validate the predictive value of those miRNAs to prompt diagnosis of IHD in T2DM patients or better identification of at-risk individuals.

Abbreviations

3'UTR

3' untranslated regions; BMI:body mass index; APOB:apolipoprotein B; CI:confidence interval; CXCL12 C-X-C: motif chemokine 12; ELISA:enzyme-linked immunosorbent assay; ERBB2:receptor tyrosine-protein kinase erbB-2; FC:fold change; FDR:false discovery rate; GEO:Gene Expression Omnibus; HbA1c:glycated haemoglobin; HCV:hepatitis C virus; HDL:high-density lipoprotein; HIV:human immunodeficiency virus; hs-cTnl:high-sensitive cardiac troponin I; IHD:ischemic heart disease; IPA:Ingenuity Pathway Analysis; LDL:low-density lipoprotein; MEF2A:myocyte-specific enhancer factor 2A; MI:myocardial infarction; MIF:macrophage migration-inhibitory factor; miRNA:micro RNA; NAMPT:nicotinamide phosphoribosyltransferase; RNA:ribonucleic acid; ROC:receiver operating characteristic curve; SCML4:Scm Polycomb Group Protein Like 4; SLC17A6:solute carrier family 17 member 6; T2DM:type 2 diabetes mellitus; TBX5:T-box transcription factor; TG:triglycerides.

Declarations

Ethics approval and consent to participate

The methods were carried out in accordance with the approved guidelines and were conducted in accordance with the ethical standards of the institutional research committee, with the 1964 Helsinki declaration and were approved by the local ethics committee of the Medical University of Bialystok, Poland (approval number:R-I-002/583/2019).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) database (accession number:GSE153593).

Competing interests

The authors declare that they have no competing interests.

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Conceptualization, A.B., M.N and A.K.; methodology, A.B., M.N., I.S., A.S., A.K.; formal analysis, A.B., M.N., I.S., W.B., investigation, A.B., A.Sz., J.R., D.O., K.G., M.N.; writing—original draft preparation, A.B.; writing—review and editing, A.K., M.N., A.S.; visualization, AB., M.N.; supervision, M.N., S.D., A.K.. All authors have read and agreed to the published version of the manuscript.

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Figures

A

B

Figure 1

The highest scoring interaction networks identified by IPA for the target genes that were connected with cardiovascular diseases.

C

D

Figure 2

The highest scoring interaction networks identified by IPA for the target genes that were connected with cardiovascular diseases.

Figure 3

Top 10 Ingenuity Pathway Analysis (IPA) canonical pathways most significantly changed in T2DM patients with IHD compared to individuals without IHD. The x-axis shows the -log of p-value calculated by the Benjamini-Hochberg (B-H) method.

Figure 4

Spearman's rank – matrix correlation between tested miRNAs, MIF, CXCL12 and clinical parameters. Blue colour indicates positive correlation and red indicate opposite.

Figure 5

The area under the curve (AUC) of the receiver operating characteristic (ROC) curves for tested miRNAs (A) and MIF and CXCL12 proteins (B). ROC analysis was carried out in order to evaluate the diagnostic potential of selected miRNA as a predictive biomarker of type 2 diabetes in comparison to MIF and CXCL12 proteins.

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