Příloha I

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Research Article

The Expression Profile of MicroRNAs in Small and Large Abdominal Aortic Aneurysms

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Background. Abdominal aortic aneurysms (AAA) are relatively frequent in elderly population, and their ruptures are related with high mortality rate. There are no actually used laboratory markers predicting the AAA development, course, and rupture. MicroRNAs are small noncoding molecules involved in posttranscriptional gene expression regulation, influencing processes on cell and tissue levels, and are actually in focus due to their potential to become diagnostic or prognostic markers in various diseases. *Methods*. Tissue samples of AAA patients and healthy controls were collected, from which miRNA was isolated. Microarray including the complete panel of 2549 miRNAs was used to find expression miRNA profiles that were analysed in three subgroups: small ($N = 10$) and large ($N = 6$) aneurysms and healthy controls ($N = 5$). Fold changes between expression in aneurysms and normal tissue were calculated including corresponding p values, adjusted to multiple comparisons. *Results*. Six miRNAs were found to be significantly dysregulated in small aneurysms (miR-7158-5p, miR-658, miR-517-5p, miR-122-5p, miR-326, and miR-3180) and 162 in large aneurysms, in comparison with the healthy control. Ten miRNAs in large aneurysms with more than two-fold significant change in expression were identified: miR-23a-3p, miR-24-3p, miR-27a-3p, miR-27b-3p, miR-30d-5p, miR-193a-3p, miR-203a-3p, miR-365a-3p, miR-4291, and miR-3663-3p and all, but the last one was downregulated in aneurysmal walls. *Conclusion*. We confirmed some previously identified miRNAs (miR-23/27/24 family, miR-193a, and miR-30) as associated with AAA pathogenesis. We have found other, yet in AAA unidentified miRNAs (miR-203a, miR-3663, miR-365a, and miR-4291) for further analyses, to investigate more closely their possible role in pathogenesis of aneurysms. If their role in AAA development is proved significant in future, they can become potential markers or treatment targets.

1. Introduction

Abdominal aortic aneurysm (AAA), the enlargement of abdominal aorta to a diameter of at least 3 cm, is a common disease in the western part of the world, mainly in developed countries. The most common occurrence is in men over 65 years, where the prevalence is around 4–7% [1]. However, a significant percentage of the deaths caused by AAA rupture are also in women [2]. Due to high mortality rate when AAA ruptures (about 70–90%), it poses a serious health and public social problem in a lot of countries [3]. Although our understanding of this issue and its mechanisms is getting better, its complete aetiology is still unclear and we are still missing the crucial trigger in majority of AAAs. There are actually no prognostic laboratory markers used in clinical practice predicting the AAA behaviour. The identification of molecules involved in the deregulation of gene expression in the process of pathogenesis of AAA could be one option. Published data showed dysregulated expression of microRNAs in AAA tissue, and presumed the microRNA can play pivotal role in AAA development [4–6].

The positive family AAA history increases the risk of developing the same condition in relatives and indicates substantial portion of the genetic component. The

heritability of AAA is over 0.7, and first-degree relatives of a patient with AAA have a 2-fold higher risk of developing an aneurysm as well [7]. Gene variants, found in genome-wide association studies (GWAS) associated with increased AAA risk, are usually not located in the coding regions and/or do not necessarily represent the causal ones. These gene variants may rather be in linkage disequilibrium with the causal alleles contributing to AAA formation, being located close to them [8]. Up to day, some risk loci on a few chromosomes were identified, but these explain only a small proportion of the heritability of AAA [7]. Positions of these genetic variants in noncoding regions suggest that they probably more often influence gene regulation than the protein structure [8]. The regulation of gene expression can be influenced in many ways including microRNAs (miRNAs).

miRNAs are short noncoding RNAs, first found and described at the very end of the last century, that are recently in focus of research due to their potential to become useful diagnostic and/or prognostic markers in a large variety of diseases. miRNAs are transcribed as long primary transcripts (pri-miRNAs) that are partly processed in the nucleus (pre-miRNAs) and finally in the cytoplasm. Mature miRNAs, about 20 nucleotides in length, associate with Argonaute family protein members and form RISCs (RNA-induced silencing complexes). RISCs interact with protein-coding mRNAs and inhibit their translation, or destabilize their molecules that are degraded and so decrease the levels of proteins coded by target mRNAs. On the other hand, in some cases miRNAs can also activate translation of target genes [9]. One miRNA can interact with many mRNAs, and, vice versa, one particular mRNA can be regulated by many different miRNAs. miRNAs can regulate gene expression either in the cell in which they were synthetized, or in other neighbouring or more distant cells, as they can be exported into circulation in the form of membrane-bound vesicles (exosomes and microvesicles), or in association with protein complexes [10].

A few thousands of miRNAs have been identified, over 2500 in the human genome. Some miRNAs, or significant changes in their levels, are associated with particular diseases, their stages, or acute events, and so became potential diagnostic or prognostic markers, for example, increased levels of miR-208a, miR-499, miR-1, and miR-133 in plasma are associated with myocardial damage [10]. The levels of miRNAs can be measured either in cells/tissue, or in circulation (serum, plasma, and blood), or other body fluids.

In this study, we aimed to evaluate the changes in 2533 miRNAs expression in small and large aneurysms compared to the normal vessel wall.

2. Materials and Methods

2.1. Patients. Our study included ten patients with small AAA (maximum diameter ≤ 5 cm), six patients with large AAA (>5 cm), and finally five "healthy controls" who were cadaveric organ donors. Only patients indicated for the AAA resection were included, either due to the size of AAA, or symptoms present. Patients with oncological diagnoses, autoimmune diseases, and mycotic or inflammatory aneurysms were excluded. Control aortic tissue samples were from the dead brain and heart-beating donors (without warm ischemia or any other tissue injury) and were collected at the same time as the kidneys were harvested and immediately frozen. All patients with AAA have signed an informed consent; cadaveric donors have been used based on the principle of presumed consent (approved by the local Ethical committee; decision from the 12th of August 2014).

2.2. Tissue Samples. Tissue samples were collected within the aortic surgery. AAA samples during the open aneurysm resection, approximately 1 cm^3 tissue sample from the anterior aneurysmal wall; control group samples from cadaveric donors, from the same region of aorta, were collected. Samples were immediately washed with physiological saline solution, cleaned from intraluminal thrombus, frozen with liquid nitrogen, and stored in the freezer (−80 degrees of centigrade).

2.3. miRNA Isolation. Sample aliquots (approximately 0.125 cm^3) were frozen in liquid nitrogen and grinded to prepare homogeneous powder that was transferred into 1 ml of chilled TRI Reagent®RT (Molecular Research Center, Inc., Cincinnati, USA). Total RNA was isolated according to manufacturer's protocol (Manual Part Number: G4170-90011, Version 3.1.1, August 2015). Isolated RNA was dissolved in RNAse/DNAse-free water (Thermo Fisher Scientific Inc., Waltham, USA), and its concentration and purity were assessed spectrophotometrically using the Infinite M200 instrument (Tecan Trading AG, Männedorf, Switzerland). The RNA sample was stored at −80°C until further use.

2.4. miRNA Array. RNA samples were prepared for hybridization using miRNA Complete Labelling and Hybridization Kit (Agilent, Santa Clara, USA), following manufacturer's protocol, without optional spike-in control and purification of labelled RNA. Samples were randomized and hybridized for 20 hours on SurePrint G3 Unrestricted miRNA arrays with 8 × 60 K fields and Amadid 070156 (Agilent, Santa Clara, USA).

2.5. Data Analysis. After hybridization, microarrays were scanned using the Agilent G 4900DA scanner, and the resulting image was processed using Agilent Feature Extractor software version 11.5.1.1. Resulting data were processed using R version 3.5.1 with limma package version 3.36.3. The weights for imported probes showing saturation, nonuniformity, or significantly higher signal over background were set to zero. The background was then corrected using the normexp method with offset set to 50. The "cyclicloess" with "affy" method was used for normalization to allow comparison of samples between arrays. Probes annotated with systematic name of detected miRNA were considered as within array replicates and combined using "avereps function" from limma package version 3.36.3 to obtain the expression of particular miRNA. Fold changes

between aneurysms and normal tissue were then calculated as well as corresponding p values. Benjamini & Hochberg method was used for adjustment of p values to multiple comparisons.

3. Results

3.1. Baseline Characteristics of Patients. Samples of 21 patients were analysed. Six were obtained from large aneurysms, ten from small aneurysms, and five from healthy controls. The baseline characteristics of patients are given in Table 1.

3.2. miRNAs Dysregulated in Small AAA. miRNAs expression in small and large aneurysm tissue was compared with healthy tissue. In small aneurysms, six miRNAs were detected with significantly different expression in comparison with the healthy aortic wall, including miR-7158-5p, miR-658, miR-517-5p, miR-122-5p, miR-326, and miR-3180 (Figure 1(a)). All of them were also significantly changed in large aneurysms. All but miR-326 were downregulated in the AAA tissue. In both large and small aneurysms the difference between the aneurysmal and healthy aortic wall was less than two-fold, and both had the same direction. Interestingly, fold changes in small and large aneurysms against healthy tissue were significantly correlated (Pearson's coefficient = 0.9521; p value = 0.0034).

3.3. miRNAs Dysregulated in Large AAA. In the large aneurysms, 162 differently expressed miRNAs were found (Figure 1(b); Table 2). Six of them were found to be also differently expressed in small aneurysms as mentioned in Section 3.2. Ten miRNAs with more than two-fold significant change in expression in large aneurysms were identified. These miRNAs included miR-23a-3p, miR-24-3p, miR-27a-3p, miR-27b-3p, miR-30d-5p, miR-193a-3p, miR-203a-3p, miR-365a-3p, miR-4291, and miR-3663-3p and all, but the last one was downregulated in aneurysmal walls.

4. Discussion

Based on miRNAs expression profiles, we have proved our assumption that some miRNAs are differently expressed in small and/or large AAA as compared to healthy tissue. In this analysis, we found six miRNAs dysregulated in small aneurysms in comparison with healthy controls (miR-7158-5p, miR-658, miR-517-5p, miR-122-5p, miR-3180 downregulated, and miR-326 upregulated). Twenty-seven times more miRNAs were found to be differentially expressed in large aneurysms, suggesting increasing miRNA levels dysregulation related to the disease progression. Despite higher number of small aneurysms than the large ones (10 vs. 6), we were unable to detect any other significantly changed miRNAs in comparison with healthy tissue, when correction for multiple comparison was applied. This suggests that small aneurysms are relatively close to the normal aortic wall regarding miRNAs expression.

As the number of differently expressed miRNAs is relatively high, we focus on miRNAs with more than two-fold significant change in expression (miR-23a-3p, miR-24-3p, miR-27a-3p, miR-27b-3p, miR-30d-5p, miR-193a-3p, miR-203a-3p, miR-365a-3p, miR-4291, and miR-3663-3p). Majority of data about these miRNAs come from studies focused on various types of cancer, where their roles in cell division, proliferation, migration, invasion, and apoptosis were identified. Some of them were analysed in cardiovascular diseases and miR-23/27/24 family and miR-193a also in AAAs.

4.1. MicroRNA-23/27/24 Family. miRNAs miR-27a, miR-23a, and miR-24-2 form an intergenic cluster on chromosome 19 (19p13.13), and miRNAs miR-23b, miR-27b, and miR-24-1 form an intronic cluster on chromosome 9 (9q22.32) [11]. Except for miR-23b, all were found to be downregulated in our samples of aneurysmal tissue. If no correction for multiple comparisons was applied, miR-23b-3p would be found significantly ($p = 0.004$), more than two-fold, downregulated as well. All cluster members are highly conserved among vertebrates, and mature sequences of miR-24-1 and 24-2 are identical [11]. The miR-23/27/24 cluster members play a role in cell cycle control, proliferation, differentiation, and apoptosis. All of them are involved in regulation of angiogenesis; proangiogenic miR-23 regulates cardiomyocyte cell growth, promotes VSMCs proliferation, and inhibits VSMCs apoptosis by targeting the BCL2L11 (BIM) gene [12]; proangiogenic miR-27 is enriched in endothelial cells and highly vascularized tissues, inhibits endothelial cell growth targeting Rb, SEMA6A, and SEMA6D genes, negatively regulates MAPK and VEGFR2 signalling pathways [11, 13], and might contribute to plaque formation in atherosclerosis influencing MMP-13 (matrix metalloproteinase 13 or collagenase-3) expression in endothelial cells [13]; antiangiogenic miR-24 plays a pivotal role in endothelial cells apoptosis and angiogenesis, mediates contractile phenotype in vascular smooth muscle cells, and is upregulated in cardiac endothelial cells after cardiac ischemia and hypoxic condition [11]. To the validated miR-24 targets belong the endothelium-enriched transcription factor GATA2, the p21-activated kinase PAK4, the RAS p21 protein activator RASA1, and the histone-coding gene H2AFX that are involved in endothelial cells biology [11].

This miR-24 also affects smooth muscle cells proliferation, function, and apoptosis and inhibits proliferation, migration, and sprouting of human umbilical venous endothelial cells (HUVECs) [14]. Derived from the above-mentioned facts, decreased levels of miR-23 attenuate its proangiogenic effect and downregulation of miR-27 and miR-24 results in the cell growth, angiogenesis, and loss of contractile phenotype. Maegdefessel et al. studied miR-24 in aneurysmal tissue and identified it as a key regulator of vascular inflammation and AAA pathology [15]. miR-24 was found to be colocalized with activated macrophages in aneurysmal aortic mouse tissue and visualized its expression in the aneurysm intimal-medial region. miR-24 downregulation was proinflammatory in macrophages [10]. The authors found miR-24 downregulated in both animal model and human AAA tissue (-1.9 ± 0.09-fold) versus control,

Table 1: Baseline characteristics of patients (number of patients and percentage in subgroups).

	Small aneurysms	Large aneurysms	Control
Number of patients	10	6	5
Age (years); mean/range	63 (54–75)	75 (63–83)	52 (37–63)
Man	9 (90)	4 (67)	4 (80)
Smoking	9 (90)	5 (83)	3 (60)
Arterial hypertension	7 (70)	5 (83)	2 (50)
Coronary artery disease	4 (40)	5 (83)	0 (0)
Peripheral arterial disease	5 (50)	1 (17)	0 (0)
Diabetes mellitus	2 (20)	1 (17)	0 (0)
Hyperlipidaemia	6 (60)	3 (50)	0 (0)
Chronic obstructive pulmonary disease	4 (40)	1 (17)	0 (0)

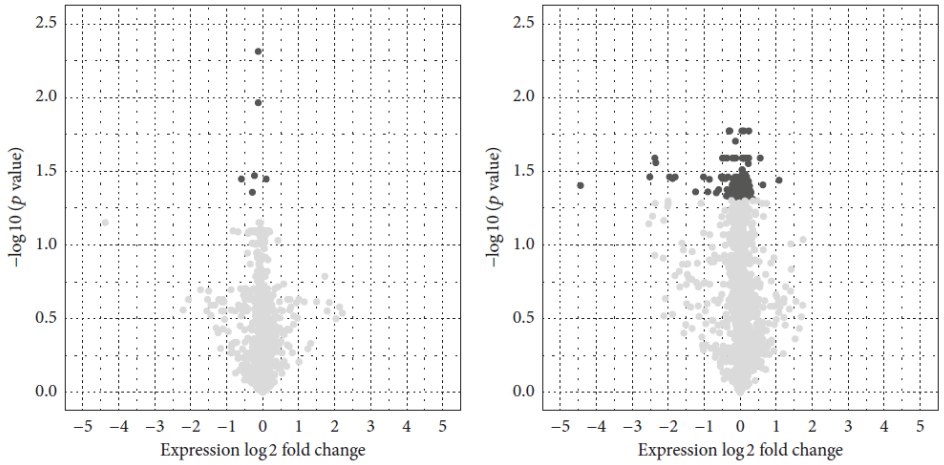

Figure 1: Comparison of miRNA expression in small (a) and large (b) aneurysms with healthy controls. Grey miRNAs (• dots) = nonsignificant; black miRNAs (• dots) = significant.

and they found no significant differences between the small (52–67 mm; $n = 12$) and large AAA (69–115 mm, $n = 10$) but suggested a trend towards lower miR-24 expression with larger AAA. This downregulation is in good agreement with our findings in large aneurysms, as our subgroup of "large" AAA (diameter over 50 mm) involves both large and small Maegdefessel's groups. In addition, miR-27b was found to be downregulated in AAA in their study [15], which is in line with our results. Furthermore, levels of both miR-24 and miR-23b were significantly lower also in the intracranial aneurysmal tissue than in the healthy controls [16]. Our findings support the hypothesis that the downregulation of miRNA-23/27/24 family dysregulates angiogenesis, smooths muscle cells proliferation, function, and apoptosis, and together with their proinflammatory effect can contribute to the AAA development/progression.

4.2. miR-30d Family. The miRNA-30 family includes five miRNAs (miR-30a–e), and six mature miRNA molecules (miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, and miR-30e) coded by six genes located on chromosome 1, 6, and 8, sharing a common seed sequence close to their 5′ ends [17]. The members of miRNA-30 family are involved in the process of angiogenesis via δ-like ligand 4 (DLL4) expression regulation [17].

We found miR-30b-3p and miR-30d-5p downregulated in large AAA tissue. If no correction for multiple comparisons was applied, miR-30a-3p, miR-30a-5p, miR-30b-5p, miR-30c-2-3p, and miR-30d-3p would be significantly downregulated as well ($p = 0.004$). It is in line with published results of Spear et al., who found miR-30a-5p downregulated in the aneurysmal tissue of AAA patients in comparison with control tissues, and in addition, levels of circulating miR-30a-5p in plasma were also lower in AAA patients compared to patients PAD (periphery artery disease) and nonaneurysmal atherosclerosis (0.8-fold; $p = 0.04$) [18].

In the cardiovascular system, the downregulation of miR-30 family contributes to endoplasmic reticulum (ER) stress and is associated with the upregulation of glucose-regulated protein 78 (GRP78) [19]. ER stress plays fundamental roles in the development and progression of various cardiovascular diseases such as ischemic heart diseases, heart failure, atherosclerosis, hypertension, and stroke [19]. Artificial

Table 2: List of 162 differently expressed miRNAs in large aneurysms, in comparison with healthy controls ($p < 0.05$).

hsa-let-7f-1-3p	hsa-miR-634	hsa-miR-4296	hsa-miR-6760-3p
hsa-let-7b-3p	hsa-miR-648	hsa-miR-4307	hsa-miR-6765-3p
hsa-miR-23a-3p	**hsa-miR-658**	hsa-miR-4310	hsa-miR-6769b-3p
hsa-miR-23c	hsa-miR-671-3p	hsa-miR-4317	hsa-miR-6775-3p
hsa-miR-24-3p	hsa-miR-767-3p	hsa-miR-4328	hsa-miR-6776-3p
hsa-miR-27b-3p	hsa-miR-939-3p	hsa-miR-4433a-5p	hsa-miR-6782-3p
hsa-miR-27a-3p	hsa-miR-944	hsa-miR-4436b-5p	hsa-miR-6785-3p
hsa-miR-30b-3p	hsa-miR-1227-3p	hsa-miR-4482-5p	hsa-miR-6792-3p
hsa-miR-30d-5p	hsa-miR-1238-5p	hsa-miR-4486	hsa-miR-6794-3p
hsa-miR-122-5p	hsa-miR-1238-3p	hsa-miR-4487	hsa-miR-6795-3p
hsa-miR-129-1-3p	hsa-miR-1266-5p	hsa-miR-4519	hsa-miR-6799-3p
hsa-miR-138-5p	hsa-miR-1288-5p	hsa-miR-4664-3p	hsa-miR-6800-3p
hsa-miR-181c-3p	hsa-miR-1291	hsa-miR-4666b	hsa-miR-6820-3p
hsa-miR-193a-3p	hsa-miR-1304-3p	hsa-miR-4680-5p	hsa-miR-6827-3p
hsa-miR-203a-3p	hsa-miR-1470	hsa-miR-4697-3p	hsa-miR-6830-5p
hsa-miR-206	hsa-miR-1539	hsa-miR-4714-3p	hsa-miR-6841-3p
hsa-miR-212-5p	hsa-miR-2116-3p	hsa-miR-4723-3p	hsa-miR-6844
hsa-miR-326	hsa-miR-2467-3p	hsa-miR-4732-5p	hsa-miR-6851-3p
hsa-miR-329-3p	hsa-miR-3064-5p	hsa-miR-4742-3p	hsa-miR-6855-3p
hsa-miR-365a-5p	hsa-miR-3156-3p	hsa-miR-4742-5p	hsa-miR-6865-5p
hsa-miR-365a-3p	hsa-miR-3161	hsa-miR-4750-3p	hsa-miR-6865-3p
hsa-miR-365b-5p	hsa-miR-3171	hsa-miR-4753-5p	hsa-miR-6877-3p
hsa-miR-376b-5p	hsa-miR-3173-3p	hsa-miR-4761-5p	hsa-miR-6880-3p
hsa-miR-378f	**hsa-miR-3180**	hsa-miR-4770	hsa-miR-6885-3p
hsa-miR-378b	hsa-miR-3184-3p	hsa-miR-5571-5p	hsa-miR-6889-3p
hsa-miR-425-3p	hsa-miR-3190-5p	hsa-miR-5681b	hsa-miR-6890-5p
hsa-miR-432-3p	hsa-miR-3198	hsa-miR-5691	hsa-miR-6892-3p
hsa-miR-512-3p	hsa-miR-3613-3p	hsa-miR-6069	hsa-miR-6893-3p
hsa-miR-514b-5p	hsa-miR-3614-5p	hsa-miR-6503-5p	hsa-miR-7107-3p
hsa-miR-517-5p	hsa-miR-3622a-3p	hsa-miR-6507-3p	hsa-miR-7108-3p
hsa-miR-520h	hsa-miR-3654	hsa-miR-6512-5p	hsa-miR-7111-5p
hsa-miR-548x-3p	hsa-miR-3660	hsa-miR-6513-3p	hsa-miR-7154-3p
hsa-miR-548c-3p	**hsa-miR-3663-3p**	hsa-miR-6731-5p	hsa-miR-7157-3p
hsa-miR-548ai	hsa-miR-3675-3p	hsa-miR-6732-3p	**hsa-miR-7158-5p**
hsa-miR-548e-5p	hsa-miR-3920	hsa-miR-6737-3p	hsa-miR-7162-5p
hsa-miR-550a-5p	hsa-miR-3926	hsa-miR-6738-5p	hsa-miR-7855-5p
hsa-miR-556-5p	hsa-miR-3940-3p	hsa-miR-6747-5p	hsa-miR-7856-5p
hsa-miR-563	hsa-miR-4259	hsa-miR-6752-3p	hsa-miR-7974
hsa-miR-602	hsa-miR-4265	hsa-miR-6753-5p	hsa-miR-8071
hsa-miR-613	**hsa-miR-4291**	hsa-miR-6757-3p	
hsa-miR-631	hsa-miR-4294	hsa-miR-6759-3p	

miRNAs in grey cells were downregulated and in white cells upregulated in the aneurysmal tissue; miRNAs bold and underlined were dysregulated in both, large and small aneurysms; miRNAs in bold italics are those with more than two-fold change in large aneurysms.

knockdown of miR-30 triggered the phenotypic ER stress with significant GRP78/ATF6/CHOP/caspase-12 upregulations and cell death in rat cells [19]. In patients with acute coronary syndrome were found higher levels of circulating miR-30d-5p, in comparison with healthy controls ($p < 0.001$). The authors found this miRNA suitable as a diagnostic predictor of myocardial infarction [20]. In this context, the downregulation of miR-30 family members in our AAA samples could indicate an increased ER stress, dysregulation of endothelial cell growth, and increased cell death.

4.3. miR-193a and miR-365a. We found miR-193a-3p downregulated in AAA tissue samples. Without correction for multiple comparisons, all four analysed miRNAs-193 (miR-193a-3p, miR-193a-5p, miR-193b-3p, and miR-193b-5p) would be significantly downregulated. Gene coding miR-193a is on chromosome 17, while miR-193b is in cluster with miR-365a located on chromosome 16. Downregulation of miR-193a expression has been published by a few authors in both plasma samples [6, 21] and aneurysmal tissue of AAA/TAA patients [22, 23], and its levels were lower in plasma samples of patients with aneurysms before surgery than 5–7 days after surgery [21].

miR-193a-3p downregulates human endothelial cell proliferation and migration, and negatively regulates human circulating endothelial colony-forming cell (ECFC) vasculo/angiogenesis that contribute to vascular repair [19, 24].

All miR-365 analysed (miR-365a-3p, miR-365a-5p, and miR-365b-5p) were significantly downregulated in the aneurysmal tissue, miR-365a-3p more than two-fold.

miR-365a plays an important role in atherosclerosis, as it is proatherosclerotic and proapoptotic, and induces

Table 3: miRNAs with more than two-fold significant change in expression in large aneurysm tissue, vascular biology processes in which they are involved, and their target genes.

miRNA	Process/function	Target genes	Reference no
miR-23	Angiogenesis (proangiogenic)	E2F1	[11]
	VSMCs proliferation		[12]
	Apoptosis (antiapoptotic)	BCL2L11 (BIM)	[12]
	Angiogenesis (antiangiogenic)	GATA2	[11]
miR-24	Apoptosis	GATA2, PAK4, BIM	[11]
	Mediator of contractile phenotype in VSMCs	Trb3	[11]
	Inhibits proliferation, migration and sprouting		[14]
	Vascular inflammation and AAA pathology		[10, 15]
miR-27	Angiogenesis (proangiogenic)	SEMA6A, SEMA6D, SPROUTY2, TSP-1, MMP-13	[11, 13]
	Endothelial cell growth inhibition	Rb	[11]
	Inflammation	MMP-13, VEGF, TSG-1	[13]
	Apoptosis	FADD	[13]
	Atherosclerosis	G2A	[13]
miR-30	Angiogenesis	DLL4	[17]
	Endoplasmic reticulum (ER) stress; cell death	GRP78, ATF6, CHOP, CASP12	[19]
miR-193	Angiogenesis (antiangiogenic)	HMGB1	[24]
miR-203	Inflammation	SOCS-3	[28, 29]
miR-365	Apoptosis	BCL2	[25]
	Atherosclerosis	BCL2	[25]
miR-3663	Downregulated in degenerative aortic stenosis	NA	[30]
miR-4291	NA	NA	—

ATF6: activating transcription factor 6; BCL2L11 (BIM): Bcl-2; CASP12: caspase-12; DLL4: δ-like ligand 4; E2F1: E2F1 transcription factor; FADD: Fas-associated protein with death domain; G2A: G protein-coupled receptor 132; GRP78: glucose-regulated protein 78; HMGB1: High mobility group box-1; CHOP: CCAAT-enhancer-binding protein homologous protein; MMP-13: matrix metalloproteinase 13; SEMA6A: semaphorin 6a; SEMA6D: semaphorin 6d; SPROUTY2: sprouty homolog 2; Trb3: tribbles-like protein-3; TSG-1: thrombospondin-1; TSP-1thrombospondin-1; VEGF: vascular endothelial growth factor; VSMCs: vascular smooth muscle cells.

endothelial cell apoptosis [25]. miR-365 is upregulated in endothelial cells upon oxLDL (oxidized low-density lipoprotein) treatment, targets antiapoptotic protein Bcl-2, and promotes cell death [25]. Both miR-193a and miR-365a are involved in endothelial cell metabolism, and their dysregulation might therefore contribute to the aneurysmal pathology as well. Based on their published effects, their downregulation could increase proliferation, migration, and survival of endothelial cells.

4.4. miR-203a. Levels of miR-203a were lower in aneurysmal tissue. This miRNA is dysregulated in chronic inflammatory diseases that suggest its involvement in immune-mediated diseases [26]. miR-203 is highly expressed in skin (and keratinocytes) and oesophagus and overexpressed in patients with psoriasis in comparison with healthy controls [27]. One of its targets is suppressor of cytokine signalling-3 (SOCS-3), negative regulator of the STAT3 pathway that is activated by inflammatory cytokines. SOCS-3 is involved in cell growth, differentiation, and survival and in the regulation of immunity [27]. SOCS-3, a target of miR-203a, was shown to play a critical role in AAA development and dissection [28, 29]. The long-term suppression of SOCS-3 by increased levels of miR-203 may lead to stronger or prolonged inflammation [26]. As the inflammation is one of the key players in aneurysmal pathology [18], dysregulation of miRNAs influencing this process could contribute to the aneurysm formation.

4.5. miR-3663 and miR-4291. The expression of miR-3663 was upregulated, and the expression of miR-4291 was downregulated in the analysed aneurysmal tissue. Little is known about miR-3663. miR-3663-3p was identified as downregulated in tissue samples of patients with degenerative aortic stenosis, and miR-193-3p as overexpressed in comparison with heathy subjects [30]. This information is just opposite to our findings in aortic dilated tissue of AAA patients. We have not found any published specific functions associated with hsa-miR-4291.

The majority of miRNAs, we found dysregulated in the tissue of AAA patients, are potentially related to the aneurysmal pathology, being either involved in endothelial cell or smooth muscle cell metabolism and survival, or angiogenesis, or inflammation, or atherosclerosis (Table 3). Some of them (miR-24, miR-27b, and miR-193a) were identified in previously published literature as downregulated in aneurysmal tissue, or in plasma of patients with aortic aneurysms [15, 22, 23], what is in line with our findings. In addition to these three miRNAs, we identified miR-23a, miR-27a, miR-30d, miR-365a, miR-203a, and miR-4291 to be downregulated and miR-3663 upregulated.

In our analysis, a complete panel of 2549 miRNAs was tested. We found 6 miRNAs dysregulated in small and 162 miRNAs in large aneurysms in comparison with healthy controls. The number of samples in particular groups was limited, and therefore microarray results were not verified using qPCR. To compensate this main limitation of this study, the Benjamini and Hochberg method

was used for adjustment of p values to multiple comparisons.

5. Conclusion

In our analysis, we tested the complete panel of 2549 miRNAs and we confirmed some previously identified miRNAs (miR-23/27/24 family, miR-193a, and miR-30) as associated with AAA pathogenesis. In addition, we identified other potential miRNA candidates not yet found dysregulated in AAA tissue (miR-203a, miR-3663, miR-365a, and miR-4291) for further analyses, to investigate more closely their possible role in pathogenesis of aneurysms. If their role in AAA development is proved significant in future, they can become potential markers or treatment targets.

Abbreviations

AA: Aortic aneurysm
AAA: Abdominal aortic aneurysm
ATF6: Activating transcription factor 6
CHOP: CCAAT-enhancer-binding protein homologous protein
GRP78: Glucose-regulated protein 78
HUVEC: Human umbilical venous endothelial cells
IA: Intracranial aneurysm
RISC: RNA-induced silencing complex
TAA: Thoracic aortic aneurysm.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Příloha I

2. Hromádka M, Černá V, Pešta M, Kučerová A, Jarkovský J, Rajdl D, Rokyta R, Moťovská Z. *Prognostic Value of MicroRNAs in Patients after Myocardial Infarction: A Substudy of PRAGUE-18*. Dis Markers. 2019 Nov 3;2019:2925019. doi: 10.1155/2019/2925019. PMID: 31781298; PMCID: PMC6875251.

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Research Article

Prognostic Value of MicroRNAs in Patients after Myocardial Infarction: A Substudy of PRAGUE-18

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Background. The evaluation of the long-term risk of major adverse cardiovascular events and cardiac death in patients after acute myocardial infarction (AMI) is an established clinical process. Laboratory markers may significantly help with the risk stratification of these patients. Our objective was to find the relation of selected microRNAs to the standard markers of AMI and determine if these microRNAs can be used to identify patients at increased risk. *Methods*. Selected microRNAs (miR-1, miR-133a, and miR-499) were measured in a cohort of 122 patients from the PRAGUE-18 study (ticagrelor vs. prasugrel in AMI treated with primary percutaneous coronary intervention (pPCI)). The cohort was split into two subgroups: 116 patients who did not die (survivors) and 6 patients who died (nonsurvivors) during the 365-day period after AMI. Plasma levels of selected circulating miRNAs were then assessed in combination with high-sensitivity cardiac troponin T (hsTnT) and N-terminal probrain natriuretic peptide (NT-proBNP). *Results*. miR-1, miR-133a, and miR-499 correlated positively with NT-proBNP and hsTnT 24 hours after admission and negatively with left ventricular ejection fraction (LVEF). Both miR-1 and miR-133a positively correlated with hsTnT at admission. Median relative levels of all selected miRNAs were higher in the subgroup of nonsurvivors ($N = 6$) in comparison with survivors ($N = 116$), but the difference did not reach statistical significance. All patients in the nonsurvivor subgroup had miR-499 and NT-proBNP levels above the cut-off values (891.5 ng/L for NT-proBNP and 0.088 for miR-499), whereas in the survivor subgroup, only 28.4% of patients were above the cut-off values ($p = 0.001$). *Conclusions*. Statistically significant correlation was found between miR-1, miR-133a, and miR-499 and hsTnT, NT-proBNP, and LVEF. In addition, this analysis suggests that plasma levels of circulating miR-499 could contribute to the identification of patients at increased risk of death during the first year after AMI, especially when combined with NT-proBNP levels.

1. Introduction

The in-hospital mortality rate for acute myocardial infarction is low, due to efficient antiplatelet treatment and primary percutaneous coronary intervention (pPCI); unfortunately, the risk of cardiac death increases during the chronic phase of ischemic heart disease that follows.

Decreased left ventricular systolic function with left ventricular ejection fraction (LVEF) ≤ 35% and recurrent ventricular tachycardia or ventricular fibrillation, beyond the early phase of myocardial infarction, are connected with a poor prognosis and are a potential indication for cardioverter implantation [1].

Despite the clear benefit of these widely used predictors, they seem to be inadequate for identifying all patients at risk of sudden death, since it fails to identify about 50% of patients who die suddenly [2] after acute myocardial infarction (AMI). Some of the standard laboratory markers associated

with the risk of sudden death can be used in combination with LVEF to improve the risk assessment process, but unfortunately, well-defined cut-off values are still not known.

Among factors that can be used for risk stratification after AMI, the following play an important role: elevated levels of troponin T or I (TnT or TnI) [3, 4] and a combination of (A) increased TnT and CRP plasma levels, (B) increased levels of N-terminal prohormone of brain natriuretic peptide (NT-proBNP) with LVEF < 40% [4–7], and (C) decreased clearance of creatinine (with a reduced LVEF) [8].

A promising group of new biomarkers, released from cells into circulation, is microRNAs (miRNAs), which are small noncoding RNA molecules, 20–22 nucleotides in length, involved in posttranscriptional regulation of gene expression. Mature miRNAs and Ago proteins (Argonaute proteins) form in the cytoplasm RISC complexes (RNA-induced silencing complexes) that interact with protein-coding mRNA molecules. This interaction usually leads to the inhibition of translation or directly to the degradation of mRNA molecules. One particular microRNA can regulate many genes (i.e., interacting with a variety of different protein-coding mRNAs), and one particular gene can be regulated by several different microRNAs. MicroRNAs can act directly within the cells where they are synthesized, or they can be exported, in complexes with proteins or in membrane-bound vesicles (exosomes or microvesicles), to other cells where they can also regulate gene expression. MicroRNAs are involved in the control of many processes in both healthy and infarcted myocardia, including proliferation, differentiation, apoptosis, repair, and revascularization [9]. Additionally, miRNA dysregulation has been strongly implicated in the destabilization and rupture of atherosclerotic plaques [10] as well as being involved in the process of myocardial recovery.

In cardiovascular diseases (CVD), the use of miRNAs as biomarkers for specific disease entities has been successfully investigated in numerous studies [11]. Nonetheless, it is not yet possible to use them in clinical practice [12]. miRNAs also have the potential for clinical use in CVD where protein biomarkers are not available.

More than 2500 mature miRNAs have been identified in humans. Four of them, miR-1, miR-133, miR-208a, and miR-499 have been found to be specific for the myocardium (or the myocardium and skeletal muscle) and are sometimes called "myomiRs" [13].

Many authors have shown that levels of circulating myomiRs increase significantly during the first few hours after the onset of myocardial infarction symptoms. After reaching a peak, myomiRs return to normal after a few hours or a few days [14, 15].

We decided to retrospectively measure the relative levels of circulating miR-1, miR-133a, miR-208a, and miR-499 in a well-described cohort of 122 patients with known one-year mortality, previously involved in the PRAGUE-18 study [16, 17]. The listed miRNAs were assessed alone and in combination with several standard markers in an effort to better characterize the nonsurvivor subgroup, with the goal of finding additional predictors of patients at increased risk of one-year cardiovascular death.

2. Material and Methods

2.1. Patients. The whole cohort of 122 patients was treated in the Department of Cardiology, University Hospital and Faculty of Medicine of Charles University, Pilsen, Czech Republic, which was one of the centers involved in phase IV of a multicenter, open-label, randomized, controlled clinical trial called the PRAGUE-18 study [16, 17].

The PRAGUE-18 study, which compared prasugrel and ticagrelor in the treatment of acute myocardial infarction, was the first randomized head-to-head comparison of these two active substances, with regard to efficacy and safety in patients after AMI undergoing pPCI. One of the outcomes was the combined endpoint of cardiovascular death, MI, or stroke within the first year. Prasugrel and ticagrelor had been similarly effective during the first year after AMI [16, 17]. Plasma samples from 122 patients in the study were used for this retrospective data analysis, where (I) levels of selected circulating microRNAs, (II) standard AMI biomarkers, and (III) LVEF were used to (A) look for correlations between miRNAs and standard AMI markers, (B) identify differences in biomarkers between survivors and nonsurvivors during the first year after AMI, and (C) better characterize the nonsurvivor subgroup relative to measures I, II, and III mentioned above.

2.2. Echocardiography. Two-dimensional, M-mode, and Doppler echocardiograms were acquired using an ultrasound system (Vivid 7, GE Medical Systems, Horton, Norway) using a 3.4 MHz multifrequency transducer. The systolic function of the left ventricle was determined according to the Simpson method from the apical 4-chamber view and the apical 2-chamber view (the biplane Simpson method).

2.3. Levels of Biomarkers. Data for the basic characteristics of all patients involved in the analysis were available from the PRAGUE-18 study. Levels of standard AMI biomarkers were known, including hsTnT, NT-proBNP, cystatin C, myoglobin, growth/differentiation factor 15 (GDF-15), and creatine kinase (CK) at patient admission and hsTnT also after 24 hours.

NT-proBNP was determined using the original analytical kits from Roche on a cobas® 8000 analyzer. NT-proBNP and high-sensitivity cardiac troponin were determined using the original analytical kits from Roche with the electrochemiluminiscence (ECLIA) principle on a cobas e602 analyzer. Imprecission of the hsTnT method on the 99th percentile was below 10% which is the required analytical performance specification. Growth/differentiation factor 15 (GDF-15) (RayBiotech, Norcross, USA) was determined using ELISA kits on a NEXgen Four ELISA reader (Adaltis, Rome, Italy).

Since hsTnT is the most frequently used standard biomarker of AMI and NT-proBNP is a sensitive marker of left ventricular dysfunction, we used them in combination with the potential new microRNA biomarkers, in subsequent analyses.

2.4. MicroRNA Analysis

2.4.1. RNA Isolation. MicroRNA was isolated from plasma samples taken 24 hours after admission (all patients were

already after pPCI at that time) and stored at −80°C. Total cell-free RNA was isolated from 200 μL of plasma using miRNeasy Serum/Plasma Kits (miRNeasy Serum/Plasma Kit (50), Cat no./ID 217184; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted in 14 μL of ribonuclease-free water and stored at −80°C until further analyses. MicroRNA-39 (*C. elegans* miR-39) was used as the RNA spike-in control. A fixed volume of 1 μL of this RNA eluate was used for each reverse transcription reaction.

2.4.2. Quantitative Estimation of MicroRNA Expression. For reverse transcriptions and quantitative estimations of selected microRNAs using real-time PCR reactions, TaqMan® MicroRNA Assays and master mixes were used (catalogue number 4440887: hsa-miR-133a-3p—Assay ID 002246, hsa-miR-1-3p—Assay ID 002222, hsa-miR-499a-5p—Assay ID 001352, hsa-miR-208-3p—Assay ID 000511, and cel-miR-39-3p—Assay ID 000200; TaqMan Universal MMIX II: catalogue number 4440049; and TaqMan® MicroRNA RT Kit: catalogue number 4366597). A T100TM thermal cycler (Bio-Rad, California, United States) was used for reverse transcription. The reaction volume was 15 μL. A fixed volume of 2.5 μL from this RT reaction was used into each real-time PCR reaction. Due to either too high or absent Ct values, levels of miR-208a could not be quantified and evaluated.

2.4.3. Processing of Real-Time PCR Data. Samples were assessed in technical duplicate. The Ct values were corrected using calibrators to eliminate differences between individual runs of the Stratagene Mx3000P Real-Time PCR apparatus (Agilent Technologies, CA, United States). In cases where a disagreement between results obtained from both technical duplicates was found, the sample assessment was repeated. Plasma levels for each miRNA were calculated in the form of a relative expression. This relative expression was calculated using the ΔCt method (i.e., the $2^{-\Delta Ct}$ algorithm was $\Delta Ct = Ct_{miR-x} - Ct_{miR-39}$).

2.5. Objectives. Our objectives were to find relationships between selected miRNAs and the standard biomarkers of AMI as well as to find a panel of standard and potential biomarkers that might contribute to the identification of high-risk patients after acute myocardial infarction and post-pPCI treatment. The whole cohort was split according to the primary outcome (death within 365 days after AMI) into two subgroups (survivors and nonsurvivors), and both subgroups were characterized according to their biomarker levels.

2.6. Statistical Analysis. In this analysis, standard descriptive statistics were applied; absolute and relative frequencies were used for categorical variables and medians (supplemented with the 5th and 95th percentiles) were used for continuous variables (mean, SD, and CV were also used for the description of miRs). The statistical significance of differences among groups of patients was tested using Fisher's exact test for categorical variables and the Mann-Whitney test for continuous variables. The Spearman correlation coefficient was used for the analysis of the statistical relationship between miRNAs and the standard markers. Cut-off points (cut-off values) of predictors of all-cause death during 365 days were established by ROC analysis. The point that guarantees the greatest sum of sensitivity and specificity was chosen as the best point. Risk factors for all-cause death during 365 days were analyzed by a Cox regression model of proportional hazards. Analysis was performed in IBM SPSS Statistics 24.0 with 5% level of significance.

3. Results

3.1. Baseline Characteristics. The analysis involved 122 adult patients (78.7% men and 21.3% women) with AMI followed by pPCI; the median age was 61.1 years. All patients used either prasugrel (53.3%) or ticagrelor (46.7%) for antiplatelet therapy. The cohort of patients was split into two subgroups: nonsurvivors ($N = 6$) and survivors ($N = 116$). Only six patients died within one year after AMI (three patients from the prasugrel and three from the ticagrelor group): five died suddenly and one died while in the hospital from an unconfirmed diagnosis of pulmonary embolism. All patients in this subgroup had an LVEF ≥ 40% at their control visit, which was 2–3 months after discharge from the hospital. The baseline characteristics of all patients, and both subgroups, including their comparison, are shown in Table 1.

3.2. Correlation of miRNAs with Standard Biomarkers. The relative levels of all three miRNAs were related to the levels of standard biomarkers: hsTnT (at admission), hsTnT (24 hours after admission), NT-proBNP, GDF-15, cystatin C, and LVEF.

miR-133a and miR-1 weakly positively correlated with hsTnT at admission and strongly positively correlated with hsTnT 24 hours after admission (Figure 1). miR-499 moderately correlated with hsTnT 24 hours after admission. A strong negative correlation was found between all three miRNAs and the LVEF (Figure 1). A strong positive correlation was identified between both miR-133a and miR-499 and NT-proBNP, and a moderate positive correlation was found between miR-1 and NT-proBNP (Figure 1).

No correlation was found between any of the miRNAs and GDP-15 or cystatin C.

3.3. The Relationship between miRNAs and One-Year Mortality. The assessment of the prognostic potential of the selected biomarkers, for the identification of patients at increased risk of death, was based on their peripheral plasma levels and one-year survival.

Median relative levels of miRNAs were higher in the nonsurvivor subgroup. But the total number of patients in this subgroup was small in comparison with that in the group of survivors (six vs. one hundred and sixteen), and the differences found did not reach statistical significance for any of the tested microRNAs (Figure 2).

The calculated cut-off values for miR-1, miR-133a, and miR-499 were 0.031, 0.330, and 0.088, respectively. Relative miRNA concentrations below these cut-off values were described as "low," and those above the value were described as "high."

Table 1: Baseline characteristics.

	All patients	Survivors	Nonsurvivors	p values
	Median (5th-95th percentile)			
Number of patients	122	116	6	
Age (years)	61.1 (40.4–76.8)	61.1 (40.1–76.7)	65.7 (56.1–81.0)	0.166
Men (number, %)	96 (78.7%)	91 (78.4%)	5 (83.3%)	0.999
BMI	27.6 (22.2–34.3)	27.6 (22.1–34.3)	26.7 (24.7–44.1)	0.929
Drug used: prasugrel (number, %)	65 (53.3%)	62 (53.4%)	3 (50.0%)	0.999
Drug used: ticagrelor (number, %)	57 (46.7%)	54 (46.6%)	3 (50.0%)	
STEMI (number, %)	121 (99.2%)	115 (99.1%)	6 (100.0%)	0.999
Left bundle branch block (LBBB) (number, %)	1 (0.8%)	1 (0.9%)	0 (0.0%)	0.999
Right bundle branch block (RBBB) (number, %)	1 (0.8%)	1 (0.9%)	0 (0.0%)	0.999
Hyperlipidaemia (number, %)	36 (29.5%)	35 (30.2%)	1 (16.7%)	0.669
Obesity (number, %)	23 (18.9%)	22 (19.0%)	1 (16.7%)	0.999
Arterial hypertension (number, %)	56 (45.9%)	52 (44.8%)	4 (66.7%)	0.412
Smoking (number, %)	84 (68.9%)	80 (69.0%)	4 (66.7%)	0.999
Diabetes mellitus (number, %)	17 (13.9%)	16 (13.8%)	1 (16.7%)	0.999
Time since the first symptoms to admission (hours)	3.0 (0.5–36.0)	3.0 (0.5–12.0)	6.0 (3.0–72.0)	0.061
Left ventricular ejection fraction (%)	50.0 (30.0–60.0)	55.0 (30.0–60.0)	45.0 (30.0–50.0)	0.054
Laboratory values (median (5th-95th percentile))				
hsTnT (at admission) (ng/L)	86.0 (12.0–1325.0)	84.0 (12.0–1325.0)	201.5 (27.0–4978.0)	0.257
hsTnT (24 hours after admission) (ng/L)	2432.0 (377.0–9651.0)	2324.0 (368.0–9651.0)	4306.5 (1526.0–15114.0)	0.201
Myoglobin (at admission) (μg/L)	198.0 (30.0–1385.0)	176.0 (30.0–1547.0)	652.0 (161.0–1317.0)	0.066
Creatine kinase (at admission) (μkat/L)	3.9 (1.4–23.7)	3.8 (1.3–23.7)	6.4 (2.6–26.8)	0.097
NT-proBNP (at admission) (ng/L)	757.0 (105.0–4142.0)	666.5 (104.0–4285.0)	1373.5 (904.0–3096.0)	0.074
Cystatin C (at admission) (mg/L)	121; 0.99 (0.80–1.47)	1.00 (0.79–1.49)	0.92 (0.85–1.09)	0.417
GDF-15 (at admission) (ng/L)	807.1 (372.8–1827.7)	796.3 (372.8–1827.7)	1044.9 (357.3–1848.8)	0.305

GDF-15 = growth/differentiation factor 15; hsTnT = high-sensitivity troponin T; NT-proBNP = N-terminal prohormone of brain natriuretic peptide; STEMI = acute myocardial infarction with ST-segment elevation.

Comparisons of the number of patients with low and high concentrations of particular miRNAs were made in both subgroups; in the nonsurvivor group, the relative frequency of high concentrations was higher, and in the case of miR-133a and miR-499, this difference reached statistical significance (Table 2, microRNAs). All 6 nonsurvivors had a high concentration of miR-499, whereas, in the survivor subgroup, only 46% of patients had a high concentration.

3.4. Relationship between Standard Biomarkers and One-Year Mortality. For the standard markers hsTnT and NT-proBNP, cut-off values were found in the same way as for miRNAs, and values were then described as either "low" or "high." The cut-off value for hsTnT was 154.5 ng/L, and for NT-proBNP, it was 891.5 ng/L.

The number of patients with low and high concentrations of these two biomarkers was compared in both subgroups, and in the nonsurvivor group, the frequency of high marker levels was higher; in the case of NT-proBNP, this difference was statistically significant (Table 3, standard biomarkers). All patients who died within one year had a high concentration of NT-proBNP, whereas in the survivor group, only 43% had a high concentration of NT-proBNP.

3.5. Combinations of Biomarkers. Using the estimated cut-off values, two or three biomarkers were combined, in an effort to better describe the nonsurvivor subgroup and identify patients at risk of death. Combinations included (A) combinations of different microRNAs, (B) combinations of standard markers, and (C) combinations of microRNAs and standard markers. All tested combinations are shown in Table 3.

Based on a combination of NT-proBNP and miR-499 levels, a test group of 39 "at-risk" patients was created, which was 32% of the entire (survivor+nonsurvivor) cohort. The NT-proBNP and miR-499 combination criteria put all six nonsurvivors in the "at-risk" group, where they represented 15% of the "at-risk" group.

4. Discussion

In patients with a proven increased risk of death based on cardiovascular risk stratification during hospitalization, treatment with ACE inhibitors (or angiotensin AT1 blockers), beta-blocker therapy, and aldosterone antagonists are indicated when EF LK is ≤40% and/or there is heart failure [1]. Implantation of cardioverter-defibrillator (ICD) in a

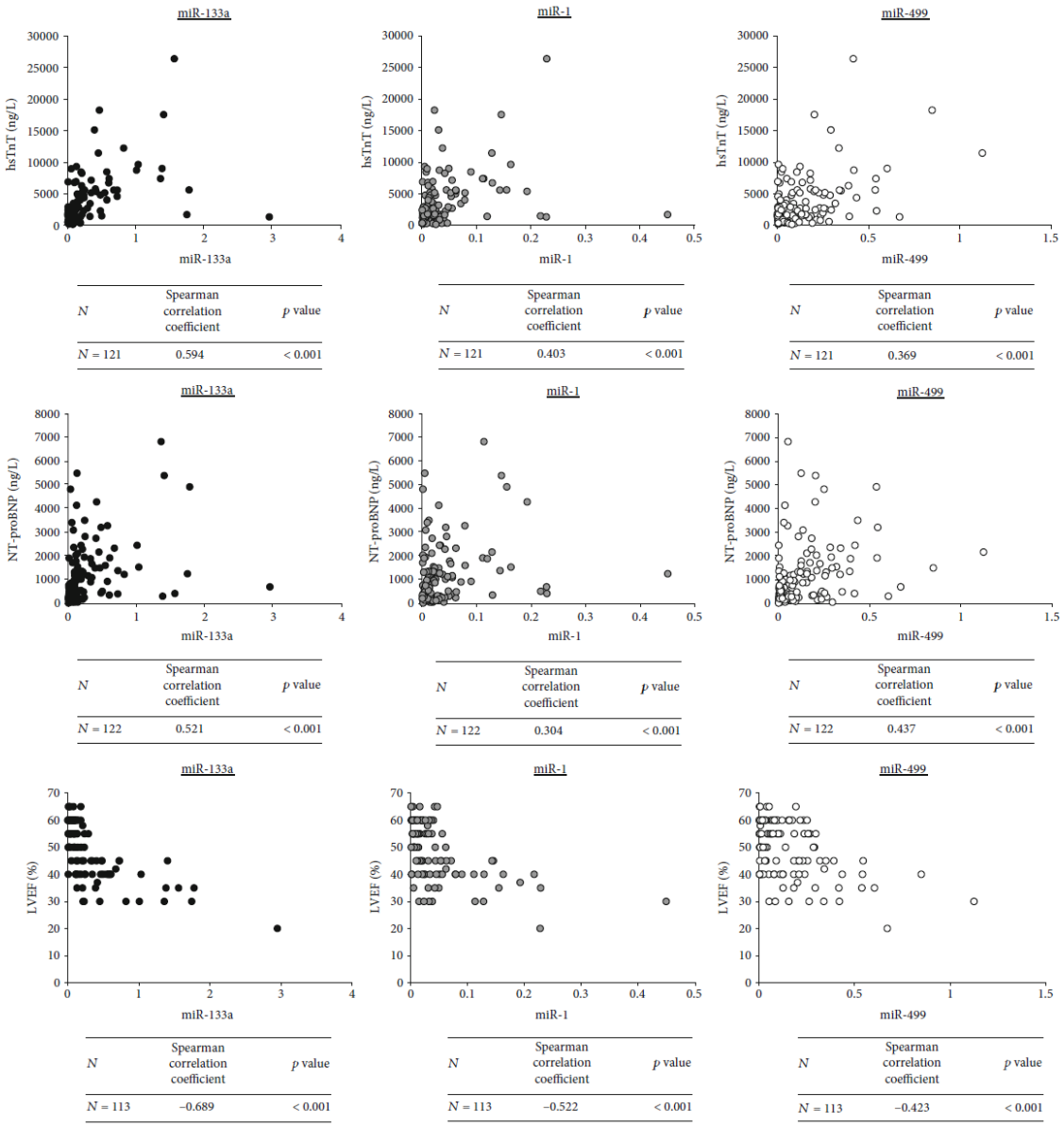
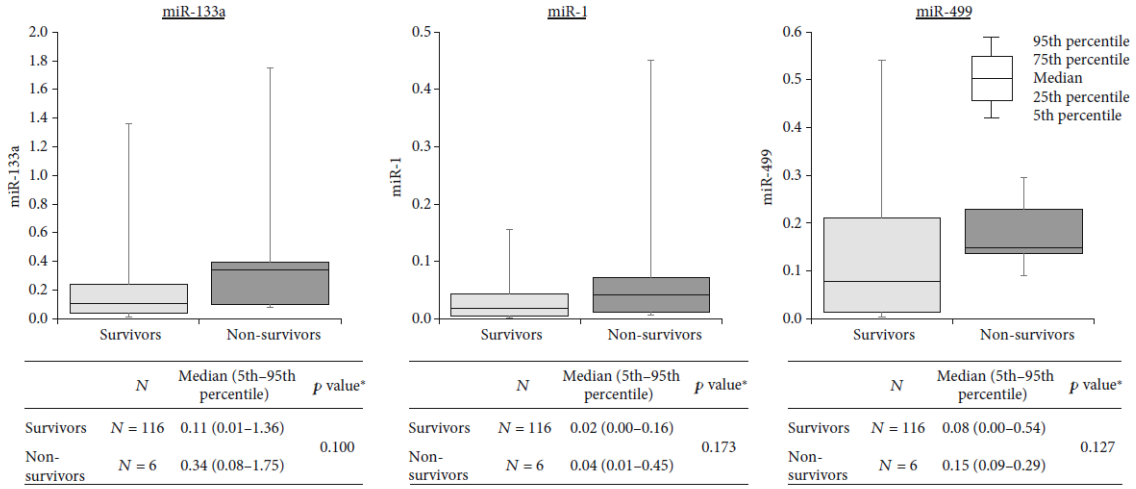


FIGURE 1: Correlations between particular miRNAs (relative expression) and hsTnT levels 24 hours after admission, NT-proBNP, and LVEF. hsTnT = high-sensitivity troponin T; LVEF = left ventricular ejection fraction; NT-proBNP = N-terminal prohormone of brain natriuretic peptide.

selected patient population is indicated when the indication criteria are met [1].

Despite the risk stratification of patients after myocardial infarction, ischemic complications recur even at low calculated risk, and these events can be fatal. miRNAs, as a group of the potential new markers, could help in the stratification of these patients. Then, if an increased miRNA value and usual risk parameters including LVEF are found without significant pathology, supplementation of the Holter ECG to exclude ventricular arrhythmias and careful follow-up of these patients should be considered.

For this reason, we used a well-defined and very homogeneous cohort of AMI patients after pPCI and tested the prognostic value of three cardiomyo-specific miRNAs (miR-1, miR-133, and miR-499) in one-year cardiovascular mortality and their relation to standard laboratory markers. We proved correlations between levels of miR-1, miR-133, and miR-499 with hsTnT, NT-proBNP, and LVEF in this cohort of



	N	Median (5th–95th percentile)	p value*
Survivors	N = 116	0.11 (0.01–1.36)	0.100
Non-survivors	N = 6	0.34 (0.08–1.75)	

	N	Median (5th–95th percentile)	p value*
Survivors	N = 116	0.02 (0.00–0.16)	0.173
Non-survivors	N = 6	0.04 (0.01–0.45)	

	N	Median (5th–95th percentile)	p value*
Survivors	N = 116	0.08 (0.00–0.54)	0.127
Non-survivors	N = 6	0.15 (0.09–0.29)	

FIGURE 2: Relationship between miRNAs (relative expression) and one-year mortality. *Mann-Whitney test.

TABLE 2: Relationship between individual marker levels and one-year mortality.

Marker	Gene Locus (OMIM)	Concentration	Patients, number (%)	Survivors, number (%)	Nonsurvivors, number (%)	p value*
MicroRNAs (relative concentration)						
miR-133a	MIR133A1/MIR133A2	<0.330	93 (76.2)	91 (78.4)	2 (33.3)	**0.028**
	18q11.2/20q13.33	≥0.330	29 (23.8)	25 (21.6)	4 (66.7)	
miR-1	MIR1-1/MIR1-2	<0.031	78 (63.9)	76 (65.5)	2 (33.3)	0.187
	20q13.33/18q11.2	≥0.031	44 (36.1)	40 (34.5)	4 (66.7)	
miR-499	MIR499	<0.088	63 (51.6)	63 (54.3)	0 (0.0)	**0.011**
	20q11.22	≥0.088	59 (48.4)	53 (45.7)	6 (100.0)	
Standard biomarkers (concentration in ng/L)						
hsTnT	TNN2	<154.5	77 (63.6)	75 (65.2)	2 (33.3)	0.189
	1q32.1	≥154.5	44 (36.4)	40 (34.8)	4 (66.7)	
NT-proBNP	NPPB	<891.5	66 (54.1)	66 (56.9)	0 (0.0)	**0.008**
	1p36.22	≥891.5	56 (45.9)	50 (43.1)	6 (100.0)	

*Fisher exact test. NT-proBNP = N-terminal prohormone of brain natriuretic peptide; hsTnT = high-sensitivity troponin.

patients. In addition, we found a possible relationship between combined levels of miR-499 with NT-proBNP and increased one-year mortality risk in these patients on dual antiplatelet therapy that has not been published yet.

4.1. MyomiR Levels after Myocardial Infarction. Many authors focus on miRNA levels during cardiovascular events and their possible contribution to the diagnostics or differential diagnostics [14, 18]. Published papers found that levels of miR-1 and miR-133a/b increase soon after AMI, reaching a peak shortly before TnI and returning to baseline within five days, while miR-499 peaks later, about 12 hours after the onset of the first symptoms [14]. miR-499 levels are naturally very low in healthy people and increase after AMI, with levels being higher in acute myocardial infarction with ST-segment elevation (STEMI) compared to non-STEMI patients [18], and provide a comparable diagnostic value to that of hsTnT [18]. Concentrations of miR-499 are higher in patients after AMI compared to patients with unstable angina [19]. miR-499 remains increased 24 hours after MI and then slowly decreases to original levels over 7 days [19]. Increased levels of circulating miR-499 and miR-208 after AMI reflect the cardiac damage caused by the AMI [19]. miR-208 levels are usually under the limits of detection in healthy individuals but rapidly increase after AMI. The peak is observed 3 hours after reperfusion, which is then followed by a rapid fall in concentration back to initial levels within 24 hours [20]. Since our samples were taken 24 hours after admission to

Table 3: Relationship between various combinations of marker levels and one-year mortality.

Markers and their levels	Patients, number (%)	Survivors, number (%)	Nonsurvivors, number (%)	p value*
MicroRNAs				
miR-133a+miR-1				
Both low	75 (61.5)	73 (62.9)	2 (33.3)	
One low and one high	21 (17.2)	21 (18.1)	0 (0.0)	**0.045**
Both high	26 (21.3)	22 (19.0)	4 (66.7)	
miR-133a+miR-499				
Both low	57 (46.7)	57 (49.1)	0 (0.0)	
One low and one high	42 (34.4)	40 (34.5)	2 (33.3)	**0.004**
Both high	23 (18.9)	19 (16.4)	4 (66.7)	
miR-1+miR-499				
Both low	47 (38.5)	47 (40.5)	0 (0.0)	
One low and one high	47 (38.5)	45 (38.8)	2 (33.3)	**0.019**
Both high	28 (23.0)	24 (20.7)	4 (66.7)	
miR-133a+miR-1+miR-499				
All low	47 (38.5)	47 (40.5)	0 (0.0)	
Minimum one low, minimum one high	55 (45.1)	53 (45.7)	2 (33.3)	**0.003**
All high	20 (16.4)	16 (13.8)	4 (66.7)	
Standard biomarkers				
hsTnT+NT-proBNP				
Both low	42 (34.7)	42 (36.5)	0 (0.0)	
One low and one high	58 (47.9)	56 (48.7)	2 (33.3)	**0.006**
Both high	21 (17.4)	17 (14.8)	4 (66.7)	
MicroRNAs and standard biomarkers				
hsTnT+miR-133a				
Both low	63 (52.1)	62 (53.9)	1 (16.7)	
One low and one high	43 (35.5)	41 (35.7)	2 (33.3)	**0.024**
Both high	15 (12.4)	12 (10.4)	3 (50.0)	
hsTnT+miR-1				
Both low	53 (43.8)	52 (45.2)	1 (16.7)	
One low and one high	48 (39.7)	46 (40.0)	2 (33.3)	0.094
Both high	20 (16.5)	17 (14.8)	3 (50.0)	
hsTnT+miR-499				
Both low	39 (32.2)	39 (33.9)	0 (0.0)	
One low and one high	61 (50.4)	59 (51.3)	2 (33.3)	**0.005**
Both high	21 (17.4)	17 (14.8)	4 (66.7)	
NT-proBNP+miR-133a				
Both low	59 (48.4)	59 (50.9)	0 (0.0)	
One low and one high	41 (33.6)	39 (33.6)	2 (33.3)	**0.003**
Both high	22 (18.0)	18 (15.5)	4 (66.7)	
NT-proBNP+miR-1				
Both low	51 (41.8)	51 (44.0)	0 (0.0)	
One low and one high	42 (34.4)	40 (34.5)	2 (33.3)	**0.014**
Both high	29 (23.8)	25 (21.6)	4 (66.7)	
NT-proBNP+miR-499				
Both low	46 (37.7)	46 (39.7)	0 (0.0)	
One low and one high	37 (30.3)	37 (31.9)	0 (0.0)	**0.001**
Both high	39 (32.0)	33 (28.4)	6 (100.0)	

*Fisher exact test: difference between both subgroups. hsTnT = high-sensitivity troponin T; NT-proBNP = N-terminal prohormone of brain natriuretic peptide.

the hospital, the concentration of miR-208 was either under the detection limit or too low to be quantified, so this miRNA was not included in our analysis, and only levels of miR-1, miR-133a, and miR-499 were measured.

4.2. Correlations of miRNAs with Standard Markers. We focused on the correlation with selected standard biomarkers and found a significant positive correlation of the three microRNAs with hsTnT and NT-proBNP. Our findings agree with other published papers, where levels of miR-499 were found to be positively correlated with levels of troponin T and I [14, 19, 21], despite minor differences in methods, the cohort of MI patients, and time of sampling. A positive correlation ($r = 0.596$, $p < 0.001$) between miR-133a and cTnI was previously published [19, 22] and reported a similar trend in levels of both markers in the early phase of AMI [22]; another work described an early miR-1, miRNA-133a, and miR-133b peak that occurred at a similar time as the TnI peak, whereas miR-499-5p exhibited a slower time course [14]. A correlation was also found between miR-499 and creatinine kinase (CK) [18, 19].

All the three analyzed miRNAs were found to have a moderate or strong positive correlation with NT-proBNP, which was published to be an important independent predictor of poor outcomes [23]. Furthermore, we found a strong negative correlation between all the three miRNAs and LVEF, which is in line with several other authors who found a similar negative correlation of miR-499 with LVEF ($r = -0.36$, $p = 0.008$) [16] or a weak negative correlation of miR-499-5p with LVEF (-0.11, $p = 0.037$) and miR-1 with LVEF ($r = -0.16$, $p = 0.003$) [21].

4.3. miRNAs in One-Year Prognosis. Finally, we looked for differences in the levels of laboratory markers between patients at an increased risk of death (nonsurvivors) and survivors. We found that all nonsurvivors had high levels of NT-proBNP and high levels of miR-499. Levels of NT-proBNP were measured in all AMI patients shortly after admission to the hospital before pPCI; in addition, the levels of microRNAs were also measured as potential new biomarkers. The choice of microRNAs was based on promising assessments for diagnostics or prognostics in recently published literature [14, 21, 22, 24].

Current risk stratification is based primarily on left ventricular dysfunction, measured as left ventricular ejection fraction [1, 2]. Many studies have found a clear relationship between reduced LVEF and mortality, which increases when LVEF falls under 50% and progressively increases when LVEF declines under 40% [2]. Despite this important predictor, about 50% of patients who die suddenly do not meet the abovementioned LVEF criteria [2]. Also, in our cohort, only 2 patients out of 6 in the nonsurvivor subgroup had an LVEF ≤ 35% during hospitalization and none at the time of follow-up. Our goal was to find a combination of laboratory markers that could contribute to the better identification of patients at increased risk of death after myocardial infarction and thus decrease the relatively high post-AMI mortality that reaches 7–20% at one year, 24–38% at five years, and 40–56% at ten years [2].

In our work, we analyzed cardio-enriched microRNAs, measurable 24 hours after patient admission to the hospital, to see if some of them could potentially fit into such a panel of biomarkers. Our results found that miR-499 in combination with NT-proBNP was best able to characterize the nonsurvivor subgroup. The number of papers dealing with myomiRs and AMI patients' prognosis is relatively limited. A recently published work confirms increased levels of cardio-enriched miRNAs (miR-499 and miR-208) in the blood of AMI patients and establishes an association of increased miRNA levels with reduced systolic function after AMI and risk of death or heart failure within 30 days [21]. Another work found that circulating levels of miR-133a and miR-208b were associated with all-cause mortality at 6 months, but this did not add prognostic information to hsTnT, the standard marker of AMI [25]. miR-133 was also studied in the high-risk STEMI patient cohort, where its levels provided prognostic information but do not add independent prognostic information to traditional markers of AMI [26].

In spite of the undeniable advantages of a well-defined and very homogeneous cohort of patients, this analysis was limited by the low number of patients in the nonsurvivor subgroup and by its retrospective character.

5. Conclusion

One-year mortality in patients after AMI treated with pPCI was very low (4.9%). A positive correlation was found between miRNA-1, miR-133a, and miR-499 and hsTnT (24 hours after admission) and NT-proBNP, and a negative correlation with LVEF. Further, this work suggests that plasma levels of circulating miR-499 might contribute to the identification of patients at increased risk of death, especially when combined with NT-proBNP levels. Further analyses are needed to determine if miR-499 or some other miRNAs can be effectively used in practice to better identify at-risk patients, to better understand the roles of these miRNAs in AMI, and to thus improve the clinical management of patients after AMI.

Data Availability

The data (miRNA Ct values and values of hsTnT and NT-proBNP) used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

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Příloha I

3. Mayer O Jr, Seidlerová J, Černá V, Kučerová A, Vaněk J, Karnosová P, Bruthans J, Wohlfahrt P, Cífková R, Pešta M, Filipovský J. *The low expression of circulating microRNA-19a represents an additional mortality risk in stable patients with vascular disease*. Int J Cardiol. 2019 Aug 15;289:101-106. doi: 10.1016/j.ijcard.2019.05.008. Epub 2019 May 6. PMID: 31085080.

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The low expression of circulating microRNA-19a represents an additional mortality risk in stable patients with vascular disease

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ABSTRACT

Background: Secondary prevention of atherosclerotic vascular diseases represents a cascade of procedures to reduce the risk of future fatal and non-fatal cardiovascular events. We sought to determine whether the expression of selected microRNAs influenced mortality of stable chronic cardiovascular patients.
Methods: The plasma concentrations of five selected microRNAs (miR-1, miR-19, miR-126, miR-133 and miR-223) were quantified in 826 patients (mean age 65.2 years) with stable vascular disease (6–36 months after acute coronary syndrome, coronary revascularization or first-ever ischemic stroke). All-cause and cardiovascular mortality rates were followed during our prospective study.
Results: Low expression (bottom quartile) of all five miRNAs was associated with a significant increase in five-year all-cause death, even when adjusted for conventional risk factors, treatment, raised troponin I and brain natriuretic protein levels [hazard risk ratios (HRRs) were as follows: miR-1, 1.65 (95% CI: 1.16–2.35); miR-19a, 2.27 (95% CI: 1.59–3.23); miR-126, 1.64 (95% CI: 1.15–2.33); miR-133a, 1.46 (95% CI: 1.01–2.12) and miR-223, 2.05 (95% CI: 1.45–2.91)]. Nearly similar results were found if using five-year cardiovascular mortality as the outcome. However, if entering all five miRNAs (along with other covariates) into a single regression model, only low miR-19a remained a significant mortality predictor; and only in patients with coronary artery disease [3.00 (95% CI: 1.77–5.08)], but not in post-stroke patients [1.63 (95% CI: 0.94–2.86)].
Conclusions: In stable chronic coronary artery disease patients, low miR-19a expression was associated with a substantial increase in mortality risk independently of other conventional cardiovascular risk factors.

1. Introduction

The majority of therapeutic interventions in secondary prevention of cardiovascular diseases target conventional risk factors (RFs) of atherosclerosis, e.g. smoking, hypertension, dyslipidemia and impaired glucose metabolism [1]. However, individual risk of cardiovascular patients is directly modulated by several other pathophysiological mechanisms. These mechanisms include pro-thrombotic activity and individual platelet aggregability, inflammatory status, calcification of coronary arteries and other tissues, ischemia/reperfusion injury, fibrosis and myocardial cell apoptosis as well as left ventricular remodeling and heart failure. Circulating biomarkers of these pathophysiological processes may potentially improve prediction of future cardiovascular events and also stimulate research along completely new lines.

Micro-ribonucleic acid (miRNA) consists of short sequences of non-coding RNA (around 18–23 nucleotides). Tissue-specific miRNAs modulate the expression of the complementary messenger RNAs. From a clinical view, expression of a specific miRNA may represent a specific pathophysiologic or reparatory mechanism [2,3]. To date, literary thousands of circulating miRNAs have been identified; however, their role has not been fully elucidated yet. Several miRNAs have been found to be involved in various processes occurring within the cardiovascular system, including cardiac organogenesis, heart remodeling and myocardial cell apoptosis or regenerative responses to various types of myocardial injury [4]. However, the majority of studies investigating the role of miRNAs in coronary artery disease (CAD) are experimental, whereas clinical studies are rare, relatively small or address the acute phase of CAD only [5]. In the present analysis, we aimed to assess the predictive

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power of selected miRNAs on mortality risk in stable, chronic vascular disease (CAD or post-stroke) patients.

2. Methods

All procedures performed in this study were in accordance with the principles of Good Clinical Practice and ethical standards formulated in the 1964 Declaration of Helsinki and its later amendments. The study protocol was approved by the local Ethics Committee of the University Hospital in Pilsen. Written informed consent was obtained from all participants included in the study.

2.1. Design and study population

The present study is a secondary analysis of EUROASPIRE survey data from the Czech Republic, a prospective follow-up of two pooled independent cohorts (EUROASPIRE III and EUROASPIRE III-Stroke Survey) [6,7] examined in 2006/2007 (both surveys were conducted in the same two centers in the Czech Republic: University Hospital in Pilsen and the Heart Center of the Institute of Clinical and Experimental Medicine in Prague).

The study population consisted of subjects examined within two well-designed surveys in patients with stable CAD or those after their first-ever ischemic stroke. In the first step, patients were retrospectively identified from hospital records, with recruitment started using the most recent hospital record and proceeding backward until the required cohort was obtained. The main inclusion criteria for the cohort of CAD patients [6] were age < 81 years *plus* at least one of the following final discharge diagnoses (qualifying coronary event): first coronary artery bypass graft (CABG), first percutaneous transluminal coronary angioplasty (PTCA), acute myocardial infarction or documented acute myocardial ischemia. Stroke patients were selected in the same manner [7]; however, the inclusion criteria were age ≤ 80 years *plus* the main qualifying event, their first-ever acute ischemic stroke (with its etiology verified by CT or MRI scan). A total of 600 CAD and 507 post-stroke consecutive patients were selected, of which number 493 CAD and 341 post-stroke patients were interviewed. After excluding 42 CAD and 77 post-stroke patients dying between the qualifying event and the survey, the overall response rates were 88.4% and 79.3%, respectively. The interview and clinical examination of patients were performed between 6 and 36 months after the qualifying vascular event (i.e., hospitalization for acute coronary syndrome, coronary revascularization or first-ever ischemic stroke) and considered, for the purpose of the present prospective cohort study, baseline visit.

2.2. Clinical examinations and biochemical measurements

Information on personal and demographic characteristics, personal and family history of CAD, lifestyle and pharmacotherapy were obtained at the interview. The following clinical examinations were performed: height and weight were measured in light indoor clothes without shoes using SECA 220 scales (SECA, Hamburg, Germany) and measuring sticks, respectively. Waist circumference was measured using a steel tape measure. Blood pressure (BP) was measured twice in the sitting position on the right arm using a standard mercury sphygmomanometer. Breath carbon monoxide was measured by an EC50 Smokerlyser device (Bedfont Scientific, Upchurch, UK) to verify the reported smoking status. Venous blood samples were drawn after at least 12 h of overnight fasting.

All laboratory examinations were performed in series from aliquots stored at −80 °C and included estimation of serum total (TCHOL) and high-density lipoprotein (HDL) cholesterol, using an ARCHITECT c800 analyzer (Abbott Laboratories, Wiesbaden, Germany) and commercially available DOT Diagnostics kits (Brno, Czech Republic); the same analyzer was used for measuring serum triglycerides (TG) and glucose (GLU), whereas brain natriuretic peptide (BNP) was measured in EDTA plasma using Abbott commercial kits (Abbott, Wiesbaden, Germany). Troponin I (cTnI) was estimated using a commercially available AccuTnI kit on UniCel DxI 800 platform (Beckman-Coulter Inc., Brea, CA, USA), while HbA1c by ionex liquid chromatography using a G7 analyzer (TOSOH, Tokyo, Japan).

2.3. Circulating microRNAs quantification

Circulating miRNAs were again estimated in series from frozen plasma samples (the choice of the initially estimated 10 miRNAs was based on literature search).

Total cell-free RNA was isolated from 200 μl of EDTA plasma using the miRNeasy Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted in 14 μL of ribonuclease-free water and stored at −80 °C until further analyses. MicroRNA-39 (*C. elegans* miR-39) was used as a spike-in control. A fixed volume of 1 μL of this RNA eluate was used for each reverse transcription reaction. For reverse transcriptions and quantitative estimations of selected microRNAs by real-time PCR reactions, TaqMan® microRNA assays and master mixes were used (hsa-miR-133a – Assay ID 002246; hsa-miR-1 – Assay ID 002222; hsa-miR-21 – Assay ID 000397; hsa-miR-34a – Assay ID 000426; hsa-miR-126 – Assay ID 002228; hsa-miR-208b – Assay ID 002290; mmu-miR-499 – Assay ID 001352; hsa-miR-223 – Assay ID 002295; hsa-miR-197 – Assay ID 000497; hsa-miR-19a – Assay ID 000395; hsa-miR-214 – Assay ID 002306; cel-miR-39 – Assay ID 000200; TaqMan universal MMIX II and TaqMan® microRNA RT kit), while a T100™ thermal cycler (BIORAD, Hercules, CA, USA) was used for reverse transcription. Reaction volume was 15 μL. A fixed volume of 2.5 μL from this RT reaction was used to each real-time PCR reaction. Samples were assessed in technical duplicates. The Ct values were corrected using calibrators to eliminate differences between individual runs of the Stratagene Mx3000P Real-Time PCR device (Agilent Technologies, Santa Clara, CA, USA). In cases showing disagreement between results obtained from both technical duplicates, sample assessment was repeated. Relative expression of investigated miRNAs was calculated using the ΔCt method ($2^{-\Delta Ct}$ algorithm was $\Delta Ct = Ct_{miR\text{-}x} - Ct_{miR\text{-}39}$).

In the first step, we estimated the 10 initially selected miRNAs (miR-1, miR-19, miR-21, miR-34a, miR-126, miR-133a, miR-197, miR-214, miR-223 and miR-499) in a pilot cohort of 100 patients [mean age 64.6 (±SD 6.71) years, 75% of males], 50 of whom had deceased during follow-up (both subgroups, i.e. dead versus alive, were age- and gender-matched). Statistical differences between the two subgroups were found for miR-1, miR-19, miR-126, miR-133 and miR-223; these 5 miRNAs were estimated in a full cohort and used for any further analysis.

2.4. Data management

Of the 834 CHD patients or post-stroke patients attending the initial interview, eight subjects were excluded because of incomplete follow-up data or miRNA estimation unfeasible for technical reasons.

For statistical analyses, we used STATISTICA 8 (StatSoft Inc., Tulsa, OK, USA) and STATA 8 (STATA Corp LP, College Station, TX, USA) software. Standard statistical methods (descriptive statistics, multiple linear regression and Cox proportional hazard regression) were used. Conventional RFs were dichotomized by cut-off points as proposed by the 3rd Joint European Guidelines for Cardiovascular Prevention (valid at the time of interview) [8]. The cut-offs for BNP and cTnI were >100 ng/L and ≥0.04 ng/mL, respectively. "Overt heart failure" was defined as the presence of at least one of the following criteria: NYHA functional class ≥ II, known systolic dysfunction (ejection fraction <40%), known history of hospitalization for heart failure before interview, chronic treatment with furosemide and/or spironolactone, BNP ≥ 500 ng/L. MicroRNAs were dichotomized using their quartiles with "low expression" ones considered those in the bottom quartile, i.e. ≤0.0014 for miR-1, ≤0.298 for miR-19a, ≤0.720 for miR-126, ≤0.0061 for miR-133 and ≤5.25 for miR-223 (all miRNAs are given in their relative expression ratios).

We ascertained the vital status of patients through May 31, 2012 using the National Mortality Registry of the Czech Institute for Medical Information and Statistics. We used death certificates and data in hospital information systems to specify the cause of death and calculate five-year all-cause or cardiovascular mortality. Using a Cox proportional hazard model, univariate analysis was performed to determine the crude relation between exposure (low or high miRNA) and total/cardiovascular mortality (outcome). As a second step, we adjusted all models for basic confounders (age, gender and primary diagnosis) and subsequently for other cardiovascular RFs, treatments with a presumable effect on cardiovascular mortality, as well as a history of coronary revascularization (before inclusion in the study), sub/clinical heart failure (increased BNP) and subclinical coronary ischemia (increased cTnI). Censored data were used for final analysis.

3. Results

Baseline characteristics of the 487 CAD patients and 339 post-stroke patients analyzed in this follow-up study are listed in Table 1. During a median follow-up of 2050 days (5.6 years), 167 patients deceased, with 126 of these fatal events considered of cardiovascular origin (baseline characteristics by these outcomes are also listed in Table 1). The corresponding five-year all-cause and cardiovascular mortality rates were 18.3% and 13.8%, respectively.

3.1. Identification of microRNAs and their relation to conventional risk factors

We analyzed the association of each miRNA (miR-1, miR-19, miR-126, miR-133 and miR-223) with conventional RFs, treatments and other characteristics (BNP, cTnI; Table 2). In a multiple linear regression analysis, we identified LDL and HbA1c as an independent positive determinant of all five miRNAs (and these results were repeated when using fasting glycemia instead of HbA1c - not shown in table). Moreover, while miR-19a, miR-133a and miR-233 were positively associated with stroke as primary qualifying diagnosis, inverse relation was found if miR-1 was the dependent variable. Finally, male gender was additional independent determinant of miR-19, while mean arterial pressure and troponin I of miR-133a.

3.2. Mortality analysis

We assessed the predictive power of each miRNA in a multivariate Cox model (Table 3) along with potential covariates (only factors with known physiological role, evident effect in cardiovascular prevention and strongly related to mortality risk were chosen). In the first step was tested the relative expression of miRNAs as continuous log-

Table 1
Basic characteristics of patients [mean (standard deviation) or *factor proportion*].

	Full cohort	All-cause death	CV death	Survived	p_1	p_2
n	826	167	126	659		
Age [years]	65.2 (9.3)	69.2 (8.8)	70.0 (8.6)	64.1 (9.2)	<0.0001	<0.0001
Gender [% of males]	*70.6*	*69.0*	*65.1*	*71.3*	0.56	0.24
Qualifying diagnosis [% of post-stroke patients]	*41.0*	*56.3*	*59.5*	*36.7*	<0.0001	<0.0001
Coronary revascularization [%]	*56.1*	*41.1*	*38.1*	*60.2*	<0.0001	<0.0001
Time to interview[#] [years; median (IQR)]	1.39 (0.83–1.74)	1.30 (0.83–1.75)	1.33 (0.82–1.80)	1.37 (0.84–1.73)	0.18	0.51
Current smoking [%]	*18.8*	*15.2*	*12.7*	*19.6*	0.17	0.052
Body mass index [kg/m^2]	29.3 (4.8)	28.3 (4.9)	28.4 (4.9)	29.4 (4.7)	0.19	0.020
Body mass index ≥ 30 kg/m^2[%]	*37.8*	*36.3*	*32.0*	*38.4*	0.55	0.20
Systolic blood pressure [mmHg]	136.4 (17.9)	136.1 (18.7)	136.3 (19.1)	136.4 (17.6)	0.92	0.76
Diastolic blood pressure [mmHg]	80.4 (10.1)	78.9 (10.3)	79.4 (10.5)	80.8 (10.0)	0.030	0.33
Raised blood pressure [%]	*44.2*	*39.9*	*41.3*	*45.0*	0.26	0.41
tx/w antihypertensive drugs [%]	*92.1*	*94.9*	*96.0*	*91.4*	0.14	0.10
tx/w betablockers [%]	*69.8*	*64.5*	*68.3*	*71.0*	0.17	0.77
tx/w ACEis or ARBs [%]	*71.8*	*74.1*	*76.2*	*70.9*	0.39	0.25
LDL-cholesterol [mmol/L]	2.84 (0.96)	2.82 (1.03)	2.82 (1.07)	2.84 (0.94)	0.81	0.85
LDL-cholesterol ≥ 2.5 mmol/L	*60.1*	*58.9*	*58.7*	*60.9*	0.69	0.73
tx/w statins [%]	*70.2*	*63.9*	*63.5*	*71.5*	0.052	0.06
Fasting glycemia [mmol/L]	6.86 (2.54)	6.94 (2.42)	7.00 (2.64)	6.84 (2.56)	0.75	0.69
Hemoglobin A1c [mmol/mol]	42.6 (12.7)	43.2 (11.3)	43.8 (11.7)	31.4 (46.5)	0.14	0.051
Overt diabetes[§] [%]	*39.4*	*47.5*	*48.4*	*37.7*	0.029	0.019
Inadequate glycemic control[§] [%]	*33.6*	*43.9*	*41.9*	*31.4*	0.003	0.015
tx/w antidiabetics [%]	*21.7*	*32.3*	*35.7*	*19.3*	0.0004	<0.0001
Brain natriuretic peptide [ng/L]	120.1 (190.9)	217.3 (303.5)	227.8 (312.8)	96.8 (144.1)	<0.0001	<0.0001
Troponin I [ng/mL]	*0.022 (0.181)*	*0.022 (0.026)*	*0.024 (0.028)*	*0.021 (0.202)*	<0.0001	<0.0001
Brain natriuretic peptide ≥ 100 ng/L	*32.2*	*51.3*	*53.6*	*27.5*	<0.0001	<0.0001
Troponin I ≥ 0.04 ng/mL	*6.2*	*14.1*	*15.3*	*4.1*	<0.0001	<0.0001
miR-1	−2.37 (0.73)	−2.57 (0.69)	−2.58 (0.71)	−2.32 (0.73)	<0.0001	0.0003
miR-19a	−0.05 (0.61)	−0.16 (0.68)	−0.18 (0.76)	−0.04 (0.60)	0.006	0.017
miR-126	0.30 (0.63)	0.16 (0.67)	0.14 (0.67)	0.33 (0.62)	0.003	0.002
miR-133a	−1.70 (0.72)	−1.82 (0.74)	−1.83 (0.76)	−1.68 (0.71)	0.010	0.012
miR-223	1.17 (0.69)	1.01 (0.70)	0.98 (0.72)	1.21 (0.69)	0.0007	0.0002

CV, cardiovascular; IQR, interquartile range; tx/w, treatment with…; ACEis, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II receptor blockers LDL, low density lipoprotein; miRNA, microRNA; p_1, all-cause death versus survived; p_2, CV death versus survived (Mann-Whitney *U* test);
[#] time between qualifying cardiovascular event (acute coronary syndrome/coronary revascularization or stroke and baseline visit); [§]fasting glycemia ≥7 mmol/L and/or treatment with antidiabetics; [§]fasting glycemia ≥7 mmol/L and/or HbA1c ≥ 48 mmol/mol; miRNAs are depicted in log-transformed relative plasma expression units;

transformed variables (model A). Each of five estimated miRNAs was inversely associated with a significant increase in both all-cause and cardiovascular mortality risk even after adjustment for conventional RFs and other potential confounders. In the next step, we entered all five log-transformed miRNAs, into one regression model (along with other potential covariates again). The significant inverse relationship remained only for miR-19a (model A, bottom part of Table 3), either with all-cause or cardiovascular mortality.

Consequently we tested predictive power of estimated miRNAs as categories (model B). Low expression (i.e. bottom quartile) of each of five estimated miRNAs was associated with a significant increase in both all-cause and cardiovascular mortality risk, independently of other potential confounders (of which, only age, troponin $I \geq 0.04$ ng/mL and brain natriuretic peptide ≥100 ng/L entered the model as additional significant mortality predictors). As in continuous manner (model A), if all five miRNAs were included into one regression model, only low miR-19a remained significant predictor of both all-cause and cardiovascular mortality (Table 3, bottom part, model B).

Finally, we ran subgroup analyses to investigate a potential interaction of miR-19a with other factors. Low miR-19a predicted all-cause mortality risk significantly only in CAD but not in post-stroke patients (Fig. 1) [fully adjusted HRR for all-cause mortality were in CAD and post-stroke patients 3.00 (95% CI: 1.77–5.08), $p < 0.0001$ and 1.63 (95% CI: 0.94–2.86), $p = 0.080$, respectively]. Significant predictive power was observed in men, while not in women [HRR 3.15 (95% CI: 2.03–4.88), $p < 0.0001$ versus 1.34 (95% CI: 0.69–2.60), $p = 0.513$, respectively], and we also observed a higher predictive power of low miR-19a in younger patients (≤65 years) compared with older ones.

Similar results were obtained in analyses stratified by age, gender and primary diagnosis for the other four miRNAs. In addition, low miR-1, miR126, miR-133a and miR-223 predicted all-cause mortality only in patients with overt heart failure and only in diabetic patients (see Supplementary Table 1).

4. Discussion

In the present study, we found that low miR-19a expression was associated with more than a four-fold risk of a future fatal cardiovascular event independently of conventional RFs or other important characteristics (presence of heart failure, subclinical ischemia, etc.). To our knowledge, no study has to date examined the association between miR-19 and mortality risk in stable chronic vascular disease patients.

In a prospective study with 1112 CAD patients, Karakas and colleagues [9] observed that an increase in miR-19 by one standard deviation was associated with about a doubling of cardiovascular mortality, hence a finding quite opposite to ours. Similarly, miR-1 and miR-133a were negatively associated with troponin I in our dataset, while in another studies were these miRNAs considered as markers of acute coronary ischemia (i.e. positively associated with troponin) [10,11] However, the discrepancy between Karakas's and our findings could be possibly explained by the different study populations assessed and by different behavior of miR-19a (and other miRNAs) in acute or chronic phase of CAD. We proposed a hypothesis that miR-19a may represent an acute response and reparatory mechanisms (i.e., while a more severe injury leads to a greater acute response, it is generally associated with a poorer outcome). The study by Karakas [9] examined patients early after coronary revascularization (for acute coronary syndrome or stable angina pectoris). Conversely, our study involved stable vascular patients and low miR-19a may herald long-term failure of protective mechanisms leading to higher risk of fatal outcome. This hypothesis is supported by the recently uncovered physiological role of miR-19. As part of the "miR-17/92 cluster", miR-19 was initially identified as a

Table 2
Multivariate association between selected miRNA's and cardiovascular risk characteristics.

Dependent variable	miR-1		miR-19a		miR-126	
	Beta coeff. (SE)	p	Beta coeff. (SE)	p		
Age	−0.0003(0.0030)	0.920	0.0011 (0.0026)	0.666	−0.0013 (0.0027)	0.633
Male gender	0.0276 (0.0585)	0.638	**0.1048 (0.0501)**	**0.037**	0.0930 (0.0510)	0.069
Stroke	**−0.1425 (0.0703)**	**0.043**	**0.2149 (0.0601)**	**<0.0001**	0.0860 (0.0612)	0.161
Time to interview	0.0217 (0.0390)	0.579	0.0491 (0.0334)	0.141	0.0789 (0.0639)	0.200
Current smoking	−0.0388 (0.0662)	0.558	−0.0253 (0.0566)	0.654	0.0245 (0.0576)	0.672
Body mass index	0.0030 (0.0056)	0.587	0.0033 (0.0047)	0.484	0.0061 (0.0048)	0.205
MAP	0.0030 (0.0023)	0.193	0.0020 (0.0020)	0.309	0.0032 (0.0019)	0.105
LDL-cholesterol	**0.0783 (0.0280)**	**0.005**	**0.0743 (0.0240)**	**0.002**	**0.0630 (0.0243)**	**0.010**
HbA1c	**0.0793 (0.0219)**	**<0.0001**	**0.0646 (0.0188)**	**0.001**	**0.0759 (0.0198)**	**<0.0001**
Troponin I	**−0.2811 (0.1382)**	**0.042**	0.0050 (0.1182)	0.966	−0.1528 (0.1204)	0.205
BNP	−0.0002 (0.0001)	0.060	−0.0001 (0.0001)	0.359	−0.0002 (0.0001)	0.065
Statins	0.0220 (0.0616)	0.721	0.0473 (0.0527)	0.369	0.0544 (0.0537)	0.311
Betablockers	−0.0715 (0.0066)	0.279	−0.0214 (0.564)	0.705	−0.0737 (0.0575)	0.200
ACEi or ARBs	−0.0320 (0.0582)	0.583	−0.0049 (0.0498)	0.921	−0.0208 (0.0507)	0.682
Antidiabetics	−0.0871 (0.0662)	0.189	−0.0705 (0.0567)	0.214	−0.1037 (0.0577)	0.073
Const.	*−3.1411 (0.3666)*	*<0.0001*	*−1.1216 (0.3135)*	*<0.0001*	*−0.7465 (0.3191)*	*0.020*

Dependent variable	miR-133a		miR-223	
	Beta coeff. (SE)	p	Beta coeff. (SE)	p
Age	0.0014 (0.0029)	0.646	0.0009 (0.0029)	0.754
Male gender	0.0580 (0.0566)	0.306	0.0780 (0.0565)	0.168
Stroke	**0.3381 (0.0680)**	**<0.0001**	**0.1370 (0.0679)**	**0.044**
Time to interview	−0.0430 (0.0377)	0.255	0.0246 (0.0376)	0.514
Current smoking	−0.0275 (0.0640)	0.668	0.0401 (0.0639)	0.531
Body mass index	0.0057 (0.0054)	0.294	0.0093 (0.0054)	0.083
MAP	**0.0054 (0.0022)**	**0.015**	0.0035 (0.0022)	0.115
LDL-cholesterol	**0.0759 (0.0270)**	**0.005**	**0.0827 (0.0270)**	**0.002**
HbA1c	**0.0798 (0.0212)**	**<0.0001**	**0.0852 (0.0211)**	**0.0001**
Troponin I	**−0.2768 (0.1337)**	**0.039**	−0.0964 (0.1334)	0.470
BNP	−0.0002 (0.0001)	0.240	−0.0002 (0.0001)	0.106
Statins	−0.0095 (0.0510)	0.873	0.0594 (0.0595)	0.318
Betablockers	−0.0182 (0.0638)	0.776	−0.0142 (0.0637)	0.824
ACEi or ARBs	−0.0396 (0.0563)	0.483	−0.0014 (0.0562)	0.981
Antidiabetics	−0.1062 (0.0641)	0.098	−0.0927 (0.0639)	0.147
const.	*−3.0556 (0.3545)*	*<0.0001*	*−0.2359 (0.3536)*	*0.505*

Multiple linear regression [beta coefficient and standard error].
MAP, mean arterial pressure; HbA1c, hemoglobin A1c (glycohemoglobin); BNP, brain natriuretic peptide; ACEi, angiotensin converting inhibitors, ARB, angiotensin II receptor blockers; (log-transformed values of each miRNA relative expression were used as dependent variables).

human oncogene, with miR-17, miR-18a, miR-19b-1, miR-20a, and miR-92a-1 being the other members of this cluster. Several researchers have proposed that miR-19a is its key component [12]. A number of reports have highlighted the role of this cluster in the heart, particularly in terms of cardiomyocyte proliferation [12–18]. While the mammalian heart was long considered a post-mitotic, terminally differentiated organ lacking a post-natal proliferative capability, this view has been changing recently. An experimental study by Chen and colleagues [13] demonstrated that transgenic overexpression of the miR-17/92 cluster induces cardiomyocyte proliferation in the post-natal and adult mice

Table 3
Fully adjusted 5-year all-cause and cardiovascular mortality risk associated with selected miRNAs as continuous or categorized predictors.

	All-cause mortality				Cardiovascular mortality			
	Model A (continuous)		Model B (categorized)		Model A (continuous)		Model B (categorized)	
	HRR (95% CI)	p	HRR (95% CI)	p	HRR (95% CI)	p	HRR (95% CI)	p
Each miRNA in own model:								
miR-1	0.73 (0.58–0.93)	0.001	1.65 (1.16–2.35)	0.005	0.63 (0.48–0.84)	0.002	1.68 (1.10–2.56)	0.016
miR-19a	0.62 (0.46–0.83)	<0.0001	2.27 (1.59–3.23)	<0.0001	0.44 (0.31–0.63)	<0.0001	2.87 (1.88–4.39)	<0.0001
miR-126	0.72 (0.54–0.95)	0.002	1.64 (1.15–2.33)	0.006	0.59 (0.42–0.81)	0.001	1.80 (1.18–2.74)	0.006
miR-133a	0.72 (0.57–0.92)	0.003	1.46 (1.01–2.12)	0.046	0.67 (0.51–0.89)	0.005	1.77 (1.15–2.71)	0.010
miR-223	0.70 (0.56–0.88)	<0.0001	2.05 (1.45–2.91)	<0.0001	0.59 (0.46–0.74)	<0.0001	2.26 (1.49–3.42)	<0.0001
All five miRNAs in one model:								
miR-1	0.70 (0.40–1.24)	0.220	1.24 (0.77–2.00)	0.372	1.00 (0.53–1.87)	0.991	1.14 (0.66–1.99)	0.638
miR-19a	0.44 (0.23–0.84)	<0.0001	1.93 (1.20–3.13)	0.007	0.47 (0.24–0.91)	0.025	2.70 (1.54–4.61)	<0.0001
miR-126	1.01 (0.99–1.02)	0.095	0.76 (0.43–1.32)	0.322	1.01 (1.00–1.02)	0.091	0.64 (0.34–1.18)	0.157
miR-133a	0.96 (0.59–1.56)	0.862	0.90 (0.54–1.48)	0.673	1.02 (0.57–1.82)	0.948	1.09 (0.61–1.95)	0.773
miR-223	0.69 (0.44–1.08)	0.106	1.58 (0.91–2.76)	0.106	0.84 (0.45–1.58)	0.588	1.47 (0.78–2.76)	0.230

HRR, hazard risk ratio; CI, confidence intervals.
Cox proportional hazard model, miRNAs included either as log-transformed continuous (model A) or as categorized variable (i.e. 1st versus 2nd to 4th quartiles, model B); the following covariates were additionally included into full models: age, gender, stroke, coronary revascularization, time to interview, current smoking, body mass index ≥30 kg/m^2, systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥ 90 mmHg, LDL-cholesterol ≥2.5 mmol/L, fasting glycemia ≥7 mmol/L and/or HbA1c ≥ 48 mmol/mol, troponin I ≥ 0.04 ng/mL, brain natriuretic peptide ≥100 ng/L, treatment with statin, betablockers, angiotensin converting enzyme inhibitors/angiotensin II receptor blockers or with antidiabetics.

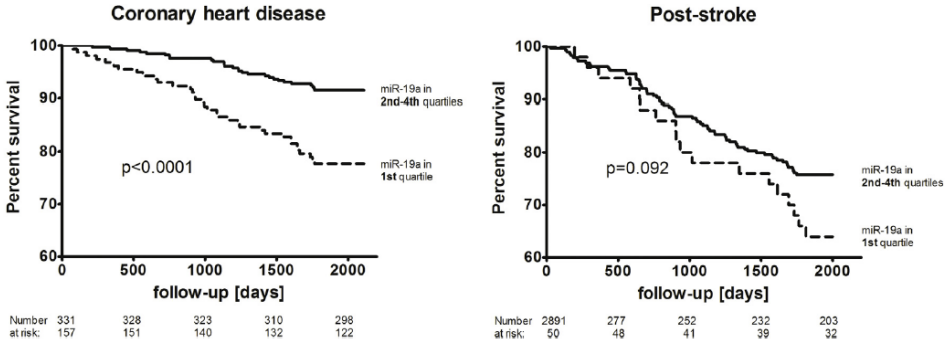

Fig. 1. Kaplan-Meier survival curves for all-cause mortality according to miR-19a expression subgroups and in coronary heart disease/post-stroke patients separately (p value by Mantel-Cox log-rank test).

hearts, implying that the miR-17/92 cluster (with miR-19a as its member) could help protect the heart from ischemic injury. Further experimental studies revealed the role of the miR-17/92 cluster as a protective factor for cardiomyocytes [12–18]. Yan and colleagues reported that miR-17/92 cluster overexpression may protect against hypoxia-induced apoptosis in a tumor tissue model [14]. Anti-apoptotic properties of miR-19a/b (notably under conditions of myocardial ischemia/reperfusion injury) have been identified and reported in other experimental studies [15,16]. Furthermore, the miR-17/92 cluster seems to be involved in cardiac aging and its increased expression has been reported to prevent cardiac senescence in a murine model [17,18]. Additionally, miR-19a has been shown to protect the murine heart from hypertension-induced cardiomyopathy [19] with miR-19a replacement therapy correcting inherited heart abnormalities (Holt-Oram syndrome) in zebrafish embryos [20].

The relative abundance of experimental studies dealing with miR-19a and its impact on the cardiovascular system is not balanced with an equivalent pool of data from human studies. In fact, with the exception of the above study by Karakas and colleagues [9], we found only one paper reporting that CAD patients compared with healthy controls showed lower expression of several miRNAs, including miR-19a [21].

Indeed, while each of the other four miRNAs examined in our present study (miR-1, miR-126, miR-133 and miR-223) were also associated with all-cause or cardiovascular mortality, their predictive power disappeared when entered into one regression model together with miR-19. A possible explanation is that miR-19 is responsible for the majority of presumptive pathophysiological mechanisms while the other four miRNAs are only bystanders. However, an interaction of low miR-1, miR126, miR-133a and miR-223 expression and mortality risk was observed in our cohort, particularly in patients with heart failure, a finding in agreement with several experimental studies (rat models). Increased miR-1 expression in the myocardium was found to be cardioprotective in chemotherapy-induced heart injury [22,23], while a protective role of miR-223 was observed in hypoxia-induced heart injury in a rat model [24]. Aberrant expression of miR-133 was accompanied with by cardiac hypertrophy and heart failure [25]. Thus, direct involvement of these four miRNAs in the pathophysiology of heart failure cannot be excluded.

A similar possible interaction in terms of increased mortality risk was noted for miR-126 and miR-133a in our subgroup of diabetes mellitus patients. In agreement with this observation, Barutta and colleagues reported that miR-126 expression was inversely associated with vascular complications of type 1 diabetes, particularly with proliferative retinopathy [26], while Chen and colleagues reported that cardiac miR-133a overexpression prevented early diabetes-induced cardiac fibrosis in a rat model of diabetes [27]. All this and our results taken together, one may speculate that miR126 and miR-133a expression play also a role in diabetes-induced chronic myocardial injury.

An alternative explanation of the observed inverse association between all five miRNAs and mortality risk can be that the expression (biosynthesis?) of miRNAs are generally (unspecific) impaired in patients with more advanced disease and, consequently, poor prognosis. On the other hand, the predictive potential of low miR-19a was observed, in our study, in CAD but not in post-stroke patients facing more than a doubled mortality risk. Hence, it is seems more likely that low expression of miRNAs reflects a specific pathophysiologic process while not being only an "innocent" general indicator of a patient's poor status.

In the present study we found also that all five miRNAs were positively correlated with LDL and glucose (HbA1c) concentration, which may seem paradoxical to inverse association of these miRNAs and mortality risk. On the other hand, despite the both, LDL and glucose metabolism are undisputable factors in CAD etiology or prognosis of patients after cardiovascular event, in our sample was not associated with increased mortality risk. The reason is probably that our subjects were more-than-less appropriately treated (with lipid-lowering drugs and antidiabetics) and our sample is not sufficiently large to discriminate mortality risk according to the relative distinct differences in conventional risk factors. This discrepancy additionally supports our hypothesis that the possible pathophysiologic role of miRNAs in this context is completely different and independent from usual atherosclerotic risk factors. We can also speculate (in line with previously stated hypothesis), that increased expression of these miRNAs, for example in diabetic patients represents compensatory and primary protective reaction.

Our study had several limitations. First, the interviews took place at least 6 months (median ≈ 1.4 years) after the qualifying cardiovascular event. This means that the most severely ill patients either died before the interview or failed to respond because of their poor functional status. This is evident from the relatively low mortality rate. Quite paradoxically, this bias supports our results in terms of clinical relevance because of the possibly higher potential of secondary prevention in stable, only moderately affected cardiovascular patients. Second, no non-fatal cardiovascular events data were available to us. Likewise, our cohort was relatively heterogeneous with regards to the type of vascular event (i.e., a mix of post-acute coronary syndrome, post-revascularization and post-stroke patients). Despite the fact that atherothrombotic origin could be expected in both of qualifying events, the observed phenomenon is far more pronounced in CAD patients.

Finally, a recent paper by de Ronde and colleagues (published in September 2018) [29] reappraised the methodology of miRNA quantification in terms of adapted calculation of expression rate. However, we were unable to re-calculate our results according to these new recommendations (as this would require the presence of calibration curves in each single assay). Nevertheless, the ΔCt method is an approach generally used and accepted in many studies. In addition, because our Ct values come from the initial phase of the amplification curve, we do

not assume that the new calculation methodology could significantly impact our final results.

5. Conclusions

The key finding of our study is that low expression of circulating miR-19a reflected a substantial additive mortality risk in stable cardiovascular patients. The exact mechanism seems to be parallel to conventional risk indicators in secondary prevention (risk factors of atherosclerosis). Assuming a role of miR-19a, it may reflect failure of the reparative capability of cardiomyocytes under conditions of post-ischemic injury. From the practical point of view, while quantification of expression of individual miRNAs is of no use in everyday clinical practice, our results at least confirmed data from experimental studies and may help stimulate future research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.05.008.

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Conflicts of interests

None.

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Příloha I

4. Slouka D, Windrichova J, Rezackova H, Houfkova K, Kucera R, Cerna V, Kostlivy T, Topolcan O, Pesta M. *The potential of miR-499 plasmatic level as a biomarker of obstructive sleep apnea syndrome*. Biomark Med. 2021 Aug;15(12):1011-1019. doi: 10.2217/bmm-2020-0826. Epub 2021 Jul 22. PMID: 34289701.

Research Article

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Biomarkers in Medicine

The potential of miR-499 plasmatic level as a biomarker of obstructive sleep apnea syndrome

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Background: Obstructive sleep apnea syndrome (OSAS) is one of the most common sleep-related breathing disorders. The aim of this study was to improve diagnostics in OSAS using blood circulating biomarkers. We consider the potential of cardiac-specific miRNAs in the diagnosis and risk assessment of cardiovascular complications. **Materials & methods:** Plasmatic levels of miR-1-3p, miR-133a-3p and miR-499a-5p were measured by reverse transcription-PCR and compared with the clinical status of OSAS patients and controls. **Results:** The level of miR-499 was higher ($p = 0.0343$) in OSAS patients (mean expression: 0.00561) compared with the controls (mean expression: 0.00003), using the multivariate logistic regression. **Conclusion:** The role of miR-499 in gene expression regulation during hypoxia and our findings indicate that miR-499 could be a new diagnostic biomarker for OSAS.

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Keywords: biomarker • cardiovascular disease • miRNA • miR1 • miR133a • miR499 • sleep apnea syndrome

Background

Obstructive sleep apnea syndrome (OSAS) is one of the most common sleep-related breathing disorders. In the adult population, it affects approximately 17% of women and 30% of men [1,2]. This type of sleep-disordered breathing has been shown to be associated with a higher incidence of hypertension [3], ischemic heart disease [4], heart failure [5], cerebrovascular [6] and metabolic disorders [7].

Repeated sleep apnea leads to a disorder of sleep architecture during which the patient's body is intermittently in hypoxia leading to oxidative stress, endothelial dysfunction, potentiation of atherosclerosis or lipid management disorders [8–10]. Negative intrathoracic pressure rises and arousal reactions are associated with the repeated activation of the sympathetic nervous system. These factors potentiate the beginning, or progression, of cardiovascular disease and heart muscle damage [11]. Obesity is cited as the strongest risk factor for OSAS [12].

The diagnosis of OSAS is made on the basis of polysomnographic examination [13] or limited polygraphy (PG) [14]. Limited PG provides smaller set of data in comparison with polysomnographic examination, but still enables a complete diagnosis of OSAS and its significant advantage is cheaper and easier management because the patients are monitored at home [15].

Common therapeutic steps recommended for all levels of sleep apnea manifestation are weight loss, body position changes in sleep, adopting a healthy lifestyle, etc. Milder forms of OSAS may be indicated for surgical treatment, severe forms are treated conservatively by applying positive pressure (PAP) to the upper respiratory tract [16–18].

The aim of the presented study was to improve and simplify diagnostics in sleep apnea patients via a more convenient approach that makes use of blood-circulating biomarkers. Despite the extensive diagnostic possibilities, OSAS remains an underdiagnosed disease in a part of the population [2]. The current diagnostic process is time

Future Medicine

Table 1. Baseline characteristics of obstructive sleep apnea syndrome patients and control cohorts.

Patient group	n	Variable	Mean	SD	Median	Lower quartile	Upper quartile	Minimum	Maximum
OSAS	194 (sex: 130 male and 64 female)	Age	60.5	11.3	62.5	53.0	69.0	27.0	88.0
		BMI	36.0	7.2	35.0	31.5	40.2	16.4	65.8
		AHI	45.5	21.3	41.6	26.9	62.4	5.0	104.9
		ODI	49.0	24.4	47.3	28.9	66.8	1.7	111.5
		Mean SaO_2	90.9	4.0	92.0	90.0	93.0	73.0	96.0
		T90	24.4	25.8	15.0	4.0	37.0	0.0	100.0
Control	50 (sex: 37 male and 13 female)	Age	61.3	8.7	60.5	55.0	67.0	41.0	81.0
		BMI	27.9	4.1	27.8	25.7	29.7	20.3	40.0

AHI – the average number of apnea–hypopnea events per hour of sleep; ODI – average number of SaO_2 dips in 1 h of sleep, mean SaO_2 (average saturation) – average oxygen saturation over the duration of sleep; T90 – percentage of sleep time in less than 90% oxygen saturation.
AHI: Apnea–hypopnea index; ODI: Oxygen distress index; OSAS: Obstructive sleep apnea syndrome; SD: Standard deviation.

consuming and requires the centralization of OSAS patients in specialized departments [13,19]. The goal was to find biomarkers that are able to detect obstructive sleep apnea syndrome and our results were published in 2019 [20] point in the direction of protein biomarkers. In the present work, we studied the relationship between myomiRs and obstructive sleep apnea syndrome in patients indicated to continous positive airway pressure (CPAP) treatment and non-OSAS probands of the same age. Three circulating miRNAs–miR-1-3p (miR-1), miR-133a-3p (miR-133a), miR-499a-5p (miR-499) – previously found to be specific for the cardimomyocytes (myomiRs) [21], were measured in plasma and their levels were compared with their clinical status in sleep apnea patients and in the control group.

Materials & methods

Patient group

Sleep apnea syndrome consecutive patients (n = 194, median age 62.5; 130 men and 64 women) with newly diagnosed moderate or severe obstructive sleep apnea syndrome (apnea–hypopnea index [AHI] ≥ 15) were monitored. At the time of study all patients were without any received treatment. Inclusion criteria: AHI ≥15, no previous OSAS treatment, complete sleep monitoring results, sleep monitoring by limited PG. Exclusion criteria: incomplete documentation of sleep monitoring, chronic obstructive pulmonary disease, previous OSAS treatment, previous upper respiratory tract surgery, patient noncompliance. Clinical parameters of sleep monitoring were defined for each patient: apnea is defined as the cessation of airflow for at least 10 s. Apnea has to be followed by desaturation ≥3% (related to the level before decrease) with arousal or desaturation ≥4% without arousal. Hypopnea is defined as a reduction of airflow ≥30% with desaturation ≥4% lasting 10 s or more or a reduction of airflow ≥50% with desaturation ≥3%. AHI – the average number of apnea–hypopnea events per hour of sleep, Oxygen Distress Index (ODI) – average number of SaO_2 dips in 1 h of sleep, mean SaO_2 (average saturation) – average oxygen saturation over the duration of sleep, T90 – percentage of sleep time in less than 90% oxygen saturation.

The OSAS group was compared with a control cohort of 50 patients (median age 60.5; 33 men, 13 women) with no history of sleep apnea syndrome and with outcome of low risk of sleep apnea syndrome in Berlin Questionnaire. The control cohort consist of patients who underwent minor noncardiovascular surgery. The blood samples and clinical data were collected before intervention. The age of the participants in the two groups did not differ significantly. The occurrence of common cardiovascular complications was described for patients from both groups, OSAS and control, including hypertension (occurrence in apnea group: n = 122, i.e., 62.9%; in control group n = 20, i.e., 40%), heart attack (apnea n = 4, i.e., 2.1%, control: n = 0); ischemic heart disease (apnea n = 14, i.e., 7.2%; control n = 0) stroke (apnea n = 8, i.e., 4.1%, control n = 1, i.e., 2%); cardiac arrhythmia (apnea n = 13, i.e., 6.7%, control n = 0). For detailed group characteristics, see Table 1.

Blood sampling

The peripheral blood samples (4 ml) were taken routinely by venipuncture from the cubital vein using K_3EDTA Vacutainer tubes (Greiner Bio-One, Kremsmünster, Austria). Plasma was separated after centrifugation at 1700× *g* (relative centrifugal force) for 10 min. Plasma samples were stored frozen at -80°C up to analysis.

Table 2. Characterization of the studied miRNAs.

miRNA	miRBase accession number	RNA sequence of mature miRNAs	Assay ID	Catalog number†
hsa-miR-1-3p	MI0000651	UGGAAUGUAAAGAAGUAUGUAU	002222	4427975
hsa-miR-133a-3p	MI0000450	UUUGGUCCCCUUCAACCAGCUG	002246	
hsa-miR-499a-5p	MI0003183	UUAAGACUUGCAGUGAUGUUU	001352	
Spike cel-miR-39-3p	MI0000010	UCACCGGGUGUAAAUCAGCUUG	000200	

†TaqMan miRNA assays available from Thermo Fisher Scientific (CA, USA).

Measurement of miRNAs plasma levels

The plasma levels of the selected three candidates for cardiac-specific miRNAs, miR-1, miR-133a and miR-499 (see Table 2 for specification), were quantified using a reverse transcription real-time polymerase chain reaction (real-time RT-PCR). Total RNA (including miRNAs fraction) was extracted manually using miRNeasy® Serum/Plasma Kit (Qiagen, Hilden, Germany) from 200 µl of blood plasma with an additional 3.5 µl of a 1.6 $\times 10^8$ copies/µl working solution of cel-miR-39 (Qiagen). Exogenous cel-miR-39 was used as a spike-in control to adjust the extraction efficiency.

A quantitative estimation of selected miRNAs was performed by the real-time RT-PCR assays only target mature miRNAs, not their precursors. The analysis consisted of a cDNA template preparation by RT reaction using TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, CA, USA) with TaqMan miRNA Assays (Thermo Fisher Scientific), followed by cDNA quantification by PCR using TaqMan miRNA Assays (Thermo Fisher Scientific) in technical duplicates on a LightCycler® 96 System (Roche, Basel, Switzerland). The PCR thermal profile followed manufacturer protocol. Inter-run calibrators were used to avoid plate-to-plate variation [22]. The deltaCt approach was used to calculate the plasma levels of the miRNAs of interest. The results are presented as relative values of expression calculated as 2 – (Ct of miRNA of interest – Ct of normalizer). Cel-miR-39, an exogenous reference, was used as a normalizer [23–25].

Statistics

The groups' characteristics were described. An ROC analysis was performed and ROC curve was plotted in order to assess the differences in levels of studied miRNAs between the OSAS group and the control group. A multivariate logistic regression model – stepwise selection with all three studied miRNAs and the BMI was applied to describe the relation of studied miRNAs and OSAS diagnosis. Further univariate logistic regression models adjusted for BMI were used to evaluate the relationship between miRNAs levels, diagnosis and clinical parameters. The Spearman rank correlation was used among markers and the following clinical parameters: AHI, ODI, mean SaO_2, T90 and age.

Results

We have found BMI in apneic patients (OSAS group – BMI mean 36.0 ± 7.2, median 35.0) to be significantly ($p < 0.0001$) higher than in the control group (BMI mean 27.9 ± 4.1, median 27.8). Moreover, 82.3% of apneic patients (OSAS group) had BMI 30 or higher, contrary to 24.4% of the patients in the control group ($p < 0.0001$). The statistical methods were selected based on these findings.

The multivariate logistic regression model stepwise selection shows that of the studied miRNAs, only miR-499 contributes to the distinction between OSAS and the control group ($p = 0.0343$), with higher levels of expression in OSAS group (miR-499 level OSAS group mean: 0.00561; control group: 0.00003). The OSAS group exhibited 44% miR-499 expression positivity, whereas the control group exhibited a more 2%. See Table 3 for the levels of all the studied miRs in individual groups and Figure 1 for miR-499. The univariate logistic regression model adjusted for BMI confirms this fact with the same p-value ($p = 0.0343$).

The ROC analysis provides values of area under the curves (AUCs) for miR-499 as: AUC = 0.7105 (95% CI: 0.6714–0.7495) (see Figure 2), miR-133a: AUC = 0.5533 (95% CI: 0.4676–0.6390) and miR-1: AUC = 0.5982 (95% CI: 0.5075–0.6889). At the 98% specificity level, the sensitivity of 40% (95% CI: 33–47%) was observed with cut-off expression value >0.0002 or the sensitivity of 44% (95% CI: 37–51%) with cut-off expression value >0.

A correlation among the three miRNAs was observed within the apneic group (see Table 4). Furthermore,

Table 3. Plasma expression levels of the studied miRNAs in obstructive sleep apnea syndrome and control group.

Patient group	n	Variable	Mean	SD
OSAS	194	miR-1	0.00148	0.00310
		miR-133a	0.06585	0.14149
		miR-499	0.00561	0.01737
Control	50	miR-1	0.00247	0.00490
		miR-133a	0.03016	0.04820
		miR-499	0.00003	0.00021

OSAS: Obstructive sleep apnea syndrome; SD: Standard deviation.

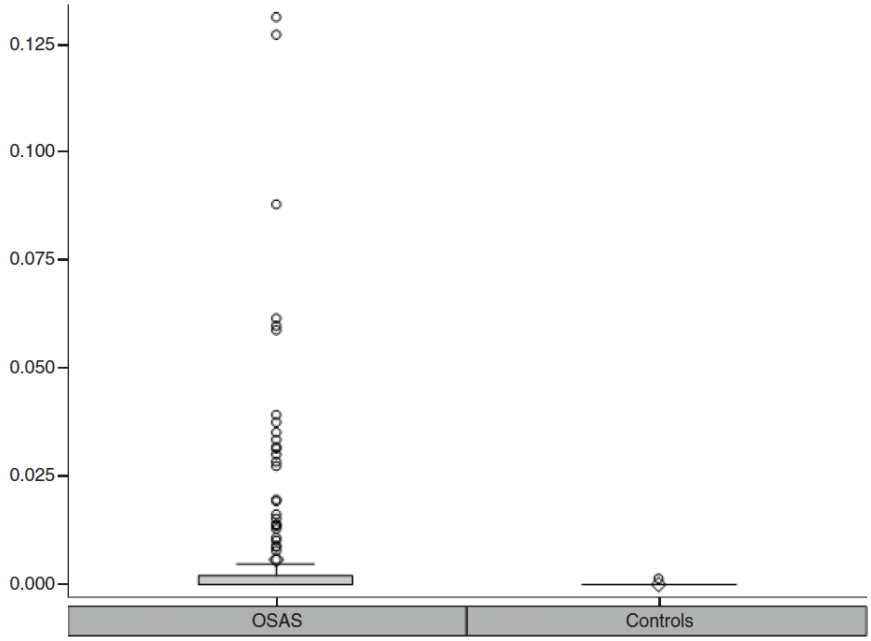

Figure 1. Distribution of miR-499 in group of obstructive sleep apnea syndrome patients and control group.

Table 4. Spearman rank correlation - correlation coefficients of miRNA expression level and hypertension within obstructive sleep apnea syndrome group.

miRNAs	Hypertension (Spearman correlation coefficients/p-value)	miR-1 (Spearman correlation coefficients/p-value)	miR-133a (Spearman correlation coefficients/p-value)	miR-499 (Spearman correlation coefficients/p-value)
miR-1	-0.08140, p = 0.2592		0.57201†, p < .0001	0.25199†, p = 0.0004
miR-133a	-0.18481†, p = 0.0099	0.57201†, p < .0001		0.34127†, p < .0001
miR-499	-0.05367, p = 0.4573	0.25199†, p = 0.0004	0.34127†, p < .0001	

†Significant correlation.

miR-133a correlates with hypertension (see Table 4), but this relation was not proven in logistic regression with adjustment to BMI. Within the control group, a correlation was shown only between the levels of miR-133a and miR-1 (Spearman correlation factor: 0.62403, <0.0001). No relationship was found between the clinical severity parameters of OSAS (AHI, ODI, mean-SaO_2, T90) and levels of either miR-1, miR-133a or miR-499. No correlation was found between age or BMI and miRNA expression levels either.

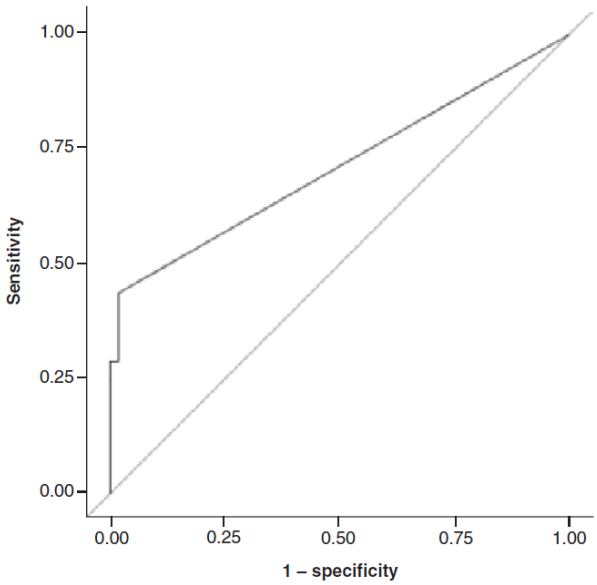

Figure 2. ROC curve for miR-499. Area under the curve is 0.7105.

Discussion

The goal of the study was to find easily determinable biomarkers, which could be used to identify patients with OSAS in a group of suspect patients and which could complement the procedures traditionally used in the diagnostics of OSAS. If the biomarkers will have been shown as beneficial, they could even replace polysomnographic examination and limited polygraphy. We have decided to search for these biomarkers in a class of a small regulatory noncoding RNA: miRNA. miRNA is easily measurable in periphery blood derivate (plasma) in low levels. In this article, we have focused on a cardiac-specific group of miRNAs: miR-1, miR-133a and miR-499. The decision to focus on these specific miRNAs was based on the hypothesis that these molecules may be released into the blood circulation from cardiomyocytes damaged by short-term hypoxia episodes during OSAS. Simultaneously, a strong link between obstructive sleep apnea and cardiovascular diseases was described [26].

In our study, the plasmatic levels of miR-499 distinguish between the OSAS and control group ($p = 0.0343$), with higher levels of expression in the OSAS group and AUC = 0.7105. The miR-499 molecule is an intronic miRNA encoded by the myosin gene family member: *Myh7b*. It is constitutively expressed at high levels in the heart myocardium. Jointly with miR-208a and miR-208b, it is assigned to a group of myoMiRs. miR-499 is differentially regulated and plays a role in heart development; mainly in controlling the maturation of cardiac progenitor cells [27]. The expression of miRNA-499 and some cardiac enriched transcription factors are linked by a positive feedback loop [28].

We assume that a significant pathophysiological link exists between higher levels of miR-499 and apnea and suspect a strong connection between oxidative stress and the role of miR-499. The culprit in OSAS pathophysiology is intermittent hypoxia, which causes high levels of oxidative stress via mitochondrial dysfunction, NADPH oxidase or xanthine oxidase, resulting in higher levels of superoxide anion radical, which, in turn, further interacts with biomolecules and the antioxidant defense system. Obesity is very often linked with OSAS and is another source of oxidative stress. Superoxide dismutase transforms further superoxide into a reactive oxygen species – hydrogen peroxide [29]. MiR-499 was shown to protect cardiomyocytes from hydrogen peroxide-induced apoptosis and so has a protective role in oxidative injury of cardiomyocytes during heart infarction via its suppressive effect on Pdcd4 and Pacs2 expression in rats. Vice versa, higher hydrogen peroxide levels upregulated the expression of miR-499 by phosphorylating the c-Jun regulator of the *Myh7b* gene promoter [30]. The effect of oxygen radicals in cardiac injury causes cellular membrane disruption and the irreversible alteration of cardiac integrity [31], which could be followed by a release of cytoplasmatic miRNAs. Also Yingqing Li and his team found that MiR-499-5p protects cardiomyocytes against ischemic injury via anti-apoptosis by targeting *Pdcd4* [32]. Yujie Shi *et al.* further

complement that miR-499 plays a role in the process of cardiomyocytes injury induced by hypoxia/reoxygenation. miR-499-5p-inhibited hypoxia/reoxygenation-induced cardiomyocytes injury by targeting *SOX6*. MiR-499-5p could upregulate the level of Bcl-2 and downregulate the expression levels of Bax and caspase-3. However, *SOX6* partially reversed these effects of miR-499-5p [33].

There could also be another pathophysiological link: the higher levels of miR-499 in apnea patients could be a result of the reactivation of the fetal miR program in cardiovascular lesions caused by apnea. Thuma *et al.* describe this phenomenon as occurring after cardiac transcriptome analyses that reveals the expression analogy between fetal and failing human heart tissue [34]. Our study has, however, failed to demonstrate a correlation between miR-499 levels and the cardiovascular conditions of patients.

Though a Santamaria-Martos *et al.* found 14 OSAS biomarker candidates, chosen from 188 studied miRNAs in their study. After validation and adjustment to BMI and age, miR181-a, miR-199b, miR-345, miR-133a, miR-340 and miR-486-3p were confirmed to be differentially expressed in non-OSAS and OSAS patients. Furthermore, a combination of NoSAS score and miRNAs was proposed to be the best model for discriminating the OSAS patients with an AUC above 0.8 [35].

The expression levels of miR-485-5p, miR-107 and miR-199-3p were described by Li *et al.* to be downregulated and miR-574-5p was upregulated in the OSAS patients compared with controls [36]. These miRNAs are known to be regulators in hypoxia via HIF-1 or in metabolism.

Li Kun *et al.* have shown serum levels of miR-664a-3p to be downregulated in OSAS patients compared with a control group and demonstrated that its expression level was correlated with AHI, lowest oxygen saturation and carotid intima media thickness in OSAS patients [37].

The potential role of circulating higher plasmatic levels of miR-499 as a diagnostic in acute myocardial infarction patients were proposed by several studies [28,38–43]. The diagnostic value seems to be universal in terms of the myocardial infarction type concerned: ST elevation myocardial infarction patients [41], or geriatric non-ST elevation myocardial infarction patients [43]. The potential use of miR-499 as a diagnostic marker in OSAS patients is not in conflict with its elevation in acute myocardial infarction disease where miR-499 is increased only during the acute stage.

On the other hand, lower levels of miR-499 are potential clinical biomarkers for noncardiovascular conditions – for example, an independent prognostic biomarker in non-small-cell lung cancer patients [44] or distinction of depression episodes in bipolar patients [45].

In our study, higher expression of miR-499 seems to be OSAS marker with high specificity but with lower sensitivity. Anyway this could be precisely judged on further studies with higher numbers of participants and different (various) control groups. No relationship was found between the clinical severity parameters of OSAS and cardiac miRNAs levels. Although the correlation between miRNAs and cardiac disease history was explored in our study, and a correlation was indeed found between miR-133a and hypertension, it was nevertheless not proven using logistic regression adjusted to BMI and is, therefore, not discussed further.

Study limitations

Although it is true that the patients and control groups used for the comparison of novel biomarker levels should be comparable in their elementary parameters, in other words, age, sex, BMI, the study of OSAS biomarkers inevitably involves a group of OSAS patients that exhibits higher levels of severe obesity due to the disease's pathophysiology. It is, therefore, very difficult to obtain a comparable control group. In our study, apneic patients (OSAS group) are age and sex matched to controls but have a higher BMI. The statistical methods used were selected in order to overcome this discrepancy. This is a challenge that applies to OSAS studies in general and has received mention from other authors, for example, Santamaria-Martos *et al.* [35]. Sleep apnea syndrome in the control group was eliminated using the Berlin Questionnaire (published sensitivity is 58.8–76% and specificity 40.5–74%). Another limit of our work can be the focusing of the study on the patients indicated to the CPAP treatment (OSAS, AHI $\geq$ 15).

Conclusion

In our analysis we have shown that the plasma level of miR-499 is significantly higher in obstructive sleep apnea syndrome patients in comparison with the control group. Based on the role of miR-499 in gene expression regulation during hypoxia and our findings, we propose that miR-499 could be a new diagnostic biomarker for obstructive sleep apnea syndrome.

Future perspective

We have focused on cardiac-specific miRNAs as potential biomarkers in OSAS. Future research would benefit from focusing on other pathophysiological processes linked to hypoxia that can potentially affect the miRNA gene expression regulation and the release of miRNAs to bloody fluids. The clinical usefulness of miRNA-499 as a diagnostic biomarker for OSAS has to be proven in subsequent studies with an emphasis on proband requirements in the control group and taking in to account the higher BMI of OSAS patients.

Summary points

What is already known about this subject?

- miRNAs could be potential biomarkers in apnea diagnostics [35].
- miR-499, miR-133, miR-1 are described as cardiac-specific miRNAs.
- miR-499 play a role in the cause of hypoxia.

What is this study's contribution to the existing body of knowledge?

- This analysis is the first to demonstrate that the levels of miR-499 are significantly higher in obstructive sleep apnea syndrome patients in comparison to a control group.
- Levels of miR-499, miR-1 and miR-133a were not shown to be connected to the clinical severity parameters of obstructive sleep apnea syndrome.

Author contributions

The conceptualization is done by D Slouka, M Pesta and T Kostlivy. R Kucera, M Pesta and J Windrichova worked on the methodology. D Slouka, J Windrichova, H Rezackova, K Houfkova and T Kostlivy done the investigation. D Slouka, J Windrichova, M Pesta and H Rezackova worked on the writing – original draft preparation. O Topolcan, V Cerna and R Kucera worked on the writing – review and editing.

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Ethical conduct of research

The study was carried out with the approval no.: 130708 of the Ethics Committee, University Hospital and Faculty of Medicine, Charles University, Pilsen. Informed consent has been obtained from the participants involved.

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Letter to the editor

miR-133a AND myocardial ischemia/reperfusion injury. Response to letter by Wang

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Dear Editor.

We are grateful for the interest in our article [1]. Dr. Wang mentioned the fact than miR-133a decrease was repeatedly found to be associated with acute myocardial ischemia or reperfusion injury [2,3]. In spite that we found [1] an inverse relationship between miR-133a and mortality risk in our study, this association disappeared after complex adjustment (only miR-19a remained independent mortality predictor). However, this finding is not contradictory to studies dealing with miR-133a and ischemia/reperfusion injury [2,3], to our opinion. Our study was conducted in stable chronic patients (≈1 year after cardiovascular event) where the biological role of the miR-133a may be different.

Regarding the expression of miR-133a during acute ischemia/reperfusion injury, we have own confirmatory results. In a pilot prospective study (not yet published) we followed 30 patients immediately after surgery in extracorporeal circulation (elective bypass or valve replacement). Relative expressions of miR-133a steeply decreased in first 24 h, but day 10 were roughly equal to baseline (pre-surgery) values. Troponin T concentrations showed inverse trend. [mean(SD) relative expression of miR-133a was before surgery 8.97 (12.15), 1 h after 5.95 (4.70), 3 h 7.42 (5.56), 9 h 6.31 (5.20), 24 h 4.11 (2.81) and after 10 days 8.05 (8.36), while troponin T concentrations at the same time points 16 (8), 506 (379), 973 (644), 870 (643), 610 (437) and 20 (9), respectively].

Therefore, we agree that miR-133a seems to be a promising marker of myocardial injury in acute phase and further research in this field is warranted.

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Declaration of competing interest

None.

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Letter to the Editor

Response to letter by Yu and Chen☆

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Dear editor,

We thank Dr. Yu and Dr. Chen for their interesting comments demonstrating alternative pathophysiological explanation of our recent observation [1]. We agree that observed association between mortality risk and miR-126 can be connected via HOTAIR (lncRNA HOX antisense intergenic RNA) expression accompanying myocardial ischemia or reperfusion [2,3]. This is indirectly supported by the fact, that we observed significant association between miR-126 and 5-years mortality risk in post-myocardial infarction patients [with HRR 2.12 (95%CI: 1.24–3.63)], but not anymore in post-stroke patients [HRR 1.19 (95% CI: 0.69–2.04)] (i.e. subjects with much higher mortality risk, but in several cases mediated by different cardiovascular pathophysiologic mechanism). In spite that predictive power of miR-126 disappeared in our study, if added into one regression model along with miR-19a, coincidence of two different mechanisms (one related with mi-126 and the second with miR19a) cannot be fully excluded.

Another problem may be that Zampetaki and colleagues [2] reported positive association between miR-126 and incident myocardial infarction, while we observed inverse relation to mortality risk. However this discrepancy can be explained by completely different setting of our sample. Our study was intentionally realized in well-stable subjects (≈1 year after their vascular attack), while both above mentioned studies [2,3] in very acute phase. Increased expression of HOTAIR/miR-126 axis in ischemia/reperfusion can be physiologically primary the protective response to acute myocardial injury, while decrease in chronic phase failure of this mechanism. In any case, this hypothesis has yet to be confirmed by another study ("2nd cohort").

Declaration of competing interest

None.

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