ORIGINAL RESEARCH

MicroRNA Expression in Circulating Microvesicles Predicts Cardiovascular Events in Patients With Coronary Artery Disease

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***Background*—**Circulating microRNAs (miRNAs) are differentially regulated and selectively packaged in microvesicles (MVs). We evaluated whether circulating vascular and endothelial miRNAs in patients with stable coronary artery disease have prognostic value for the occurrence of cardiovascular (CV) events.

***Methods and Results*—**Ten miRNAs involved in the regulation of vascular performance—miR-126, miR-222, miR-let7d, miR-21, miR-20a, miR-27a, miR-92a, miR-17, miR-130, and miR-199a—were quantified in plasma and circulating MVs by reverse transcription polymerase chain reaction in 181 patients with stable coronary artery disease. The median duration of follow-up for major adverse CV event–free survival was 6.1 years (range: 6.0–6.4 years). Events occurred in 55 patients (31.3%). There was no significant association between CV events and plasma level of the selected miRNAs. In contrast, increased expression of miR-126 and miR-199a in circulating MVs was significantly associated with a lower major adverse CV event rate. In univariate analysis, above-median levels of miR-126 in circulating MVs were predictors of major adverse CV event–free survival (hazard ratio: 0.485 [95% CI: 0.278 to 0.846]; P=0.007) and percutaneous coronary interventions (hazard ratio: 0.458 [95% CI: 0.222 to 0.945]; P=0.03). Likewise, an increased level of miR-199a in circulating MVs was associated with a reduced risk of major adverse CV events (hazard ratio: 0.518 [95% CI: 0.299 to 0.898]; P=0.01) and revascularization (hazard ratio: 0.439 [95% CI: 0.232 to 0.832]; P=0.01) in univariate analysis. miRNA expression analysis in plasma compartments revealed that miR-126 and miR-199a are present mainly in circulating MVs. MV-sorting experiments showed that endothelial cells and platelets were found to be the major cell sources of MVs containing miR-126 and miR-199a, respectively.

***Conclusion*—**MVs containing miR-126 and miR-199a but not freely circulating miRNA expression predict the occurrence of CV events in patients with stable coronary artery disease. **(*J Am Heart Assoc*. 2014;3:e001249 doi: 10.1161/JAHA.114.001249)**

Key Words: coronary artery disease • microRNA • microvesicles • prognosis

MicroRNAs (miRNAs) are small (22-nucleotide) noncoding RNAs regulating gene expression at the post-transcriptional level by binding to the target mRNA, leading either to mRNA degradation or to translational repression.[1]

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MiRNAs have emerged as key regulators of several physiological and pathophysiological processes in cardiovascular (CV) health and disease.[2,3] Besides their intracellular function, recent studies demonstrate that miRNAs can be exported or released by cells and circulate in the blood in a remarkably stable form.[4]

The discovery of circulating miRNAs opens up fascinating possibilities to use circulating miRNA patterns as biomarkers for CV diseases (CVDs).[5,6] Altered levels of circulating miRNAs have been reported in patients with heart failure, coronary artery disease (CAD), and diabetes.[7–9] Evidence is accumulating that microvesicles (MVs) represent major protective transport vehicles for miRNAs by separating them from circulating ribonuclease (RNase).[10] Moreover, recent studies suggest that MV-associated miRNAs not only represent passively released cellular debris but also may contribute to intercellular signaling mechanisms.[11,12] In this context, MVs containing secreted monocytic miR-150, which are

increased in the plasma of patients with atherosclerosis, were shown to be taken up by recipient endothelial cells and to regulate cell migration and expression of the target gene c-Myb.[13] Furthermore, we and others have demonstrated that endothelial cell-derived MVs mediate vascular protection and endothelial regeneration in a miR-126–dependent mechanism.[14,15] These findings suggest that circulating MV-packaged miRNAs, in addition to their function as biomarkers, represent a functional mediator in CVD.

Previously, circulating vascular and endothelial cell–derived miRNAs were shown to be significantly regulated in patients with CAD compared with healthy subjects.[8] Whether circulating miRNAs can also be used as a prognostic biomarker is largely unknown. Because MVs represent major transport vehicles for circulating miRNAs, we aimed to assess whether the expression of 10 vascular and endothelial cell–derived miRNAs involved in vascular biology in the plasma or in isolated MVs is associated with CV outcomes in patients with stable CAD.

Methods

Study Subjects

Between March and November 2003, 200 patients undergoing coronary angiography were screened for stable CAD for inclusion in this study. Nineteen patients with clinical presentation of acute or subacute myocardial infarction (MI) were excluded from the study. Patients with malignant, inflammatory diseases or severe hepatic or renal dysfunction were also excluded from the study. Informed consent was obtained from all patients, and the ethics committee of the University of Saarland (Saarbrücken, Germany) approved the study protocol.

Patients in the second study cohort were included between August 2012 and July 2013. Sixty patients undergoing coronary angiography were screened for stable CAD for inclusion. Six patients with clinical presentation of acute or subacute MI were excluded from the study. Patients with malignant, inflammatory diseases or severe hepatic or renal dysfunction were also excluded from the study. Informed consent was obtained from all patients, and the ethics committee of the University of Bonn (Bonn, Germany) approved the study protocol.

Coronary Angiography

Cardiac catheterization was performed according to the guidelines for coronary angiography of the American College of Cardiology and the American Heart Association. The extent of CAD was scored by at least 2 independent interventional cardiologists. Angiographic CAD was defined as stenosis of 50% in at least 1 major epicardial coronary artery. Biplane ventriculography was performed in standard projections. The ejection fraction was calculated by dividing the end-diastolic and end-systolic left ventricular areas with an automated computer system (digital cardiac imaging software; Philips).

Preparation of Blood Samples

Arterial blood was drawn under sterile conditions from the femoral artery before cardiac catheterization and was buffered using sodium citrate. Additional blood samples for routine analyses were obtained. Blood was drawn prior to heparin application, so there was no confounding effect of heparin on miRNA analysis.

Blood was centrifuged at 1500*g* for 15 minutes followed by centrifugation at 13 000*g* for 2 minutes to generate platelet-deficient plasma. The deprived plasma samples were immediately stored at −80°C. Annexin V- and CD31-positive microparticle levels were freshly measured with flow cytometry by using annexin V-FITC and CD31-PE (BD Pharmingen).

Platelet-deficient plasma was stored at −80°C until miRNA levels were analyzed. Previous studies have shown that miRNAs in frozen plasma remain stable for years and are reliable biomarkers of CVD.[16,17]

Separation of Plasma Compartments and RNA Isolation

RNA was isolated from plasma, circulating MVs, exosomes, and vesicle-free supernatant (ie, the remaining supernatant after exosome isolation) by using a TRIzol-based miRNA isolation protocol. For each patient, 250 μL total plasma was diluted in 750 μL TRIzol LS (Life Technologies) to measure plasma miRNA levels. An additional 250 μL total plasma was centrifuged at 20 000*g* for 30 minutes at 4°C to pellet circulating MVs.[18] The pellet was diluted in 250 μL RNase-free water and then diluted in 750 μL TRIzol LS to measure MV miRNA levels. *Caenorhabditis elegans* miR-39 (cel-miR-39; 5 nmol/L; Qiagen) was spiked in TRIzol for normalization of miRNA content, as described previously.[8] To increase the yield of small RNAs, the RNA was precipitated in ethanol at −20°C overnight with glycogen (Invitrogen).

To analyze miRNA levels in exosomes and vesicle-free supernatant, the remaining supernatant after 20 000*g* centrifugation was collected and centrifuged at 100 000*g* for 1 hour at 4°C to pellet exosomes.[19] The pellet was diluted in 250 μL RNase-free water and then diluted in 750 μL TRIzol LS to measure exosome miRNA levels. The remaining supernatant after exosome isolation, defined as “vesicle-free supernatant,” was diluted in 750 μL TRIzol LS and processed, as described.

Sorting of Microvesicle Subspecies

For sorting of MV subspecies, 250 μL platelet-free plasma was stained with CD31-PE, CD42b-APC, and annexin V-FITC

(BD Pharmingen) and the corresponding isotype and negative controls. Stained plasma was incubated for 45 minutes in the dark at room temperature according to the manufacturer's suggestions.

To sort MV subspecies, a FACSAria III flow cytometer (BD Biosciences) was used. Annexin V–positive vesicles between 100 and 1000 nm in diameter were gated for sorting. CD31+/CD42b−, CD31+/CD42b+ and CD31−/CD42b− MV were gated, sorted, and collected. RNase-free water was added to the sorted MVs to reach a total volume of 250 μL, which was diluted in 750 μL TRIzol LS to measure MV miRNA levels. Cel-miR-39 (5 nmol/L; Qiagen) was spiked in TRIzol for normalization of miRNA content, as described previously.[8] To increase the yield of small RNAs, the RNA was precipitated in ethanol at −20°C overnight with glycogen (Invitrogen).

Quantification of MicroRNAs by Quantitative Polymerase Chain Reaction

RNA was quantified using NanoDrop spectrophotometer, and 10 ng of the total RNA was reversely transcribed using a TaqMan miRNA reverse transcription kit (Applied Biosystems), according to the manufacturer's protocol. MiR-126, miR-222, miR-let7d, miR-21, miR-20a, miR-27a, miR-92a, miR-17, miR-130, and miR-199a in plasma and MVs were detected by using Taqman miRNA assays (Applied Biosystems) on a 7500 HT real-time polymerase chain reaction machine (Applied Biosystems). Cel-miR-39 was used as an endogenous control. For all miRNAs, a Ct value >40 was defined as undetectable. The ΔCt method was used to quantify relative miRNA expression. Values were normalized to cel-miR-39 and are expressed as $2^{-[Ct(miRNA)-Ct(cel\text{-}miR\text{-}39)]}$.

Previous Events, Follow-up, and Causes of Death

The classification of events and follow-up data was made on the basis of medical records and personal interviews. The occurrence of a first major adverse CV event (MACE), including nonfatal MI, revascularization by percutaneous coronary intervention, or coronary artery bypass graft, and death from cardiac causes were evaluated after 6 years. Causes of death were determined by examination of hospital records, autopsy reports, and medical files of the patients' general practitioners. Deaths from CV causes included sudden deaths and deaths from acute MI, CAD, or congestive heart failure.

Statistical Analysis

All miRNAs were dichotomized into 2 categories with categorical analysis (lower than median and higher than median). Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test. Normally distributed continuous variables were presented as mean±SD. Variables with skewed distribution were transformed to their natural logarithm for the regression analysis. The Mann–Whitney *U* test was used to analyze variables with skewed distribution. Means of 2 categories were compared with the 2-tailed, unpaired Student *t* test. The chi-square test was used for categorical data that result from classifying objects. Fisher's exact test was used when any of the cells of a contingency table are <5 or are <10 when there is only 1 degree of freedom. Binary logistic regression was applied to identify factors that were associated with miR-126 and miR-199a. All models were adjusted for age, sex, body-mass index, diabetes, hypertension, hyperlipoproteinemia, chronic kidney disease, and concomitant use of angiotensin-converting enzyme inhibitors and statins. Survival analysis was determined by the Kaplan–Meier method. The log-rank test was used to determine statistical differences in terms of survival.

Statistical significance was assumed when the null hypothesis could be rejected at $P<0.05$. Statistical analysis was performed with IBM SPSS Statistics version 20.

Results

Baseline Characteristics

A total of 181 patients with clinically and angiographically stable CAD on coronary angiography were enrolled and followed up for 6 years.

From the initially included 181 patients, 5 patients (2.5%) were lost to follow-up. The mean (±SD) age of the remaining 176 patients was 66.7±10.2 years, with high prevalence of CV risk factors: hypertension (84.1%), hyperlipoproteinemia (82.4%), diabetes (28.4%), and smoking (19.3%). During the 6-year follow-up period (range: 6.0–6.4 years), 27 patients (15.3%) died; 13 (7.4%) died from CV causes, and other causes included sepsis (6 of 27), pneumonia (3 of 27), and cancer (5 of 27). A first MACE occurred in 55 patients (31.3%).

MicroRNA Selection and Detection in Plasma and Microvesicles

Ten vascular and endothelial cell–expressed miRNAs that have been shown to be regulated in patients with CAD[8] and involved in vascular biology were chosen for analysis of their prognostic relevance in patients with CAD: miR-126,[14,20–22] miR-222,[23] miR-let7d,[24] miR-21,[25] miR-20a,[26] miR-27a,[27] miR-92a,[28] miR-17,[29] miR-130,[30] and miR-199a.[31]

Because previous studies strongly suggest that circulating miRNAs are selectively packaged in MVs, levels of analyzed miRNAs were measured in plasma and in circulating MVs in all patients. Overall, miR-126, miR-222, miR-let7d, miR-21, miR-20a, miR-27a, miR-92a, miR-17, miR-130, and miR-199a in

plasma were below the limit of detection in 1.1%, 1.1%, 2.3%, 1.1%, 1.7%, 0, 0.5%, 50%, 13%, and 7.9% of the patients, respectively; in MVs, the corresponding miRNAs were below the limit of detection in 3.5%, 3.5%, 4.7%, 0.5%, 4.1%, 1.1%, 0.5%, 36.4%, 2.3%, and 4.1% of the patients, respectively.

Circulating Microvesicle Characterization

To characterize isolated MVs according to our protocol, size analysis using electron microscopy and flow cytometry revealed that the vast majority had a size between 0.1 and 1 μm (Figure 1A and 1B).

MicroRNA Level and Cardiovascular Events

To analyze the correlation of circulating miRNAs and CV events, all measured miRNAs in plasma and circulating MVs were dichotomized into 2 categories with categorical analysis (lower than median and higher than median) and associated with the CV outcome after a follow-up period of 6.1 years. There was no significant association between CV events and plasma levels of the 10 analyzed miRNAs (Table 1). In contrast, the expression of 2 miRNAs in circulating MVs was

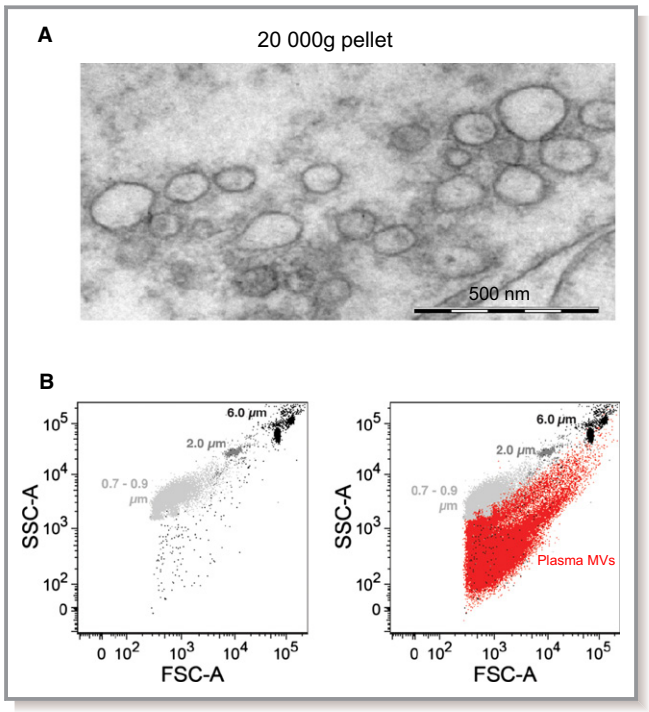

Figure 1. Circulating MVs characterization. Circulating MVs were collected by 20 000*g* centrifugation of platelet-deficient plasma. A, MVs were fixed and identified by electron microscopy. Magnification: ×46 000. B, Analyzed MVs (red) had a size <1 μm, demonstrated using fluorescent polystyrene particles with a defined size of 0.7 to 0.9 μm (light gray), 2 μm (dark gray), and 6 μm (black). FSC indicates forwad-scattered light; MV, microvesicles; SSC, side-scattered light.

Table 1. Association of MicroRNAs in Plasma and Circulating Microvesicles Categorized According to Median With Clinical Outcomes

	P Value (Chi-Square Test)	
	Cardiovascular Mortality	MACE
PmiR-126	0.150	0.255
PmiR-222	0.364	0.555
PmiR-21	0.364	0.699
PmiR-20a	0.364	0.477
PmiR-27a	0.364	0.555
PmiR-92a	0.364	0.699
PmiR-130	0.364	0.360
PmiR-let7d	0.411	0.300
PmiR-199a	0.741	0.360
PmiR-17	0.773	0.626
MmiR-222	0.741	0.215
MmiR-21	0.741	0.699
MmiR-20a	0.138	0.360
MmiR-27a	0.150	0.074
MmiR-92a	0.364	0.059
MmiR-130	0.364	0.118
MmiR-let7d	0.138	0.215
MmiR-17	0.387	0.416

MACE indicates major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles; PmiR, microRNA in plasma.
*Analysis were performed according to the median of microRNA level.

significantly associated with the risk for CV events. Patients with miR-126 levels in circulating MVs (MmiR-126) above the median had a significantly lower first MACE rate (*P*=0.006) than patients below the median. Particularly, CV mortality (*P*=0.044), nonfatal MI (*P*=0.029), and the need for revascularization by percutaneous coronary intervention (*P*=0.034) were significantly lower in patients with MmiR-126 levels above the median (Table 2). Furthermore, patients with MmiR-199a levels above the median experienced significantly fewer MACEs (*P*=0.015) than patients with levels below the median. In particular, the need for revascularization (*P*=0.009) was remarkably lower in patients with higher levels of MmiR-199a (Table 3). The remaining 8 miRNAs in circulating MRs did not show any relationship to CV events (Table 1).

MmiR-126 and MmiR-199a Level in Relation to Baseline Characteristics

We categorized the study population into 2 groups according to the median of MmiR-126 or MmiR-199a. There was no difference in baseline characteristics between groups (Tables 4 and 5). Notably, the amount of circulating annexin

Table 2. Events in 6-year Follow-up in Groups With Different MmiR-126 Levels*

Event	Total (n=176)	Low MmiR-126 Level (n=88)	High Mmir-126 Level (n=88)	*P* Value
Cardiovascular mortality	13 (7.4)	10 (11.4)	3 (3.4)	0.044
All-cause mortality	27 (15.3)	16 (18.2)	11 (12.5)	0.296
Nonfatal myocardial infarction	6 (3.4)	6 (6.8)	0 (0)	0.029
PCI	33 (18.8)	22 (25)	11 (12.5)	0.034
CABG	10 (5.7)	5 (5.7)	5 (5.7)	1
Revascularization	43 (24.4)	27 (30.7)	16 (18.2)	0.054
First MACE	55 (31.3)	36 (40.9)	19 (21.6)	0.006

Data are shown as number (percentage). CABG indicates coronary artery bypass graft; MACE, major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles; PCI, percutaneous coronary intervention.
*According to the median of MmiR-126.

V- and CD31-positive MVs did not differ between groups (*P*=0.311 for MmiR-126, *P*=0.181 for MmiR-199a). These data suggest that the observed associations of miRNA expression patterns and CV outcomes are not influenced by differences in circulating MV level.

Binary logistic regression demonstrated that higher or lower levels of MmiR-126 and MmiR-199a were not associated with baseline characteristics (Tables 6 and 7), confirming that MmiR-126 and MmiR-199a levels were not influenced by patients' comorbidities or medication.

MmiR-126 and MmiR-199a Level and Clinical Outcomes

Univariate analysis identified MmiR-126 levels above the median as a predictor of MACE-free survival (hazard ratio: 0.485 [95% CI: 0.278 to 0.846]; *P*=0.011), driven by a reduced rate of repeated percutaneous coronary interventions (hazard ratio: 0.458 [95% CI: 0.222 to 0.945]; *P*=0.035) and a trend toward lower CV mortality (Table 8).

Likewise, increased levels of MmiR-199a were associated with a reduced risk of MACE (hazard ratio: 0.518 [95% CI: 0.299 to 0.898]; *P*=0.01), driven by a lower rate of repeated percutaneous coronary interventions (hazard ratio: 0.454 [95% CI: 0.220 to 0.936]; *P*=0.03) and total revascularization (hazard ratio: 0.439 [95% CI: 0.232 to 0.832]; *P*=0.01) (Table 9).

Kaplan–Meier cumulative survival analysis further confirmed that patients with higher MmiR-126 levels had significantly lower occurrences of a first MACE (*P*=0.007) (Figure 2A) and revascularization (*P*=0.033) (Figure 2B) compared with those with lower MmiR-126 levels. Similarly, the first MACE (*P*=0.013) (Figure 2C) and the need for revascularization (*P*=0.012) (Figure 2D) were significantly lower in patients with higher MmiR-199a levels compared with those with levels below the median. For Kaplan–Meier analysis, patients were censored after a first event or when they were lost to follow-up. Moreover, 88.4% of patients had follow-up of ≥5 years, and 82.6% of patients were followed up for more than 1 year of 6 years.

Table 3. Events in 6-year Follow-up in Groups With Different MmiR-199a Level*

Event	Total (n=176)	Low MmiR-199a Level (n=88)	High Mmir-199a Level (n=88)	*P* Value
Cardiovascular mortality	13 (7.4)	8 (9.1)	5 (5.7)	0.387
All-cause mortality	27 (15.3)	13 (14.8)	14 (15.9)	0.834
Nonfatal myocardial infarction	6 (3.4)	4 (4.5)	2 (2.3)	0.682
PCI	33 (18.8)	22 (25)	11 (12.5)	0.034
CABG	10 (5.7)	7 (8)	3 (3.4)	0.193
Revascularization	43 (24.4)	29 (33)	14 (15.9)	0.009
First MACE	55 (31.3)	35 (39.8)	20 (22.7)	0.015

Data are shown as number (percentage). CABG indicates coronary artery bypass graft; MACE, major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles; PCI, percutaneous coronary intervention.
*According to the median of MmiR-199a.

Table 4. Baseline Characteristics of the Study Population With Different MmiR-126 Level*

Characteristic	Total (n=176)	Low MmiR-126 (n=88)	High MmiR-126 (n=88)	*P* Value
Age, y	66.7±10.2	66.2±10.7	67.2±9.7	0.487
Sex, no. (%)				0.25
Female	53 (30.1)	30 (34.1)	23 (26.1)	
Male	123 (69.9)	58 (65.9)	65 (73.9)	
Cardiovascular risk factors, no. (%)				
Arterial hypertension	148 (84.1)	76 (86.4)	72 (81.8)	0.410
Hyperlipoproteinemia	145 (82.4)	74 (84.1)	71 (80.7)	0.553
Diabetes	50 (28.4)	30 (34.1)	20 (22.7)	0.095
Family history of CAD	26 (14.8)	12 (13.6)	14 (15.9)	0.671
Smoking	34 (19.3)	15 (17)	19 (21.6)	0.445
Body mass index, kg/m^2	28.1±4.8	28.5±4.8	27.7±4.9	0.266
Laboratory parameters				
Cholesterol, mg/dL	192.3±44	195.9±48	188.8±39	0.364
LDL cholesterol, mg/dL	115.6±36.4	111.7±36.7	119.3±36.1	0.274
HDL cholesterol, mg/dL	50.7±13.6	49.2±11.8	52.1±15	0.260
Triglycerides, mg/dL	156.4±111	174.7±137	138.4±74.7	0.065
Serum creatinine, mg/dL	1.1±1.1	1.1±0.9	1.2±1.3	0.651
Glomerular filtration rate, mL/min	77.0±23.9	76±22.4	77.9±25.4	0.614
Leucocytes, 10^9/L	7.03±1.86	7.15±1.96	6.91±1.76	0.430
C-reactive protein, mg/L	9.5±14.7	8.6±10.9	10.4±18	0.510
Medical history, no. (%)				
Previous MI (6 months)	54 (30.7)	26 (29.5)	28 (31.8)	0.744
Previous PCI	76 (43.2)	39 (44.3)	37 (42.4)	0.761
Left ventricular ejection fraction, %	58.9±15.8	57.5±16.4	60.2±15.1	0.276
Medication on admission, no. (%)				
ACE inhibitors	102 (58)	51 (58)	51 (58)	1
Angiotensin receptor blockers	18 (10.2)	10 (11.4)	8 (9.1)	0.619
Beta blockers	115 (65.3)	58 (65.9)	57 (64.8)	0.874
Calcium channel blockers	30 (17)	19 (21.6)	11 (12.5)	0.109
Diuretics	69 (39.2)	35 (39.8)	34 (38.6)	0.877
Statins	98 (55.7)	53 (60.2)	45 (51.1)	0.225
Nitrates	57 (32.4)	30 (34.1)	27 (30.7)	0.629
Aspirin	122 (69.3)	62 (70.5)	60 (68.2)	0.744
Clopidogrel	33 (18.8)	13 (14.8)	20 (22.7)	0.176
Annexin V/CD31+ MV (log_{10} value)	3.34±0.5	3.38±0.5	3.30±0.5	0.311

Chronic kidney disease was defined as a glomerular filtration rate <60 mL/min. ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; MmiR, microRNA in circulating microvesicles; MV, microvesicles; PCI, percutaneous coronary intervention.
*According to the median of MmiR-126.

MmiR-126 Expression is Significantly Reduced in Patients With Coronary Artery Disease

After we found that increased expression of MmiR-126 and MmiR-199a was associated with a reduced risk of MACE, we aimed to explore whether CAD was linked to altered miRNA expression levels compared with healthy subjects.

We analyzed miR-126 and miR-199a expression in circulating MVs and total plasma in an age-matched study cohort (n=49; baseline characteristics are described in Table 10)

Table 5. Baseline Characteristics of the Study Population With Different MmiR-199a Level*

Characteristic	Total (n=176)	Low MmiR-199a (n=88)	High MmiR-199a (n=88)	*P* Value
Age, y	66.7±10.2	65.5±10.9	67.9±9.2	0.106
Sex, no. (%)				0.411
Female	53 (30.1)	29 (33)	24 (27.3)	
Male	123 (69.9)	59 (67)	64 (72.7)	
Cardiovascular risk factors, no. (%)				
Arterial hypertension	148 (84.1)	74 (84.1)	74 (84.1)	1
Hyperlipoproteinemia	145 (82.4)	75 (85.2)	70 (79.5)	0.322
Diabetes	50 (28.4)	28 (31.8)	22 (25)	0.316
Family history of CAD	26 (14.8)	9 (10.2)	17 (19.3)	0.089
Smoking	34 (19.3)	17 (19.3)	17 (19.3)	1
Body mass index, kg/m^2	28.1±4.8	28.7±5.4	27.5±4.1	0.087
Laboratory parameters				
Cholesterol, mg/dL	192.3±44	191.4±43.3	193.2±45.0	0.818
LDL cholesterol, mg/dL	115.6±36.4	116.5±37.0	114.7±36.1	0.789
HDL cholesterol, mg/dL	50.7±13.6	49.2±9.8	52.2±16.4	0.840
Triglycerides, mg/dL	156.4±111	176.1±138.4	137.6±72.3	0.125
Serum creatinine, mg/dL	1.1±1.1	1.18±1.16	1.12±1.02	0.730
Glomerular filtration rate, mL/min	77.0±23.9	76.4±23.7	77.6±24.1	0.737
Leucocytes, 10^9/L	7.03±1.86	6.98±1.77	7.08±1.96	0.735
C-reactive protein, mg/L	9.5±14.7	7.44±8.39	11.9±19.6	0.105
Medical history, no. (%)				
Previous MI (6 months)	54 (30.7)	24 (27.3)	30 (34.1)	0.327
Previous PCI	76 (43.2)	37 (42)	39 (44.3)	0.761
Left ventricular ejection fraction, %	58.9±15.8	58.26±16.2	59.54±15.5	0.609
Medication on admission, no. (%)				
ACE inhibitors	102 (58)	51 (58)	51 (58)	1
Angiotensin receptor blockers	18 (10.2)	9 (10.2)	9 (10.2)	1
Beta blockers	115 (65.3)	58 (65.9)	57 (64.8)	0.874
Calcium channel blockers	30 (17)	19 (21.6)	11 (12.5)	0.109
Diuretics	69 (39.2)	39 (44.3)	30 (34.1)	0.165
Statins	98 (55.7)	50 (56.8)	48 (54.5)	0.762
Nitrates	57 (32.4)	32 (36.4)	25 (28.4)	0.260
Aspirin	122 (69.3)	62 (70.5)	60 (68.1)	0.744
Clopidogrel	33 (18.8)	15 (17)	18 (20.5)	0.562
Annexin V/CD31+ MV ($\log_{10}$ value)	3.34±0.5	3.39±0.5	3.29±0.5	0.181

Chronic kidney disease was defined as a glomerular filtration rate <60 mL/min. ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; MmiR, microRNA in circulating microvesicles; MV, microvesicles; PCI, percutaneous coronary intervention.
*According to the median of MmiR-199a.

including subjects with angiographically proven or excluded CAD. Because diabetes mellitus influences miR-126 expression levels,[9] patients with diabetes mellitus were excluded from this analysis.

Importantly, MmiR-126 expression was significantly reduced in patients with CAD compared with healthy subjects (*P*=0.013). Furthermore, there was a clear trend toward reduced MmiR-199a (*P*=0.1) expression levels and reduced

Table 6. Association of the Level of MmiR-126 With Baseline Characteristics

	Exp(B) (95% CI)	*P* Value
Age	1.021 (0.983 to 1.060)	0.579
Male sex	0.586 (0.251 to 1.369)	0.248
Body mass index	0.932 (0.858 to 1.012)	0.255
Hypertension	0.967 (0.354 to 2.638)	0.611
Hyperlipoproteinemia	0.958 (0.376 to 2.445)	0.632
Diabetes	0.895 (0.397 to 2.020)	0.688
Family history of CAD	2.377 (0.874 to 6.469)	0.452
Smoking	0.903 (0.341 to 2.391)	0.463
History of myocardial infarction	1.672 (0.750 to 3.729)	0.749
Chronic kidney disease	0.867 (0.331 to 2.272)	0.881
Number of diseased vessels	0.799 (0.573 to 1.115)	0.197
Left ventricular ejection fraction	1.009 (0.987 to 1.031)	0.254
ACE inhibitors	1.074 (0.498 to 2.318)	0.232
Beta blockers	0.852 (0.381 to 1.907)	0.972
Statins	0.810 (0.355 to 1.846)	0.123
Annexin/CD31+ MV	0.629 (0.305 to 1.296)	0.881

The coefficient of the continuous variables was relative to 1-U differences. Binary logistic regression according to the median of MmiR-126 level. ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; Exp(B), exponentiation of the B coefficient; MmiR, microRNA in circulating microvesicles; MV, microvesicles.

Table 7. Association of the Level of MmiR-199a With Baseline Characteristics

	Exp(B) (95% CI)	*P* Value
Age	1.035 (0.975 to 1.099)	0.217
Male sex	0.941 (0.263 to 3.373)	0.094
Body mass index	0.942 (0.829 to 1.070)	0.947
Hypertension	1.316 (0.293 to 5.907)	0.929
Hyperlipoproteinemia	0.424 (0.102 to 1.763)	0.790
Diabetes	1.045 (0.311 to 3.505)	0.090
Family history of CAD	2.982 (0.673 to 13.210)	0.838
Smoking	2.448 (0.503 to 11.910)	0.209
History of myocardial infarction	5.484 (1.587 to 18.948)	0.772
Chronic kidney disease	0.263 (0.047 to 1.483)	0.188
Number of diseased vessels	0.721 (0.431 to 1.205)	0.444
Left ventricular ejection fraction	1.004 (0.974 to 1.035)	0.856
ACE inhibitors	0.441 (0.153 to 1.266)	0.697
Beta blockers	0.647 (0.211 to 1.985)	0.616
Statins	2.236 (0.687 to 7.278)	0.209
Annexin/CD31+ MV	0.554 (0.200 to 1.529)	0.288

The coefficient of the continuous variables was relative to 1-U differences. Binary logistic regression according to the median of MmiR-199a level. ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; Exp(B), exponentiation of the B coefficient; MmiR, microRNA in circulating microvesicles; MV, microvesicles.

Table 8. Univariate Analysis for Cardiovascular Outcomes According to the Median Level of MmiR-126

	Univariate	
	HR (95% CI)	*P* Value
Cardiovascular mortality	0.311 (0.085 to 1.130)	0.076
All-cause mortality	0.700 (0.325 to 1.508)	0.362
Nonfatal myocardial infarction	0.015 (0 to 10.924)	0.213
PCI	0.458 (0.222 to 0.945)	0.035
CABG	0.993 (0.287 to 3.433)	0.991
Revascularization	0.547 (0.295 to 1.016)	0.056
First MACE	0.485 (0.278 to 0.846)	0.011

CABG indicates coronary artery bypass graft; HR, hazard ratio; MACE, major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles; PCI, percutaneous coronary intervention.

expression in plasma (*P*=0.5 for miR-126 in plasma; *P*=0.1 for miR-199a in plasma) in patients with CAD (Figure 3). Taken together, these data strengthen the diagnostic and prognostic impact of miRNA packaged in MVs in CAD.

miR-126 and miR-199a Expression in Microvesicles, Exosomes, and Vesicle-Free Plasma

Circulating miRNAs in plasma can be transported within extracellular vesicles (exosomes, MVs, apoptotic bodies) or bound to proteins (high-density lipoprotein, Ago-2). Both routes provide remarkable stability and resistance to degradation from endogenous RNase activity. Next, we aimed to explore the plasma compartments (exosomes, MV, vesicle-free plasma) in which the analyzed miRNAs are mainly contained. Consequently, MVs, exosomes, and vesicle-free plasma were collected by series of centrifugation, and the

Table 9. Univariate Analysis for Cardiovascular Outcomes According to the Median Level of MmiR-199a

	Univariate	
	HR (95% CI)	*P* Value
Cardiovascular mortality	0.651 (0.213 to 1.993)	0.453
All-cause mortality	1.108 (0.521 to 2.359)	0.790
Nonfatal myocardial infarction	0.529 (0.097 to 2.891)	0.462
PCI	0.454 (0.220 to 0.936)	0.032
CABG	0.420 (0.108 to 1.624)	0.209
Revascularization	0.439 (0.232 to 0.832)	0.012
First MACE	0.518 (0.299 to 0.898)	0.019

CABG indicates coronary artery bypass graft; HR, hazard ratio; MACE, major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles; PCI, percutaneous coronary intervention.

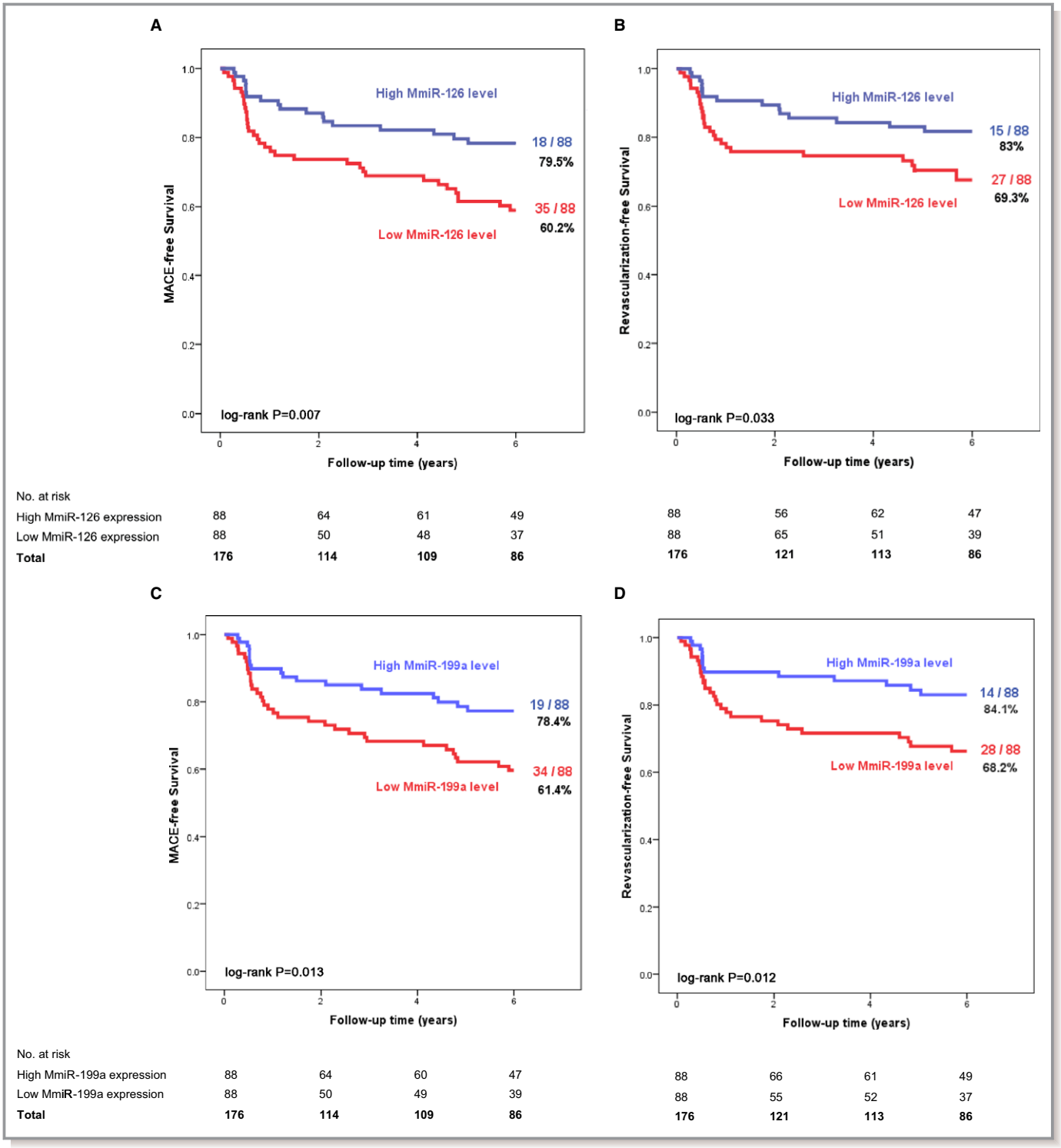

Figure 2. Kaplan–Meier cumulative survival analysis according to MmiR-126 and Mmir-199a levels. The study population was categorized into 2 groups according to the median of MmiR-126 and MmiR-199a (lower than median vs higher than median). A, Survival rate free of a first MACE based on MmiR-126 level. B, Survival rate free of a revascularization event based on MmiR-126 level. C, Survival rate free of a first MACE based on MmiR-199a level. D, Survival rate free of a revascularization event based on MmiR-199a level. MACE indicates major adverse cardiovascular event; MmiR, microRNA in circulating microvesicles.

described miRNAs were analyzed in the subcompartments of 10 patients with stable CAD. Proper isolation of exosomes according to the protocol used was confirmed by electron microscopy (Figure 4A). Levels of miR-130, miR-let7d, miR-20a, and miR-17 were undetectable in >4 samples in exosomes or in supernatant. In contrast, miR-126 and

Table 10. Baseline Characteristics of the Second Study Population

Characteristic	Healthy Control (n=15)	Stable CAD (n=34)	*P* Value
Age, y	66.3±2.44	62.2±1.72	0.194
Male	10 (66.7)	4 (11.8)	<0.001
Cardiovascular risk factors, no. (%)			
Arterial hypertension	9 (73.3)	23 (72.7)	1
Hyperlipoproteinemia	4 (28.6)	24 (72.7)	0.005
Diabetes	0	0	
Family history of CAD	1 (7.1)	7 (21.2)	0.405
Smoking	2(14.3)	14 (42.4)	0.0603
Body mass index, kg/m^2	28.1±2.1	27.7±0.6	0.848
Laboratory parameters			
Cholesterol, mg/dL	191.6±8.8	173.6±8.32	0.201
LDL cholesterol, mg/dL	114.5±7.78	106.9±6.23	0.484
HDL cholesterol, mg/dL	58.5±4.41	44.6±1.77	0.001
Triglycerides, mg/dL	120.6±13.9	154.4±18.1	0.247
Serum creatinine, mg/dL	0.86±0.25	1.0±0.24	0.062
Leucocytes, 10^9/L	6.9±0.45	7.8±0.44	0.219
C-reactive protein, mg/L	3.73±0.9	4.6±1.8	0.759
Medical history, no. (%)			
Previous MI (6 months)	0	18 (52.9)	<0.001
Previous PCI	0	29 (85.3)	<0.001
Left ventricular ejection fraction, %	58.8±2.5	53.64±2.1	0.150
Medication on admission, no. (%)			
ACE inhibitors	5 (33.3)	29 (85.3)	<0.001
Angiotensin receptor blockers	6 (40)	5 (14.7)	0.069
Calcium channel blockers	5 (33.3)	2 (5.9)	0.022
Diuretics	11 (73.3)	11 (32.4)	0.012
Statins	5 (33.3)	33 (97.1)	<0.001
Nitrates	0	2 (5.9)	1
Aspirin	1 (6.7)	34 (100)	<0.001
Clopidogrel	0	10 (29.4)	0.021

ACE indicates angiotensin-converting enzyme; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; MmiR, microRNA in circulating microvesicles; PCI, percutaneous coronary intervention.

miR-199a were found to be expressed mainly in circulating MVs (Figure 4B), whereas miR-222, miR-21, miR-27, and miR-92a were detectable mainly in vesicle-free plasma (Figure 5). These findings indicate that miR-126 and miR-199a are particularly selectively transported in MVs in CAD.

Because circulating MVs compose different subspecies of membrane vesicles released from endothelium and blood cells, we sorted endothelial-, platelet-, and other cell-derived MVs using flow cytometry to explore the cellular origins of miR-126 and miR-199a contained in MVs. Overall, miR-126 showed the highest expression in CD31+/CD42b− endothelial cell–derived MVs, whereas miR-199a was detectable mainly in CD31+/CD42b+ platelet-derived MVs (Figure 6).

Discussion

Studies show that miRNAs are powerful regulators of cellular CV processes.[29] Moreover, an increasing number of studies demonstrates that miRNAs can be detected in circulating blood and that these circulating miRNAs might be useful biomarkers in patients with CVD; however, because the first correlation between miRNAs and CVD was described only a few years ago,[1] data about the long-term prognostic value of circulating miRNAs are still scarce.

In this prospective study in 176 patients with stable CAD, we demonstrated that increased expression of miR-126 and miR-199a in circulating MVs is associated with a lower risk of future MACEs. During the observational period of 6 years, significantly reduced incidence of CV mortality and nonfatal MI and need for revascularization were observed in CAD patients with MmiR-126 levels above the median. MmiR-199a levels above the median were associated with a significantly reduced need for revascularization therapies. Interestingly, there was no association between circulating, unbound miR-126 and miR-199a within the plasma and CV outcomes.

Recently, it was demonstrated that MVs represent major transport vehicles for miRNAs in patient plasma.[32] MVs are released from activated or apoptotic cells. Accordingly, circulating MVs are increased in conditions of systemic cell damage including thrombotic thrombocytopenic purpura, lupus anticoagulant, and end-stage renal failure as well as in CVD.[33] Importantly, increasing evidence suggests that MVs represent not only biomarkers for cellular damage and apoptosis but also display vector functions that are important for the intercellular exchange of biological information.[34] Recent data suggest that the effects of MVs depend on miRNA expression in MVs.[32,35]

Accordingly, we showed that miR-126 and miR-199a levels in circulating MVs, rather than plasma, have prognostic value predicting CV events in patients with CAD. Wang et al compared profiles of miRNAs in cell-derived vesicles (ie, exosomes and MVs) with vesicle-free miRNAs (ie, supernatant fraction after ultracentrifugation) and convincingly showed that miRNA profiles within and outside these vesicles were strikingly different.[36] Diehl et al confirmed that circulating MVs represent transport vehicles for large numbers of specific miRNAs that have been associated with CVD and that miRNA profiles of MVs were significantly different from their maternal cells.[32] Our data extend these findings and suggest that

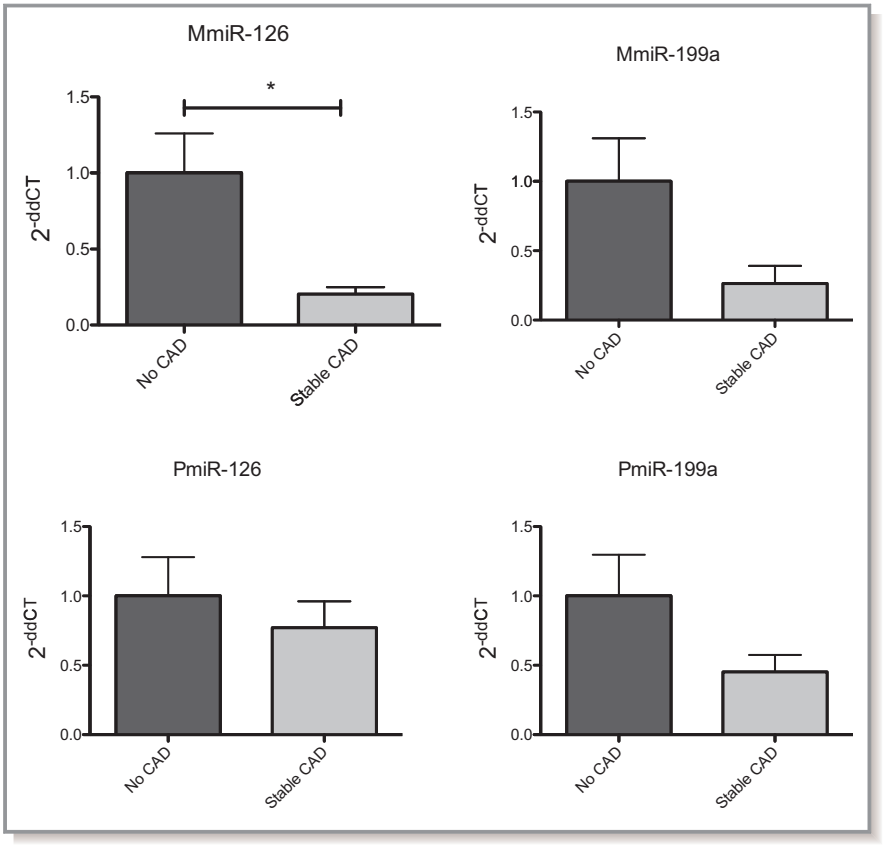

Figure 3. Expression of miR-126 and miR-199a in plasma and microvesicles in patients with and without CAD. miR-126 and miR-199a were analyzed in patients with and without CAD (n=49). **P*<0.05. Relative quantification of gene expression was determined using the comparative CT method [2-ddCT, internal control: Cel-miR-39]. CAD indicates coronary artery disease; MmiR, microRNA in circulating microvesicles; PmiR, microRNA in plasma.

miRNAs selectively packaged in MVs may play a crucial role in intercellular signaling influencing the development of CVD.

In our study, a higher level of miR-126 or miR-199a in isolated circulating MVs is associated with a reduced risk of MACEs in patients with stable CAD. Notably, the levels of circulating annexin V- and CD31-positive MVs were not associated with MmiR-126 or MmiR-199a levels. Our group, however, has previously demonstrated that high circulating CD31- and annexin V-positive MVs correlate with CV outcomes.[37] In view of these data, one could speculate that besides the quantity of circulating MVs, the enrichment of specific miRNAs, proteins, or other cell fractions may influence vascular homeostasis and subsequent CV outcomes.

In order to find further evidence for this hypothesis, we analyzed the subgroup of patients with diabetes. Diabetes is known to increase circulating MV levels, but, in contrast, these MVs seem to be severely impaired in terms of modulating biological actions.[15,38] In our cohort, CAD patients with diabetes had significantly higher annexin V- and CD31-positive MVs compared with nondiabetic patients. When comparing MmiR-126 and MmiR-199a levels in CAD patients with and without diabetes, we were able to show that the diabetic subgroup had significantly lower MmiR-126 and MmiR-199a levels compared with euglycemic patients (*P*=0.017[15] and *P*=0.034, respectively) (Figure 7). This finding fits nicely with previous findings indicating a loss of circulating miR-126 in plasma samples of diabetic patients.[9] Taken together, it appears reasonable to speculate that not only the amount but also the contents of circulating MVs define their effects on vascular health.

An increasing body of evidence has highlighted miR-126 as an important regulator of vascular integrity. Embryonic blood vessel development and angiogenic signaling have been shown to be regulated by miR-126 and repression of Spred1.[20,22] Furthermore, miR-126 has been shown to counteract atherosclerosis in a CXCL12/CXCR4-dependent way and influence inflammation by dampening VCAM1 expression.[14,39]

Analysis of circulating miRNAs in patients with CAD and diabetes showed significantly reduced levels of miR-126 in patients compared with healthy controls[8,9]; however, a com-

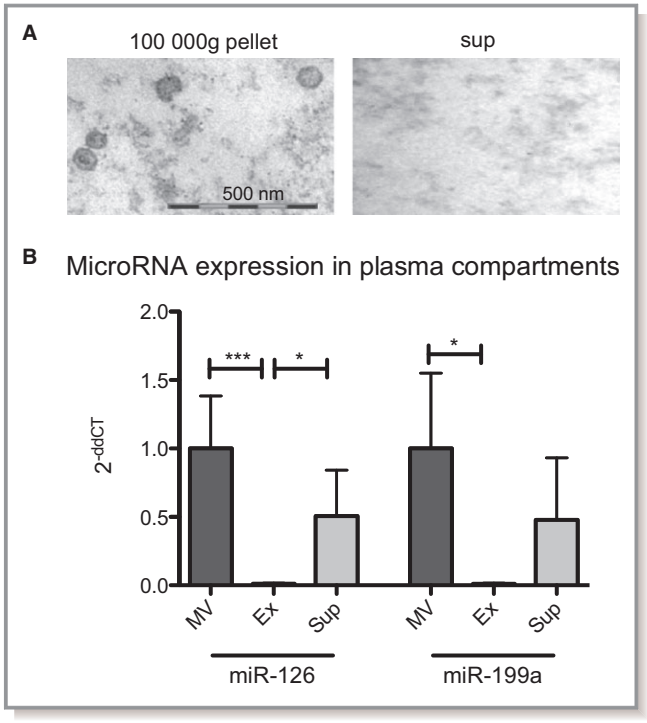

Figure 4. Expression of miR-126 and miR-199a in plasma compartments. MVs and exosomes were isolated using 20 000*g* and 100 000*g* centrifugation. A, Characterization of exosomes and the last supernatant with electron microscopy. Magnification ×46 000. B, miR-126 and miR-199a were detected in MVs, exosomes, and vesicles-free supernatant (n=6). *P<0.05. ***P<0.001. Relative quantification of gene expression was determined using the comparative CT method [2-ddCT, internal control: Cel-miR-39]. EX indicates exosomes; MV, microvesicles; Sup, supernatant.

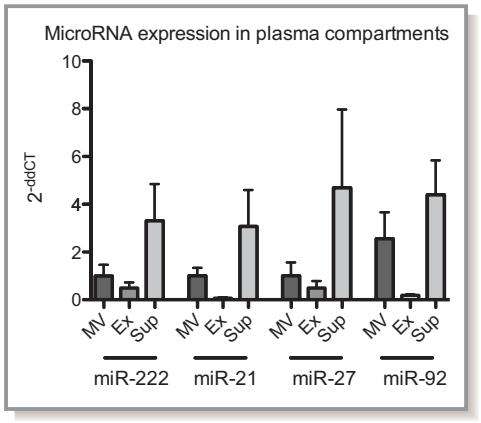

Figure 5. Expression of miR-222, miR-21, miR-27, and miR-92 in plasma compartments. A, miR-222 miR-21, miR-27 and miR-92 were detected in MVs, exosomes, and vesicle-free supernatant. There was no significant difference among MVs, exosomes, and supernatant for each microRNA. N=6. Relative quantification of gene expression was determined using the comparative CT method [2-ddCT, internal control: Cel-miR-39]. EX indicates exosomes; MV, microvesicles; Sup, supernatant.

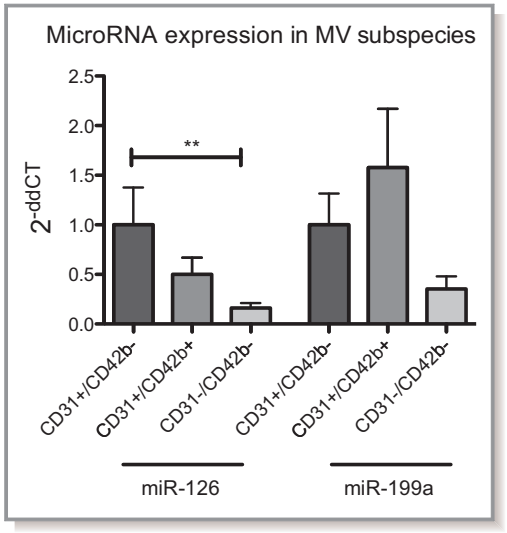

Figure 6. Analysis of microRNA in MV subspecies. Endothelial cell–derived (CD31+/CD42b−), platelet-derived, and other cell–derived MVs were sorted, and miR-126 and miR-199a expression was analyzed in MV subspecies. **P<0.01, n=10. Relative quantification of gene expression was determined using the comparative CT method [2-ddCT, internal control: Cel-miR-39]. MV indicates microvesicles.

parison of patients with unstable angina and healthy controls did not find differences in circulating miR-126 expression, whereas miR-186 could be identified as a biomarker for unstable angina.[40] Furthermore, miR-126 levels in circulating angiogenic early outgrowth cells and CD34+ peripheral blood mononuclear cells defined their regenerative capacity and were reduced in diabetic patients.[21,41] Our group recently demonstrated that endothelial cell–derived MVs mediate

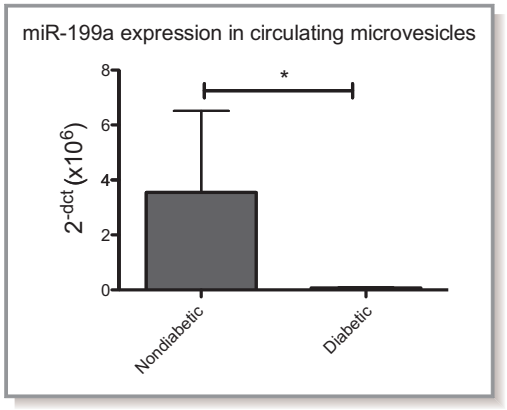

Figure 7. Expression of miR-199a in diabetic and nondiabetic patients. miR-199a expression in circulating microparticles is significantly reduced in patients with diabetes. The ΔCt method was used to quantify relative microRNA expression. Values were normalized to *Caenorhabditis elegans* miR-39 (cel-miR-39) and are expressed as $2^{-[Ct(microRNA)-Ct(cel\text{-}miR\text{-}39)]}$.

vascular regeneration in a miR-126–dependent mechanism.[15] Taken together, miR-126 seems to mediate vasculoprotective effects using different biological mechanisms. In this study, we extend these findings and demonstrate that a low expression level of miR-126 in circulating MVs influences CV events. It can be speculated that the prognostic value of MmiR-126 might result from those robust, consistent biological effects of miR-126 in MVs, which can act as biological vectors.

Although the effect of miR-126 on the vasculature has been studied in detail, the role of miR-199a in vascular and cardiac biology is less known. In endothelial cells, miR-199a has been shown to promote cell survival, proliferation,[42] and tube formation.[43] In murine cardiomyocytes, miR-199a–mediated stabilization of p53 by inhibiting HIF-1α reduced apoptosis and treatment of mice after MI with exogenous miR-199a–stimulated cardiac regeneration and led to almost complete recovery of cardiac functional parameters.[31,44] These findings from cell culture and animal experiments provide possible mechanistic insights into the cardioprotective function of miR-199a. We postulate that higher expression of miR-199a in circulating MVs in patients with stable CAD have prognostic value in terms of reducing MACEs and the need for revascularization; however, the role of miR-199a in vascular biology needs further exploration to better understand the molecular background mediating the observed effects.

Although the potential of miRNAs as biomarkers has attracted increasing attention in recent years, the prognostic value of circulating miRNAs is still largely unknown. Recently, Zampetaki et al described the association of miRNA expression patterns and the incidence of MI in the Bruneck cohort.[16] Furthermore, miR-133a and miR-208b levels were significantly associated with the risk of death in univariate and age- and sex-adjusted analyses in patients with acute coronary syndrome[45]; however, long-term follow-up studies evaluating the potential of circulating miRNAs as prediction markers for CV events do not exist, to our knowledge.

We show that miR-126 and miR-199a levels in circulating MVs but not in plasma have prognostic relevance for predicting CV events. MV-sorting experiments showed that endothelial cells and platelets were found to be the major cell sources of MVs containing miR-126 and miR-199a, respectively. Furthermore, MVs represent the major plasma compartment for miR-126 and miR-199a, whereas other analyzed miRNAs were predominantly found to be transported in the vesicle-free form. These findings are in accordance with data from Wang et al, who compared profiles of miRNAs in cell-derived vesicles (ie, exosomes and MVs) with vesicle-free miRNAs (ie, supernatant fraction after ultracentrifugation) and found that miRNA profiles within and outside these vesicles were strikingly different.[36] The notion that miRNAs selectively packaged in MVs may play a crucial role in intercellular signaling influencing CVD is supported by an increasing experimental data.[46,47] In this context, injected miRNA-containing apoptotic bodies were shown to be transported into atherosclerotic lesions, where they controlled downstream targets and promoted vascular protection. Furthermore, Hergenreider et al described an atheroprotective communication mechanism between endothelial cells and vascular smooth muscle cells via endothelial cell–derived exosomes in a miR-143/145–dependent way. Taken together, these well-performed and convincing studies demonstrated the cardioprotective potential of intercellular communication mechanisms by miRNA-containing extracellular vesicles.[14,48] Our data broaden these findings by showing a possible clinical relevance for miRNA expression patterns in circulating MVs. The incorporation of miRNAs into MVs provides protection from RNases but also may simplify the uptake of miRNAs into target cells. Based on the broad experimental data and our findings, one may speculate that miRNAs packed in MVs might be biologically more active and relevant compared with vesicle-free miRNAs.

This study has several limitations. Only a selected number of miRNAs, based on previously published data, were analyzed. Moreover, although there is profound knowledge concerning miR-126–mediated vascular protection, the role and function of miR-199a facilitating the observed effects are unclear so far. Further exploration of MVs containing miR-199a is of importance to understand their role in vascular hemostasis. In addition, exploration of selection and packaging mechanisms of miRNAs into MVs would be of interest to better comprehend the physiological and pathophysiological functions of miRNA-containing MVs in vascular biology. Finally, the relatively small sample size limits the final conclusion that can be drawn from this study.

We found that increased miR-126 or miR-199a expression in circulating MVs is associated with reduced risk of CV events in stable CAD patients. We provided evidence that the expression of potential cardioprotective miRNAs in circulating MVs has prognostic value in patients with stable CAD and may contribute to risk stratification.

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Disclosures

None.

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