Tohoku J. Exp. Med., 2024, **264**, 185-192

Plasma miR-21-5p and miRNA-93-5p Levels as Early Assessment Tools for In-Stent Restenosis Following Endovascular Stenting Treatment in Patients with Lower Extremity Atherosclerotic Disease

Zhijun Wang[1] and Shuanlin Ma[1]

[1]Interventional Vascular Department, Baoji Gaoxin Hospital, Baoji, Shaanxi, China

In-stent restenosis (ISR) still remains a leading cause of failure of interventional therapy in patients with lower extremity atherosclerotic disease (LEAD). Sensitive and reliable biomarkers to predict ISR should be identified. This study aims to investigate predictive values of two microRNAs, miR-21-5p and miR-93-5p for ISR following endovascular stenting treatment. A total of 128 LEAD patients receiving endovascular stenting treatment were included into the study and their restenotic status followed up by computed tomography angiography after 6 months to examine the incidence of ISR. The results of two-way ANOVAs showed a significant effect of ISR presence, time, and ISR × time interaction on the plasma level of miR-21-5p and miR-93-5p among LEAD patients, which reduced at the postoperative 14th day. The following multiple comparisons test showed higher plasma level of miR-21-5p and miR-93-5p at the postoperative 14th day in the ISR than the non-ISR ($P < 0.0001$). The plasma levels of miR-21-5p and miR-93-5p at 14 days after surgery used alone or combination as a test to predict ISR occurrence 6 months after surgery produced an AUC of 0.845, 0.839, and 0.906, respectively. Multiple logistic regression analysis revealed the plasma levels of miR-21-5p and miR-93-5p at 14 days after surgery were risk factors for LEAD patients developing ISR at 6 months after surgery ($P < 0.001$). Our results suggest that plasma miR-21-5p and miR-93-5p levels at 14 days after surgery may serve as potential biomarkers for developing ISR following endovascular stenting treatment among LEAD patients.

Keywords: diagnosis; hsa-miR-21-5p; hsa-miR-93-5p; in-stent restenosis; lower extremity atherosclerotic disease; prediction
Tohoku J. Exp. Med., 2024 December, **264** (4), 185-192.
doi: 10.1620/tjem.2024.J066

Introduction

Lower extremity atherosclerotic disease (LEAD), also known as lower extremity peripheral artery disease, is characterized by sclerotic stenosis or obstruction of the large arteries in the lower limbs, often caused by atherosclerotic plaque (Polonsky and McDermott 2021). The prevalence of LEAD exceeds 230 million adults across the globe and leads to a significant morbidity and mortality (Criqui et al. 2021). The most common clinical outcomes of LEAD include rest pain, impaired functional capacity, and tissue loss in the distal limbs that may result in lower extremity amputation (Levin et al. 2020). LEAD involves a broad spectrum of disease from asymptomatic to intermittent claudication or chronic limb-threatening ischemia (Shishehbor et al. 2023). LEAD similarly affects men and women, while women exhibit more atypical symptoms and receive revascularization at their older ages than men (McDermott et al. 2023). Revascularization procedures, such as angioplasty, stenting, and bypass grafting, are indicated in those with severe disease or those being not improved after non-surgical interventions (Annex and Cooke 2021). Accordingly, a new clinical entity of in-stent restenosis (ISR) become apparent. ISR is defined as ≥ 50% narrowing in the stent lumen following stent implantation and occurs in as many as 30% of all patients with place-

Received March 28, 2024; revised and accepted July 3, 2024; J-STAGE Advance online publication July 18, 2024
Correspondence: Shuanlin Ma, Interventional Vascular Department, Baoji Gaoxin Hospital, No. 19, Gaoxin Fourth Road, Baoji, Shaanxi 721000, China.
e-mail: 15378008844@163.com

ment of bare metal stents, leading to stent failure, repeated interventions, and even a significant morbidity (Nicolais et al. 2018; Muller et al. 2023). Even with drug-eluting stents, a considerable portion of patients experience ISR after stent implantation, create a critical need of non-invasive biomarkers to follow-up patients at an increased risk of developing ISR following endovascular stenting and tailor management strategies (Montelione et al. 2022).

Much evidence indicates that microRNAs (miRNAs) appear to hold promise in the identification of LEAD patients at increased risk of ISR following stent implantation due to their extraordinary stability in several bodily fluids (Stojkovic et al. 2018). The formation of ISR is largely associated with the migration and proliferation of vascular smooth muscle cells (VSMCs) (Clare et al. 2022). VSMCs migrate from the medial and adventitial layers into the intimal layer and accumulate within neointimal lesions, leading to the incidence of neointimal hyperplasia in the blood vessel wall and ultimately contributing to restenosis (Tang et al. 2022). miRNAs have long been recognized as potent regulators of VSMC intracellular or extracellular signaling during phenotypic switching, senescence, calcification, and neointimal hyperplasia (Sufianov et al. 2023). MiRNA-21-5p (miR-21-5p) was previously demonstrated with a significantly elevated expression in peripheral monocytes from patients with coronary artery disease and mouse atherosclerotic plaques (Gao et al. 2019). Compared to non-atherosclerotic left internal thoracic arteries, the miR-21-5p was found to be enriched in atherosclerotic arteries (Raitoharju et al. 2011). Interestingly, miRNA-93-5p (miR-93-5p) was revealed with a high expression in patients with peripheral arterial disease (Shu et al. 2019). Of note, miR-93-5p has also been studied as a consistently changed miRNA in ISR compared to non-ISR after coronary stent implantation (O'Sullivan et al. 2019). However, the sensitivity and stability of miR-21-5p and miR-93-5p as early biomarkers to identify patients at an increased risk of ISR after stenting is currently limited. In this study, we prospectively recruited LEAD patients scheduled to undergo surgery and determine changes of plasma levels of miR-21-5p and miR-93-5p at different evaluation times after surgery, in a bid to uncover their discriminatory abilities between ISR and non-ISR among LEAD patients after stent implantation.

Materials and Methods

Participant selection

The prospective observational study consecutively recruited symptomatic patients scheduled to undergo surgery for LEAD at our hospital. Participant recruitment started in January 2020 until the end of June 2023 and approved by the Ethics Committee of our hospital. The diagnosis of LEAD was confirmed by the cardiologist with the aid of computed tomography angiography (CTA) and according to the ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (Hirsch et al. 2006). The disease severity was assessed based on the Fontaine stage including stage I (asymptomatic), stage II (intermittent claudication), stage III (rest pain), and stage IV (severe ischemia, anabrosis and necrosis). Eligible participants should be at their first diagnosis of LEAD and aged ≥ 18 years. The following patients were excluded: patients unwilling or unable to take anti-platelet aggregation drugs; patients lost to follow up; patients with liver or renal dysfunction; patients diagnosed with malignant tumor diseases; patients with hemophilia or autoimmune disease; pregnancy or women in lactation. Eligible participants were split into ISR and non-ISR groups according to their restenotic status at 6 months after surgery. ISR was defined as ≥ 50% diameter narrowing within the stented segment and confirmed by the CTA (Lian et al. 2021). All participants have signed the informed consent.

Surgical protocols

The same team of surgeons were responsible for endovascular procedures tailored according to the preoperative CTA imaging. The femoral artery puncture was performed using the modified Seldinger method. The femoral artery puncture was performed through ipsilateral anterograde access when the stenosis or occlusion was found in the middle or distal third segment of the superficial femoral artery or in the popliteal artery. If the stenosis or occlusion was found in the common femoral artery, proximal third segment of the superficial femoral artery, or the iliac artery, the intracavitary therapy was required and retrograde or crossover femoral access was conducted for femoral artery puncture. When the bilateral femoral arteries were not applicable to puncture in this way, a brachial artery access was used. After femoral artery puncture, a sheath pipe was inserted. According to different approaches and forming methods, different types of guidewires were selected. The ev3 NanoCross and Bantam were selected for percutaneous transluminal angioplasty (PTA) microballoon. The endovascular stenting was performed using Viabahn covered stents (W.L. Gore and Assoc. Inc., Flagstaff, AZ, USA). Technical success was defined as < 30% residual stenosis assessed at the narrowest point of the treated vascular segment, absence of evident artery dissection, or no serious complication. After interventional treatment, patients were administered with aspirin (Guangzhou Baiyunshan Pharmaceutical Holdings Co., Ltd., China) (100 mg/day) combined with clopidogrel (75 mg/day) within 1 year.

Blood sample collection and mRNA measurements

Each participant was required to provide venous blood (5 mL) after overnight fasting before surgery, 7 days and 14 days after surgery. The plasma was obtained after centrifugation (3,000 × *g* for 10 min) of venous blood and stored until measurement with quantitative real time polymerase chain reaction (qRT-PCR). The collected plasma was submitted for extraction of total RNA by using the Trizol reagents (Invitrogen, Carlsbad, CA, USA), with synthetic

cel-miR-39 (1.6×10^8 copies/μL, Sigma Aldrich, Zwijndrecht, The Netherlands) spiked-in. The following cDNA synthesis was completed by the TaqMan microRNA Reverse Transcription kit (Invitrogen). The TaqMan MicroRNA Assays were employed to quantify hsa-miR-21-5p (Assay ID 000397), hsa-miR-93-5p (Assay ID 001090), cel-miR-39 (Assay ID 000200), and hsa-miR-16-5p (Assay ID 000391) (Life Technologies, USA). Amplification was performed on a Roche Lightcycler 480 (Roche, Diagnostics, Indianapolis, IN, USA). The expression levels of miR-21-5p and miR-93-5p were relative to that of hsa-miR-16-5p according to the 2-$\Delta\Delta$Ct formula (Maren et al. 2023).

Statistical analysis

We did descriptive statistics for collected data and data analysis in the GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA) and the SPSS version 20 (IBM, New York, NY, USA). Box-plot methods were applied to identify the outlying data and no outlying data existed in this study. The distribution of continuous data was determined by the Shapiro-Wilk normality test. When the data were described as mean ± standard deviation (s.d.), unpaired t test was carried out for demographic and clinical characteristics between ISR vs. non-ISR at baseline, and two-way ANOVAs with Tukey's multiple comparisons test for plasma miR-21-5p and miR-93-5p levels between ISR vs. non-ISR according to the presence of ISR and evaluation times. When the data were described as frequency or percentage, Fisher's exact test was carried out for ISR vs. Non-ISR. Regression analysis was used to determine the risk factors for ISR at 6 months after surgery. Area under the curve (AUC) using receiver operating characteristic (ROC) method were used to define cut-off points, sensitivity, and specificity, which is the Youden's index for the optimization criteria, for estimating the diagnostic values of miR-21-5p and miR-93-5p for developing ISR at 6 months after surgery. All tests were two-tailed with the probability less than 0.05 ($P < 0.05$) indicating significant difference.

Results

Demographic and lesion characteristics of patients

A total of 141 LEAD patients were recruited into this study, with 6 patients failing at screening. Resulting 135 patients underwent surgery and were followed up at 6 months after surgery. During the study period, 7 patients were lost to follow-up and excluded. Finally, 128 patients completed the study and were split into ISR (n = 45) and non-ISR (n = 83) groups according to their restenotic status at 6 months after surgery (Fig. 1). Demographic and lesion characteristics of patients are outlined in Table 1. The ISR and non-ISR groups were comparable due to no significant difference noted on their demographic and lesion characteristics before surgery ($P > 0.05$). The ISR group had a higher proportion with complete stenosis than the non-ISR group ($P = 0.026$). Among patients with ISR, there were 6 being symptomatic. Reinterventions were indicated for recurrent symptoms associated with CTA evidence of ISR. Patients with asymptomatic restenosis were followed with repeat duplex ultrasound. Patients with asymptomatic restenosis in need of reinterventions were based on progressed lesions (peak systolic velocity > 500 cm/s and distal tardus-parvus waveform). The type of reintervention was left at the discretion of the physician performing the procedure without predefined algorithm, but most often, balloon angioplasty alone or repeated stent placement. In this study, there were 10 patients (22.2%) undergoing reintervention with balloon angioplasty alone and 26 (57.8%) undergoing repeated stent placement.

The plasma level of miR-21-5p between ISR and non-ISR

The plasma levels of miR-21-5p and miR-93-5p were analyzed before surgery, 7 days, and 14 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients. The mean values of miR-21-5p level before surgery, 7 days, and 14 days after surgery were 5.16, 4.52, and 4.06 in the ISR group, 5.02, 4.26, and 3.42 in the non-ISR group. The results of two-way ANOVAs for the plasma level of miR-21-5p before surgery and at 7 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients were as follows: $P_{ISR} < 0.0001$; $P_{time} = 0.036$; $P_{interaction} = 0.528$. No significant ISR × time interaction was observed concerning the miR-21-5p expression level at the postoperative 7th day. The results of two-way ANOVAs for the plasma level of miR-21-5p before surgery and at 14 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients were as follows: $P_{ISR} < 0.0001$; $P_{time} < 0.0001$;

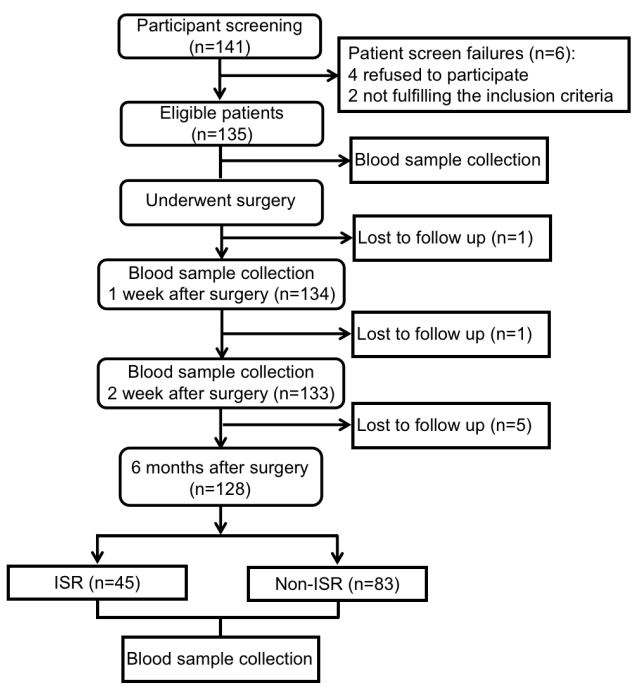

Fig. 1. Flow chart of patient selection.

Table 1. Demographic and lesion characteristics of LEAD patients undergoing surgery.

Characteristic	ISR (n = 45)	Non-ISR (n = 83)	P
Age (year, mean ± s.d.)	73.2 ± 9.4	70.6 ± 9.5	0.147
Gender distribution (male, n/%)	33 (73.3%)	52 (62.7%)	0.245
BMI (kg/m^2, mean ± s.d.)	22.5 ± 2.2	22.2 ± 2.1	0.506
Current smoker (n/%)	30 (66.7%)	52 (62.7%)	0.703
Comorbidity: diabetes (n/%)	16 (35.6%)	21 (25.3%)	0.229
Comorbidity: hypertension (n/%)	21 (46.7%)	31 (37.4%)	0.306
Hyperlipidemia	12 (26.7%)	16 (19.3%)	0.334
Location of lesion (n/%)			0.356
Unilateral	28 (62.2%)	56 (67.5%)	
Bilateral	17 (37.8%)	27 (32.5%)	
Lesion length (mm, mean ± s.d.)	51.0 ± 24.0	45.4 ± 25.5	0.233
Presence of calcification (n/%)	22 (48.9%)	35 (42.2%)	0.465
Fontaine stage (n/%)			0.155
II	15 (33.3%)	18 (21.7%)	
III	25 (55.6%)	60 (72.3%)	
IV	5 (11.1%)	5 (6.0%)	
Target vessel (n = 243)			0.435
Femoropopliteal artery	56 (59.8%)	95 (65.1%)	
Iliac artery	25 (23.7%)	34 (23.3%)	
Infrapopliteal arteries	16 (16.5%)	17 (11.6%)	
TASC II classification (n = 243)			0.216
A	11 (11.3%)	28 (19.2%)	
B	20 (20.6%)	31 (21.2%)	
C	29 (29.9%)	47 (32.2%)	
D	37 (38.2%)	40 (27.4%)	
Complete stenosis (n/%)	15 (33.3%)	13 (15.7%)	0.026
Stent mean diameter (mm, mean ± s.d.)	6.9 ± 1.3	6.5 ± 1.1	0.060

TASC, TransAtlantic Inter-Society Consensus

$P_{interaction}$ = 0.005, showing a significant reduction of miR-21-5p expression level at the postoperative 14th day. In addition, the two-way ANOVA test showed a significant interaction between group (ISR, non-ISR) and time (7 days post-surgery, 14 days post-surgery) (P_{ISR} < 0.0001; P_{time} < 0.0001; $P_{interaction}$ = 0.006), revealing a lower miR-21-5p expression level from 7 days to 14 days post-surgery. The following multiple comparisons test showed a higher plasma level of miR-21-5p in the ISR than the non-ISR before surgery vs. 14 days post-surgery and 7 days post-surgery vs. 14 days post-surgery (P < 0.05; Fig. 2A).

The plasma level of miR-93-5p between ISR and non-ISR

The mean values of miR-93-5p level before surgery, 7 days, and 14 days after surgery were 1.57, 1.38, and 1.25 in the ISR group, 1.48, 1.31, and 1.02 in the non-ISR group. The results of two-way ANOVAs for the plasma level of miR-93-5p before surgery and at 7 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients were as follows: P_{ISR} < 0.0001; P_{time} = 0.011; $P_{interaction}$ = 0.747. There was no significant effect of ISR × time on the miR-93-5p expression level at the postoperative 7th day. The results of two-way ANOVAs for the plasma level of miR-93-5p before surgery and at 14 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients were as follows: P_{ISR} < 0.0001; P_{time} < 0.0001; $P_{interaction}$ = 0.013, indicating a significant effect of ISR × time on the miR-93-5p expression level, which decreased at the postoperative 14th day. The two-way ANOVA test for time points of 7 days and 14 days post-surgery also revealed a significant interaction between group and time (P_{ISR} < 0.0001; P_{time} < 0.0001; $P_{interaction}$ = 0.001), revealing a lower miR-93-5p expression level from 7 days to 14 days post-surgery. The following multiple comparisons test showed a higher plasma level of miR-93-5p in the ISR than the non-ISR before surgery vs. 14 days post-surgery and 7 days post-surgery vs. 14 days post-surgery (P < 0.05; Fig. 2B).

Early prediction of ISR according to plasma levels of miR-21-5p and miR-93-5p

To determine the discriminatory abilities of plasma levels of miR-21-5p and miR-93-5p at 14 days after surgery between ISR and non-ISR among LEAD patients, ROC

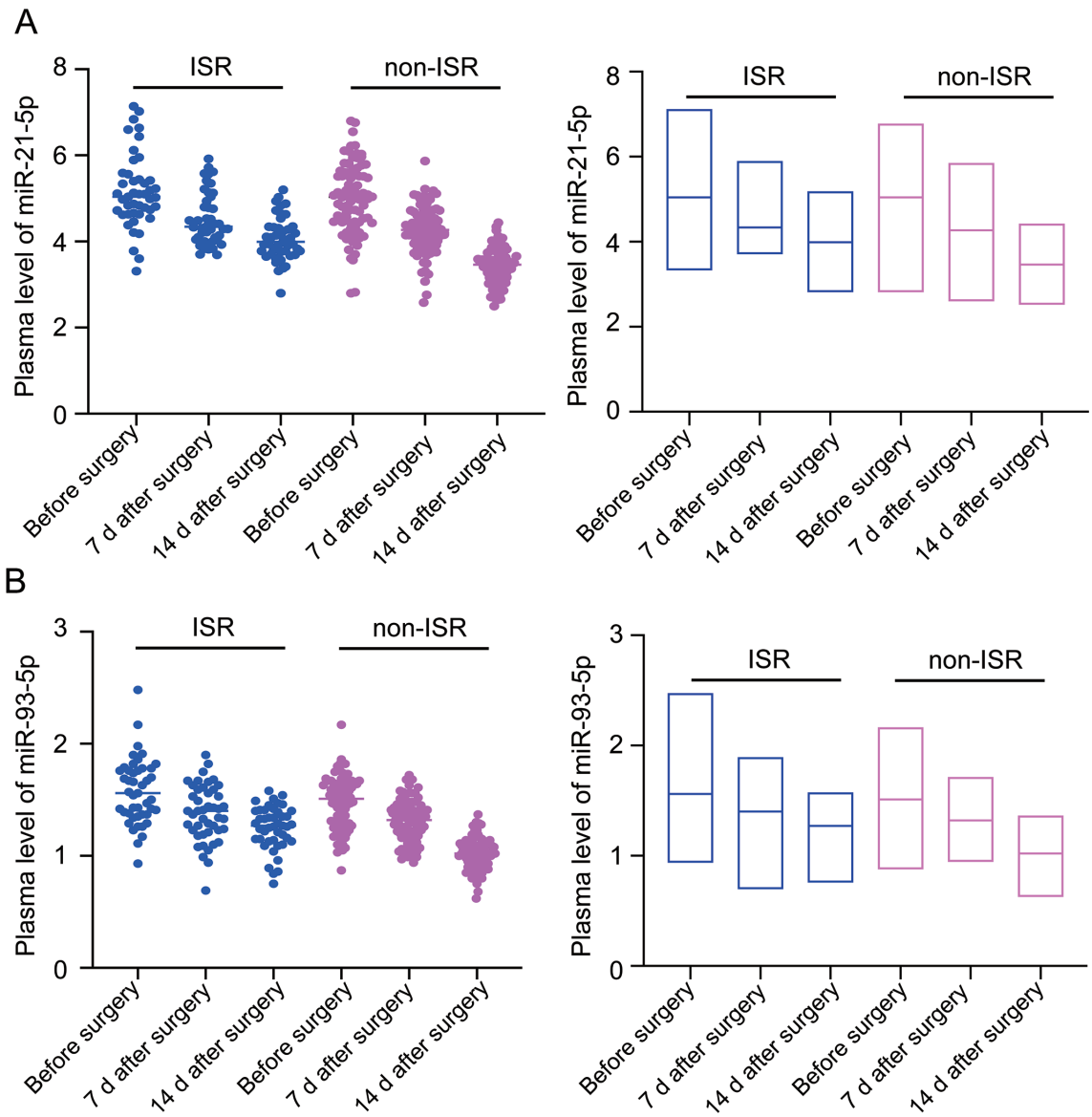

Fig. 2. The plasma level of miR-21-5p and miR-93-5p in ISR and non-ISR groups.
The plasma levels of miR-21-5p (A) and miR-93-5p (B) were determined by qRT-PCR before surgery, 7 days and 14 days after surgery according to ISR or non-ISR 6 months after surgery among LEAD patients. Results were displayed as scatter plot and box plot, respectively. Two-way ANOVAs with Tukey's multiple comparisons test were performed for statistical analysis. d, days.

methods were carried out to plot the curve and obtain the AUC. When applied as a tool to predict the incidence of ISR at 6 months after surgery, the plasma level of miR-21-5p produced an AUC of 0.845 (95% CI: 0.774 - 0.916), with sensitivity: 82.2%, specificity: 75.9%, cut-off point: 3.67, and Youden's index: 0.581 (Fig. 3A). When applied as a tool to predict the incidence of ISR at 6 months after surgery, the plasma level of miR-93-5p produced an AUC of 0.839 (95% CI: 0.756 - 0.921), with sensitivity: 80%, specificity: 78.3%, cut-off point: 1.12, and Youden's index: 0.583 (Fig. 3B). The plasma levels of miR-21-5p and miR-93-5p combining together to predict the incidence of ISR at 6 months after surgery produced an AUC of 0.906, with a sensitivity of 84.4% and a specificity of 88.0% after assignment of weight coefficients calculated by entropy methods and multicollinearity evaluation (Fig. 3C).

Plasma levels of miR-21-5p and miR-93-5p as risk factors for developing ISR at 6 months after surgery

Multiple logistic regression analysis including complete stenosis, the plasma levels of miR-21-5p and miR-93-5p was carried out to risk factors for LEAD patients developing ISR at 6 months after surgery. The results revealed the plasma levels of miR-21-5p and miR-93-5p at 14 days after surgery were risk factors for LEAD patients developing ISR at 6 months after surgery ($P < 0.001$; Table 2).

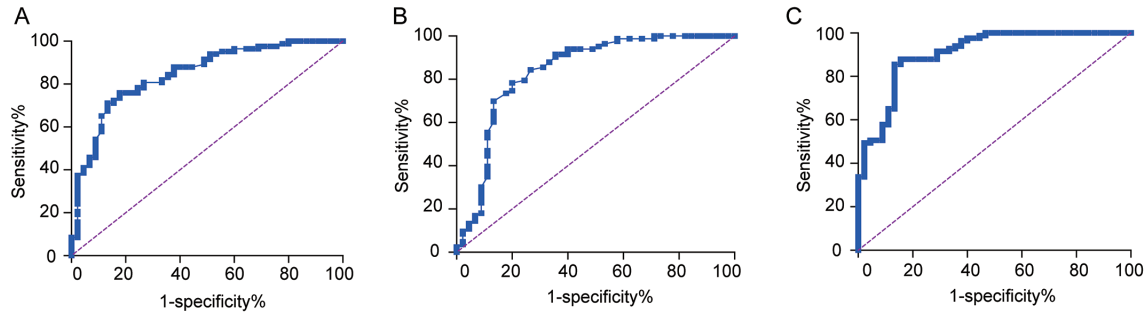

Fig. 3. Early prediction of ISR according to plasma levels of miR-21-5p and miR-93-5p.
ROC methods were carried out to plot the curve and obtain the AUC when the plasma levels of miR-21-5p and miR-93-5p at the postoperative 14th day alone or combining together were used to predict the incidence of ISR among LEAD patients at 6 months after surgery. (A) The plasma level of miR-21-5p at the postoperative 14th day was applied as a tool to predict the incidence of ISR at 6 months after surgery. (B) The plasma level of miR-93-5p at the postoperative 14th day was applied as a tool to predict the incidence of ISR at 6 months after surgery. (C) The plasma levels of miR-21-5p and miR-93-5p at the postoperative 14th day combining together as a tool to predict the incidence of ISR at 6 months after surgery.

Table 2. Multiple logistic regression analysis including complete stenosis, the plasma levels of miR-21-5p and miR-93-5p to identify risk factors for LEAD patients developing ISR at 6 months after surgery.

Variable	β	SE	Wald	P	OR	95% CI
Complete stenosis	0.434	0.418	2.316	0.265	1.523	1.198-2.028
miR-21-5p	6.159	0.863	37.077	< 0.001	32.470	15.792-78.058
miR-93-5p	4.769	0.717	30.578	< 0.001	28.485	13.270-73.250

Discussion

This study aims to investigate predictive values of two microRNAs, miR-21-5p and miR-93-5p, for ISR following endovascular stenting treatment. The findings obtained from the current study support miR-21-5p and miR-93-5p could aid in early prediction of ISR incidence among LEAD patients 6 months after surgery.

Earlier work showed that miR-21-5p has been one of miRNAs highly expressed in the vascular wall after balloon injury and promoted the proliferation and migration of VSMCs (Ji et al. 2007, Jia et al. 2019). In addition to regulating phenotypic switch of VSMCs, the overexpression of miR-21-5p also contributed to the progression of atherosclerosis by weakening macrophage infiltration and reducing smooth muscle cell hyperplasia as well as collagen content (Gao et al. 2019, Sun et al. 2019). Overexpression of miR-21-5p facilitated the spread cell area, enhanced the proliferation of human saphenous vein smooth muscle cells, and induced switch phenotypes from contractile to synthetic, promoting aberrant remodeling and intimal hyperplasia (Alshanwani et al. 2018). Our results presented a reduced expression of miR-21-5p in LEAD patients at the 14 days after stent treatment, but the ISR exhibited a higher expression of miR-21-5p compared to non-ISR, indicating upregulated miR-21-5p may be implicated in the process of LEAD. Consistent with our study, Zhang et al. (2017) demonstrated upregulated miR-21-5p the circulation of LEAD patients with predictive value for vascular restenosis after stent placement. Of note, Wang et al. (2015) and McDonald et al. (2015) demonstrated a significant inhibiting effect of modulating aberrantly elevated miR-21 levels on neo-intimal lesion formation, and the implementation of miRNAs, such as with an anti-miR-21-coated stent, effectively reduce the progression of ISR.

Previously, miR-93-5p was extensively studied as an oncogenic miRNA (Dong et al. 2023). During atherogenesis, miR-93-5p exhibited a big epigenetic change in smooth muscle cells and macrophages, highlighting its significant role in the pathogenesis of atherogenesis (Schiano et al. 2020). He et al. (2015) in their previous study provided clear evidence that serum miR-93-5p was positively correlated with the serum cholesterol level and its upregulation contributed to coronary atherosclerosis pathogenesis. As shown in our study, although LEAD patients exhibited a declined expression of miR-93-5p after stent treatment, it was elevated in ISR compared to non-ISR, suggesting miR-93-5p may be a new target for preventing ISR. Similar with our results, Feng et al. (2019) miR-93-5p was reported as a target for treating ISR as its expression was increased in VSMCs after carotid artery injury and its inhibition could reduce neointimal formation after carotid artery injury.

For both miRNAs, there was no significant effect of ISR × time interaction on the miR-21-5p and miR-93-5p expression levels at the postoperative 7th day, which suggests the plasma levels of miR-21-5p and miR-93-5p at the postoperative 14th day rather than those at the postoperative 7th day may be associated with the incidence of ISR 6

months after surgery among LEAD patients. In addition, a significant ISR × time interaction was observed on the plasma levels of miR-21-5p and miR-93-5p for time points of 7 days and 14 days post-surgery. Perhaps the cause for absence of ISR × time interaction at the postoperative 7th day is relatively small sample size. More importantly, our Multiple logistic regression analysis found that plasma levels of miR-21-5p and miR-93-5p at the postoperative 14th day as risk factors for developing ISR at 6 months after surgery.

There are several limitations needed to be taken into account. First, relatively sample size due to the difficulty of recruiting patients with ISR 6 months after surgery from a single hospital creates a need of a large-scale and multicenter study. Secondly, the definition of ISR was ≥ 50% diameter narrowing within the stented segment rather than ≥ 70% in other reports and the definition of successful interventional treatment was < 30% diameter narrowing rather than 10% in other reports, which may lead to a relatively high rate of ISR among LEAD patients 6 months after interventional therapy. Thirdly, the specific roles of miR-21-5p and miR-93-5p has not been analyzed with regard to the regulation of gene expression in human arteries. Finally, long-term prediction of circulating miR-21-5p and miR-93-5p for developing ISR among LEAD patients such as 1 year after stenting treatment should be further studied.

In conclusion, our study reveals miR-21-5p and miR-93-5p detected 14 days after stenting treatment has further circulating biomarker potential as a predictor of ISR among LEAD patients. These two miRNAs would help us to fully understand the clinical treatment and prognosis judgment in the treatment of LEAD patients and implies a miRNA-related mechanism common to development of ISR in the context of LEAD, which needs to be further explored.

Conflict of Interest

The authors declare no conflict of interest.

References

Alshanwani, A.R., Riches-Suman, K., O'Regan, D.J., Wood, I.C., Turner, N.A. & Porter, K.E. (2018) MicroRNA-21 drives the switch to a synthetic phenotype in human saphenous vein smooth muscle cells. *IUBMB Life*, **70**, 649-657.

Annex, B.H. & Cooke, J.P. (2021) New Directions in Therapeutic Angiogenesis and Arteriogenesis in Peripheral Arterial Disease. *Circ. Res.*, **128**, 1944-1957.

Clare, J., Ganly, J., Bursill, C.A., Sumer, H., Kingshott, P. & de Haan, J.B. (2022) The Mechanisms of Restenosis and Relevance to Next Generation Stent Design. *Biomolecules*, **12**, 430.

Criqui, M.H., Matsushita, K., Aboyans, V., Hess, C.N., Hicks, C.W., Kwan, T.W., McDermott, M.M., Misra, S., Ujueta, F., American Heart Association Council on, E., Prevention, Council on Arteriosclerosis, T., Vascular, B., Council on Cardiovascular, R., Intervention, et al. (2021) Lower Extremity Peripheral Artery Disease: Contemporary Epidemiology, Management Gaps, and Future Directions: A Scientific Statement From the American Heart Association. *Circulation*, **144**, e171-e191.

Dong, Z.R., Cai, J.B., Shi, G.M., Yang, Y.F., Huang, X.Y., Zhang, C., Dong, R.Z., Wei, C.Y., Li, T., Ke, A.W. & Fan, J. (2023) Oncogenic miR-93-5p/Gal-9 axis drives CD8 (+) T-cell inactivation and is a therapeutic target for hepatocellular carcinoma immunotherapy. *Cancer Lett.*, **564**, 216186.

Feng, S., Gao, L., Zhang, D., Tian, X., Kong, L., Shi, H., Wu, L., Huang, Z., Du, B., Liang, C., Zhang, Y. & Yao, R. (2019) MiR-93 regulates vascular smooth muscle cell proliferation, and neointimal formation through targeting Mfn2. *Int. J. Biol. Sci.*, **15**, 2615-2626.

Gao, L., Zeng, H., Zhang, T., Mao, C., Wang, Y., Han, Z., Chen, K., Zhang, J., Fan, Y., Gu, J. & Wang, C. (2019) MicroRNA-21 deficiency attenuated atherogenesis and decreased macrophage infiltration by targeting Dusp-8. *Atherosclerosis*, **291**, 78-86.

He, Y., Lin, L., Cao, J., Mao, X., Qu, Y. & Xi, B. (2015) Up-regulated miR-93 contributes to coronary atherosclerosis pathogenesis through targeting ABCA1. *Int. J. Clin. Exp. Med.*, **8**, 674-681.

Hirsch, A.T., Haskal, Z.J., Hertzer, N.R., Bakal, C.W., Creager, M.A., Halperin, J.L., Hiratzka, L.F., Murphy, W.R., Olin, J.W., Puschett, J.B., Rosenfield, K.A., Sacks, D., Stanley, J.C., Taylor, L.M. Jr., White, C.J., et al. (2006) ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation*, **113**, e463-654.

Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., Dean, D.B. & Zhang, C. (2007) MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ. Res.*, **100**, 1579-1588.

Jia, S., Ma, W.D., Zhang, C.Y., Zhang, Y., Yao, Z.H., Quan, X.H., Guo, X. & Wang, C.X. (2019) Tanshinone IIA attenuates high glucose induced human VSMC proliferation and migration through miR-21-5p-mediated tropomyosin 1 downregulation. *Arch. Biochem. Biophys.*, **677**, 108154.

Levin, S.R., Arinze, N. & Siracuse, J.J. (2020) Lower extremity critical limb ischemia: A review of clinical features and management. *Trends Cardiovasc. Med.*, **30**, 125-130.

Lian, W., Nie, H., Yuan, Y., Wang, K., Chen, W. & Ding, L. (2021) Clinical Significance of Endothelin-1 And C Reaction Protein in Restenosis After the Intervention of Lower Extremity Arteriosclerosis Obliterans. *J. Invest. Surg.*, **34**, 765-770.

Maren, N.A., Duduit, J.R., Huang, D., Zhao, F., Ranney, T.G. & Liu, W. (2023) Stepwise Optimization of Real-Time RT-PCR Analysis. *Methods Mol. Biol.*, **2653**, 317-332.

McDermott, M.M., Ho, K.J., Alabi, O., Criqui, M.H., Goodney, P., Hamburg, N., McNeal, D.M., Pollak, A., Smolderen, K.G. & Bonaca, M. (2023) Disparities in Diagnosis, Treatment, and Outcomes of Peripheral Artery Disease: JACC Scientific Statement. *J. Am. Coll. Cardiol.*, **82**, 2312-2328.

McDonald, R.A., Halliday, C.A., Miller, A.M., Diver, L.A., Dakin, R.S., Montgomery, J., McBride, M.W., Kennedy, S., McClure, J.D., Robertson, K.E., Douglas, G., Channon, K.M., Oldroyd, K.G. & Baker, A.H. (2015) Reducing In-Stent Restenosis: Therapeutic Manipulation of miRNA in Vascular Remodeling and Inflammation. *J. Am. Coll. Cardiol.*, **65**, 2314-2327.

Montelione, N., Catanese, V., Nenna, A., Jawabra, M., Verghi, E., Loreni, F., Nappi, F., Lusini, M., Mastroianni, C., Jiritano, F.,

Serraino, G.F., Mastroroberto, P., Codispoti, F.A., Chello, M., Spinelli, F., et al. (2022) The Diagnostic Value of Circulating Biomarkers and Role of Drug-Coated Balloons for In-Stent Restenosis in Patients with Peripheral Arterial Disease. *Diagnostics* (*Basel*), **12**, 2207.

Muller, A., Bradaric, C., Kafka, A., Joner, M., Cassese, S., Xhepa, E., Kufner, S., Kastrati, A., Laugwitz, K.L., Ibrahim, T. & Koppara, T. (2023) Prevalence and patterns of in-stent neoatherosclerosis in lower extremity artery disease. *EuroIntervention*, **18**, 1462-1470.

Nicolais, C., Lakhter, V., Virk, H.U.H., Sardar, P., Bavishi, C., O'Murchu, B. & Chatterjee, S. (2018) Therapeutic Options for In-Stent Restenosis. *Curr. Cardiol. Rep.*, **20**, 7.

O'Sullivan, J.F., Neylon, A., Fahy, E.F., Yang, P., McGorrian, C. & Blake, G.J. (2019) MiR-93-5p is a novel predictor of coronary in-stent restenosis. *Heart Asia*, **11**, e011134.

Polonsky, T.S. & McDermott, M.M. (2021) Lower Extremity Peripheral Artery Disease Without Chronic Limb-Threatening Ischemia: A Review. *JAMA*, **325**, 2188-2198.

Raitoharju, E., Lyytikainen, L.P., Levula, M., Oksala, N., Mennander, A., Tarkka, M., Klopp, N., Illig, T., Kahonen, M., Karhunen, P.J., Laaksonen, R. & Lehtimaki, T. (2011) miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. *Atherosclerosis*, **219**, 211-217.

Schiano, C., Benincasa, G., Franzese, M., Della Mura, N., Pane, K., Salvatore, M. & Napoli, C. (2020) Epigenetic-sensitive pathways in personalized therapy of major cardiovascular diseases. *Pharmacol. Ther.*, **210**, 107514.

Shishehbor, M.H., Powell, R.J., Montero-Baker, M.F., Dua, A., Martinez-Trabal, J.L., Bunte, M.C., Lee, A.C., Mugglin, A.S., Mills, J.L., Farber, A., Clair, D.G. & Investigators, P.I. (2023) Transcatheter Arterialization of Deep Veins in Chronic Limb-Threatening Ischemia. *N. Engl. J. Med.*, **388**, 1171-1180.

Shu, X., Mao, Y., Li, Z., Wang, W., Chang, Y., Liu, S. & Li, X.Q. (2019) MicroRNA-93 regulates angiogenesis in peripheral arterial disease by targeting CDKN1A. *Mol. Med. Rep.*, **19**, 5195-5202.

Stojkovic, S., Jurisic, M., Kopp, C.W., Koppensteiner, R., Huber, K., Wojta, J. & Gremmel, T. (2018) Circulating microRNAs identify patients at increased risk of in-stent restenosis after peripheral angioplasty with stent implantation. *Atherosclerosis*, **269**, 197-203.

Sufianov, A., Begliarzade, S., Kudriashov, V., Nafikova, R., Ilyasova, T. & Liang, Y. (2023) Role of miRNAs in vascular development. *Noncoding RNA Res.*, **8**, 1-7.

Sun, P., Tang, L.N., Li, G.Z., Xu, Z.L., Xu, Q.H., Wang, M. & Li, L. (2019) Effects of MiR-21 on the proliferation and migration of vascular smooth muscle cells in rats with atherosclerosis via the Akt/ERK signaling pathway. *Eur. Rev. Med. Pharmacol. Sci.*, **23**, 2216-2222.

Tang, H.Y., Chen, A.Q., Zhang, H., Gao, X.F., Kong, X.Q. & Zhang, J.J. (2022) Vascular Smooth Muscle Cells Phenotypic Switching in Cardiovascular Diseases. *Cells*, **11**, 4060.

Wang, D., Deuse, T., Stubbendorff, M., Chernogubova, E., Erben, R.G., Eken, S.M., Jin, H., Li, Y., Busch, A., Heeger, C.H., Behnisch, B., Reichenspurner, H., Robbins, R.C., Spin, J.M., Tsao, P.S., et al. (2015) Local MicroRNA Modulation Using a Novel Anti-miR-21-Eluting Stent Effectively Prevents Experimental In-Stent Restenosis. *Arterioscler. Thromb. Vasc. Biol.*, **35**, 1945-1953.

Zhang, B., Yao, Y., Sun, Q.F., Liu, S.Q., Jing, B., Yuan, C., Liu, X.Y., Jiao, T., Li, H.C. & Wang, H.Y. (2017) Circulating mircoRNA-21 as a predictor for vascular restenosis after interventional therapy in patients with lower extremity arterial occlusive disease. *Biosci. Rep.*, **37**, BSR20160502.