International Journal of Cardiology 289 (2019) 101–106

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

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

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ARTICLE INFO

Article history:
Received 28 June 2018
Received in revised form 8 March 2019
Accepted 2 May 2019
Available online 6 May 2019

Keywords:
Micro-ribonucleic acids (miRNAs)
Atherosclerotic disease
Secondary prevention
Risk prediction
EUROASPIRE

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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https://doi.org/10.1016/j.ijcard.2019.05.008
0167-5273/

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* ≥ 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.

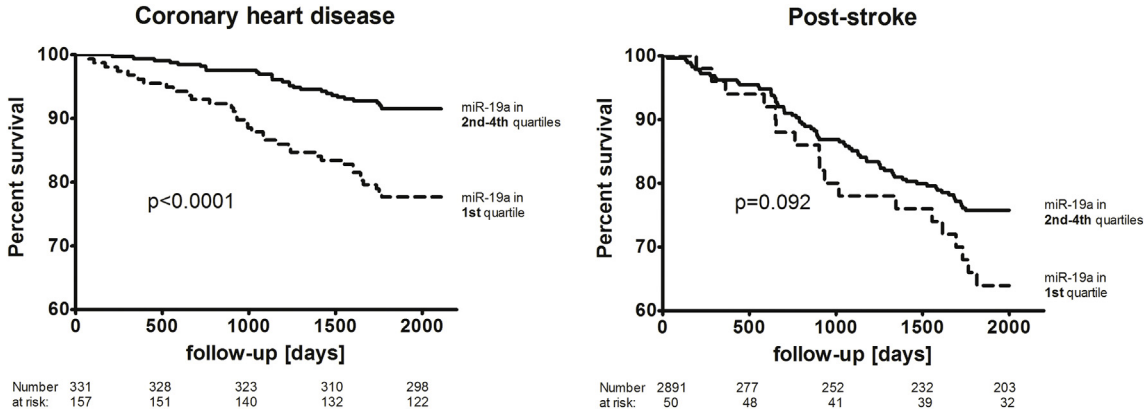

Fig. 1. Kaplan-Meier survival curves for all-cause mortality according to miR-19a expression subgroups and in coronary heart disease/post-stroke patients separately (p value by Mantel-Cox log-rank test).

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.

Sponsorship

The present study was supported by Internal Grant Agency of Ministry of Health, (project 17-29520A), Charles University Research Fund (PROGRESS, project Q39].

Conflicts of interests

None.

Funding and acknowledgment

The present analysis was supported by the Charles University Research Fund (PROGRES, project Q39), Health Development Agency of the Czech Ministry of Health [project 17-29520A] and Specific Academic Research Project of Charles University [project SVV 260 393], Czechia.

We would like to acknowledge the hard work of all investigators, study nurses, laboratory technicians and other study personnel participating in the EUROASPIRE III survey in the Czech Republic and this subanalysis in particular. We are also grateful to Mr. Rene Prahl for proofreading our manuscript.

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