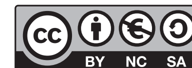
J Atheroscler Thromb, 2017; 24: 430-441.
doi: 10.5551/jat.35923

Original Article



Potential Role of Lipometabolism-Related MicroRNAs in Peripheral Blood Mononuclear Cells as Biomarkers for Coronary Artery Disease

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Aim: **To explore the relationship between lipometabolism-related microRNAs (miRNAs) in peripheral blood mononuclear cells (PBMCs) and the presence of coronary artery disease (CAD).**
Methods: **In the present study, 161 stable CAD patients and 149 health controls were enrolled. The expression levels of seven miRNAs (miR-21, miR-24, miR-29a, miR-33a, miR-34a, miR-103a, and miR-122) in PBMCs were qualified by quantitative real-time polymerase chain reaction (*q*RT-PCR). The miRNA markers that showed significant difference between the two groups were used for further analysis. The risk of miRNA contributing to the presence of CAD was estimated by univariate and multivariate logistic regression models. The area under the receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy.**
Results: **The expression levels of miR-24, miR-33a, miR-103a, and miR-122 in PBMCs were significantly increased in CAD patients compared with controls and were significantly correlated with blood lipids in both CAD patients and controls. The increased levels of miR-24 (adjusted OR=1.32, 95% CI 1.07−1.62, P=0.009), miR-33a (adjusted OR=1.57, 95% CI 1.35−1.81, $P<0.001$), miR-103a (adjusted OR=1.01, 95% CI 1.01−1.02, $P<0.001$), and miR-122 (adjusted OR=1.03, 95% CI 1.01−1.04, $P<0.001$) were associated with risk of CAD. We identified a miRNA panel (miR-24, miR-33, miR-103a, and miR-122) that provided a high diagnostic accuracy of CAD (AUC=0.911, 95% CI 0.880−0.942).**
Conclusion: **The increased expression levels of miR-24, miR-33a, miR-103a, and miR-122 in PBMCs are associated with risk of CAD. A panel of the four miRNAs has considerable clinical value in diagnosing stable CAD.**

Key words: MicroRNA, Coronary artery disease, Lipometabolism, Mononuclear leucocytes, Biomarker

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Introduction

Coronary artery disease (CAD) is currently a major cause of mortality and morbidity in both men and women in many countries, including China[1]. Atherosclerosis, which is the pathogenetic basis of CAD, is characterized by accumulation of lipid in the arterial wall resulting in narrowing of the vessel lumen[2]. Disorders of lipid homeostasis are common risk factors for cardiovascular disease, and dyslipidemia has been confirmed to play an important role in the development and progression of CAD[3]. Reductions in the levels of atherogenic lipoproteins such as low-density lipoprotein cholesterol (LDLC) are associated with substantially decreased risk for CAD[4].

MicroRNAs (miRNAs) are a class of highly conserved, noncoding small RNAs that usually comprise 19−24 nucleotides. These RNAs play a vital role in the regulation of gene expression on the post-transcriptional level by inhibiting the translation of protein from mRNA or by inducing mRNA degrada-

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Received: April 7, 2016
Accepted for publication: July 28, 2016

Table 1. Demographic and clinical characteristics of study subjects

Variable	CAD (n = 161)	Control (n = 149)	t	P
Age (year)	61.35 ± 7.10	61.08 ± 7.51	0.330	0.742
Gender (male/female)	86/75	72/77	0.804[a]	0.370
BMI	25.77 ± 3.06	24.81 ± 3.29	2.664	0.008
SBP (mmHg)	130.39 ± 16.83	128.08 ± 19.43	1.122	0.263
DBP (mmHg)	81.11 ± 10.42	77.56 ± 12.36	2.734	0.007
FPG (mmol/L)	6.01 ± 1.77	5.16 ± 0.97	5.168	<0.001
TC (mmol/L)	5.14 ± 1.04	4.30 ± 0.74	8.124	<0.001
TG (mmol/L)	1.74 ± 1.23	1.05 ± 0.55	6.239	<0.001
HDLC (mmol/L)	1.39 ± 0.51	1.69 ± 0.52	5.080	<0.001
LDLC (mmol/L)	3.13 ± 0.93	2.36 ± 0.59	8.642	<0.001
HbA1c (%)	5.21 ± 0.59	5.65 ± 1.04	4.657	<0.001
Smoking (n, %)	22, 13.66	19, 12.75	0.056[a]	0.813
Alcohol use (n, %)	30, 18.63	25, 16.78	0.182[a]	0.669
Physical activity (n, %)	105, 65.22	111, 74.50	3.154[a]	0.076

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fast plasma glucose, TC: total cholesterol, TG: triglyceride, HDLC: high-density lipoprotein cholesterol, LDLC: low-density lipoprotein cholesterol.
[a] χ^2 value

tion[5]. In recent years, accumulating data from *in vivo* and *in vitro* studies have identified that several miRNAs, including miR-33, miR-24, miR-34a, miR-103a, and miR-122, play crucial roles in regulating lipid metabolism. Several key genes involved in cholesterol synthesis/transport and fatty acid metabolism were found to be regulated by miR-33. Experiments performed in both normal and high-fat diet-fed mice demonstrated that inhibition of miR-33 results in an increase in circulating HDLC[6]. Ng R *et al.* report that the expression of miR-24 is significantly increased in the livers of high-fat diet-treated mice and in isolated human hepatocytes incubated with fatty acid. Knockdown of miR-24 in these mice resulted in impaired hepatic lipid accumulation and reduced plasma triglycerides[7]. MiR-34a is considered to be involved in nonalcoholic fatty liver disease (NAFLD). MiR-34a inhibits very low-density lipoprotein secretion and promotes liver steatosis and hypolipidemia in a hepatocyte nuclear factor 4α (HNF4α)-dependent manner[8]. Trajkovski *et al.* demonstrated that miR-103 silencing improved insulin sensitivity in adipose tissue of mice and demonstrated an association between miR-103 and body weight. The results suggest that miR-103 could increase lipolysis and elevate circulating free fatty acid levels by repression of caveolin-1[9]. Another miRNA, miR-122, was also found to have prominent effects on lipid metabolism[10].

To date, circulating miRNAs have been considered as promising novel biomarkers for cardiovascular diseases, particularly CAD[11]. Circulating monocytes are important cells that participate in practically all stages of atherogenesis[12]. Various cross-sectional and interventional studies in different patient cohorts demonstrated monocyte subset-specific interactions with different lipoprotein subclasses[13]. Considering the crucial role of hyperlipidemia and circulating monocytes in the development of CAD, dysregulation of lipometabolism-related miRNAs in peripheral blood mononuclear cells (PBMCs) may also be related to the presence of CAD. In addition, PBMCs circulating throughout the body maintain close contact with various body organs and may be considered as an appropriate source for disease related miRNA expression signature[14]. Prior studies demonstrated that plasma/serum levels of some miRNAs increase or decrease in CAD patients; however, expression level of lipometabolism-related miRNAs in PBMCs have not been evaluated.

In this study, a panel of seven miRNAs (miR-21, miR-24, miR-29a, miR-33a, miR-34a, miR-103a, and miR-122) which have been report to be associated with lipid metabolism both in patients and animal models[15, 16] was selected. To explore the clinical value including risk and diagnostic accuracy for the presence of CAD, the expression level of the seven lipometabolism-related miRNAs in PBMCs were measured in CAD patients and healthy controls. The study focused on these miRNAs because they have been report to be associated with lipid metabolism both in patients and animal models.

Table 2. Comparison of miRNAs level between CAD group and control group

	CAD group	Control group	*t*	*P*
miR-21[a]	31.87 ± 37.46	41.38 ± 54.73	1.494[b]	0.136
	17.03 (8.47-39.26)	20.11 (8.51-48.67)		
miR-24[a]	3.45 ± 2.48	2.16 ± 1.37	7.022[b]	0.001
	2.69 (1.94-3.72)	1.74 (1.30-2.68)		
miR-29a[a]	5.37 ± 4.26	6.23 ± 5.21	1.193[b]	0.234
	4.41 (2.70-6.17)	4.69 (2.55-7.86)		
miR-33a[a]	12.84 ± 4.91	8.55 ± 2.67	9.590[b]	< 0.001
	11.79 (9.79-14.78)	8.47 (6.56-10.66)		
miR-34a[a]	255.55 ± 289.18	228.65 ± 252.91	1.623[b]	0.106
	162.02 (86.17-343.59)	132.51 (66.95-283.20)		
miR-103a[a]	56.82 ± 76.78	22.21 ± 25.83	6.561[b]	< 0.001
	26.54 (11.98-56.09)	15.03 (6.70-26.23)		
miR-122[a]	49.68 ± 39.48	23.91 ± 20.73	8.152[b]	< 0.001
	38.81 (21.43-67.13)	21.66 (8.59-32.96)		

[a] Data are represented as means ± SD and median (25−75 percentiles).
[b] Skewed distributed and analyzed by log-transformed values.

Materials and Methods

Subjects

A total of 161 stable CAD patients (40−75-year old) were consecutively recruited from outpatients who admitted to the Xuanwu Hospital, Capital Medical during March−July 2015. Diagnosis of CAD was confirmed by coronary angiography[17]. Coronary angiograms were evaluated independently by two operators, who made visual estimation of luminal narrowing in multiple segments based on a modified form of the AHA/ACC classification of the coronary tree. Using these data, significant CAD was defined as at least one major epicardial vessel with > 50% stenosis, assessed by quantitative coronary angiography. Depending on the extent of epicardial coronary artery involvement, CAD patients were divided into single-, double-, and triple-vessel disease subgroups in accordance with the Coronary Artery Surgery Study classification. The exclusion criteria for CAD patients were as follows: previous history of acute myocardial infarction (AMI), elevated cardiac troponin I (cTnI) or creatine kinase (CK-MB) levels, impaired left ventricular ejection fraction (LVEF) (≤ 45%), cardiac arrhythmias and congestive heart failure. We simultaneously recruited 149 health control subjects without any evidence of CAD or dyslipidemia from those who take routine physical examination in the hospital. Cases and controls were frequency matched according to age (± 3 years), gender, smoking, and drinking. All individuals had no cardiomyopathy or congenital heart, severe hepatic and renal dysfunction, inflammatory and malignant disease, bleeding disorders, and any major operation within the previous month. Subjects with a history of using lipid lowering (statin agents) or anti-diabetic and antihypertensive drugs were also excluded. This study was approved by the university ethical committees (approval number: 2015SY27) and informed consent was obtained from each participant.

A structured questionnaire was used to collect information on demographic data, environmental exposure and medical histories. Current cigarette smokers were defined as those who smoked ≥ 1 cigarette/day. Alcohol use was defined as intake of wine/beer/cider/spirits ≥ 1 time per week. Physical activity was defined as walking or riding 15 min/day, or doing sports or physical exercise > 2 h/week, or lifting or carrying heavy objects at work daily[18].

Blood Samples Collection

Following an overnight fast, venous blood samples were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) from each subject. Blood samples were immediately centrifuged and plasma was preserved at − 80℃. PBMCs were subsequently isolated by Ficoll-Hypaque density gradient centrifugation. Immediately after, $5-8 \times 10^6$ cells per ml of Trizol (Invitrogen, USA) were stored at − 80℃ until use[19].

Laboratory Measurement

Total cholesterol (TC), triglycerides (TG), and high-density lipoprotein-cholesterol (HDLC) were

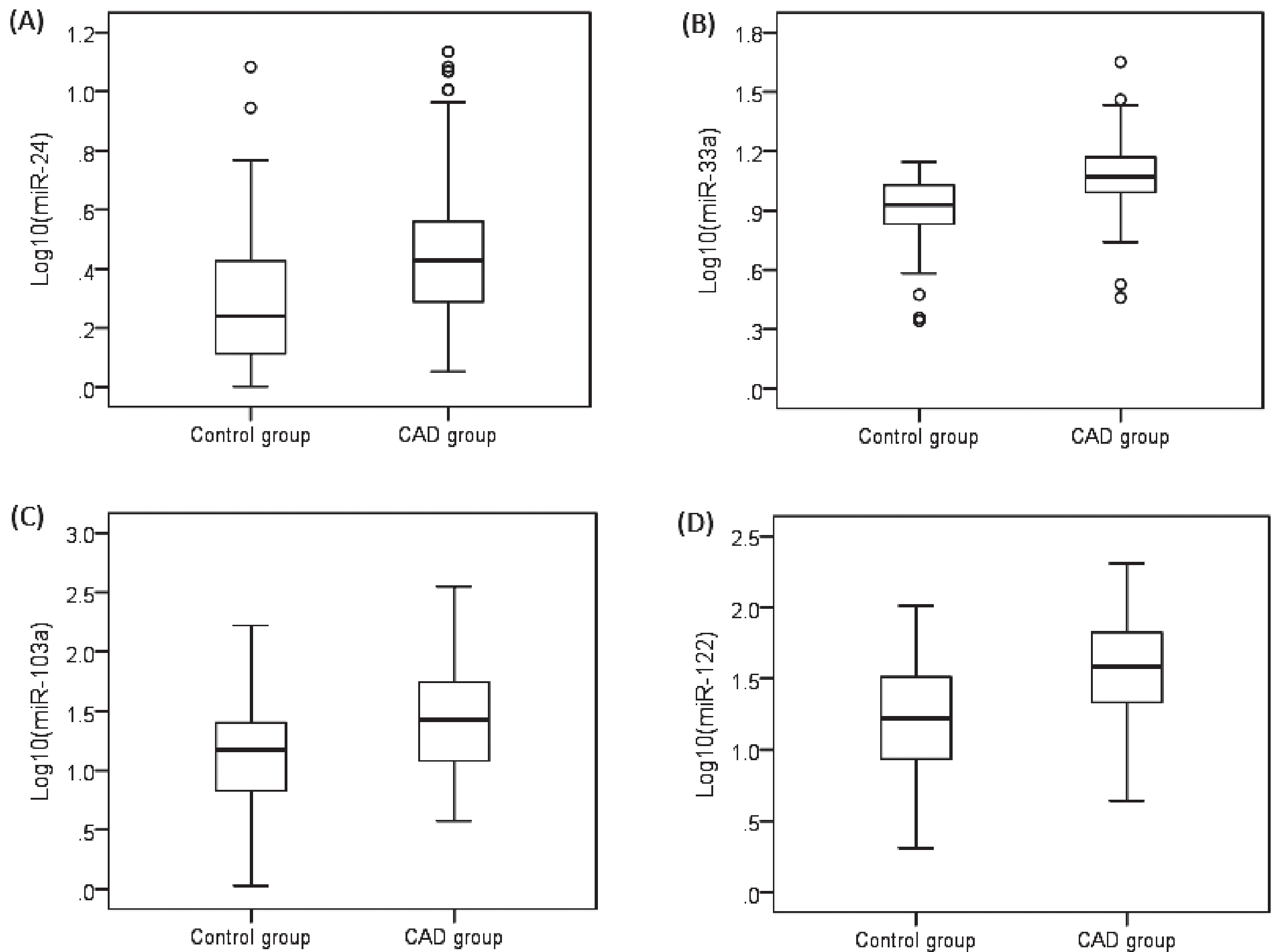

Fig. 1. Expression levels of miRNAs in PBMCs between CAD group and control group.

measured using standard laboratory methods (Hitachi autoanalyzer 7060; Hitachi, Japan). Low-density lipoprotein-cholesterol (LDLC) was calculated using the Friedewald method. Fasting serum glucose levels were measured by the glucose oxidase method. Glycated hemoglobin (HbAlc) was estimated by high-pressure liquid chromatography method (Tosoh Corporation, Tokyo, Japan).

RNA Extraction

Total RNA was extracted from PBMCs using Trizol according to the manufacturer's specifications. The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. RNA electrophoresis and the A260/280 ratio (ranging from 1.80 to 2.22) indicated that both integrity and purity of the isolated RNA samples were acceptable.

Quantitative Real-Time PCR

Quantification was performed with a two-step reaction process: reverse transcription (RT) and PCR. Each RT reaction consisted of 1 μg RNA, 4 μl of miScript HiSpec Buffer, 2 μl of Nucleics Mix and 2 μl of miScript Reverse Transcriptase Mix (Qiagen, Germany), in a total volume of 20 μl. Reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) for 60 min at 37℃, followed by heat inactivation of RT for 5 min at 95℃. The 20 μl RT reaction mix was then diluted 5× in nuclease-free water and held at −20℃.

Real-time PCR was performed using LightCycler® 480 Ⅱ Real-time PCR Instrument (Roche, Swiss) with 10 μl PCR reaction mixture that included 1 μl of cDNA, 5 μl of 2×LightCycler® 480 SYBR Green I Master (Roche, Swiss), 0.2 μl of universal primer (Qiagen, Germany), 0.2 μl of miRNA-specific primer and 3.6 μl of nuclease-free water. Reactions

Table 3. Spearman correlation among the selected miRNAs

	TC	TG	HDLC	LDLC
Total subjects				
miR-24	0.242**	0.334**	−0.120*	0.267**
miR-33a	0.161**	0.224**	−0.283**	0.311**
miR-103a	0.311**	0.208**	−0.039	0.254**
miR-122	0.349**	0.261**	−0.090	0.317**
CAD group				
miR-24	0.221**	0.159*	0.026	0.207**
miR-33a	0.173*	0.059	−0.167*	0.024
miR-103a	0.180*	0.022	−0.127	0.171*
miR-122	0.240**	0.124	0.074	0.207**
Control group				
miR-24	0.161*	0.218**	−0.054	0.016
miR-33a	0.060	0.072	−0.163*	0.239**
miR-103a	0.218**	0.113	−0.025	0.093
miR-122	0.171*	0.077	0.013	0.108

*$P<0.05$, **$P<0.001$.
TC: total cholesterol, TG: triglyceride, HDLC: high-density lipoprotein cholesterol, LDLC: low-density lipoprotein cholesterol.

were incubated in a 384-well optical plate (Roche, Swiss) at 95℃ for 10 min, followed by 40 cycles of 95℃ for 10 s, 60℃ for 30 s. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The miRNA-specific primer sequences (**Supplementary Table 1**) were designed in the laboratory and synthesized by Generay Biotech (Generay, PRC) based on the miRNA sequences obtained from the miRBase database (Release 20.0).

All samples were performed in triplicate and the mean values of the triplicates were taken as the final result. The expression levels of miRNAs were normalized to RNU6B and were calculated using the $2^{-\Delta Ct}$ method [ΔCt = mean Ct (miRNA of interest) − mean Ct (U6)].

Statistical Analysis

The differences of demographic and clinical parameters between the CAD cases and healthy controls were evaluated using Student's *t* test or χ^2 test. Logarithmic transformations were made if data were not normally distributed. Those miRNA markers that showed significant difference between the two groups were used for further analysis. For comparison of more than two groups, one-way ANOVA and LSD (least significant difference) tests were used as appropriate. The strength and direction of the relationship between miRNA markers and blood lipids were analyzed by the Spearman's correlation analysis. The risk of miRNAs contributed to the presence of CAD were estimated by computing odds ratios (ORs) and their 95% confidence intervals (95% CIs) from both univariate and multivariate logistic regression models, in which each miRNA expression level itself was used as a variable factor with or without adjustment for covariates. Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic accuracy of each miRNA. The area under the ROC curve (AUC) was used as diagnostic index. The diagnostic performance of the selected miRNA panel was further evaluated using the predicted probability of being diagnosed with CAD as a surrogate marker to construct ROC curve. A *P* value of less than 0.05 was considered statistical significance. The reported *P* values were two-tailed in all calculations. All statistical analyses were performed using SPSS 20.0 (IBM SPSS, Inc., Chicago, USA).

Results

Basic Characteristics of the Study Subjects

The demographic and clinical characteristics of the CAD patients and healthy controls were presented in **Table 1**. There was no significant difference between the two groups for a series of demographic parameters, including age, gender, smoking, alcohol use, and physical activity ($P>0.05$). BMI, DBP, plasma levels of TC, TG, and LDLC, and the FBG level were higher in subjects with CAD ($P<0.05$), whereas plasma levels of HDLC were found to be significantly lower in subjects with CAD than in controls

Table 4. Univariate analysis and multiple logistic regression analysis for the risk of CAD

Variable	OR (95%CI)	*P* value	OR* (95%CI)	*P* value
miR-24				
Univariate analysis	1.59 (1.31, 1.94)	<0.001	2.69 (1.78, 4.07)	<0.001
Multiple logistic regression model 1[a]	1.60 (1.31, 1.94)	<0.001	2.71 (1.79, 4.11)	<0.001
Multiple logistic regression model 2[b]	1.57 (1.29, 1.91)	<0.001	2.61 (1.73, 3.95)	<0.001
Multiple logistic regression model 3[c]	1.32 (1.07, 1.62)	0.009	1.76 (1.16, 2.70)	0.009
miR-33a				
Univariate analysis	1.49 (1.34, 1.65)	<0.001	6.05 (3.82, 9.58)	<0.001
Multiple logistic regression model 1[a]	1.50 (1.35, 1.67)	<0.001	6.33 (3.95, 10.14)	<0.001
Multiple logistic regression model 2[b]	1.49 (1.35, 1.66)	<0.001	6.17 (3.85, 9.91)	<0.001
Multiple logistic regression model 3[c]	1.57 (1.35, 1.81)	<0.001	7.84 (4.06, 15.14)	<0.001
miR-103a				
Univariate analysis	1.02 (1.01, 1.03)	<0.001	2.74 (1.71, 4.40)	<0.001
Multiple logistic regression model 1[a]	1.02 (1.01, 1.03)	<0.001	2.79 (1.75, 4.42)	<0.001
Multiple logistic regression model 2[b]	1.02 (1.01, 1.03)	<0.001	2.73 (1.72, 4.34)	<0.001
Multiple logistic regression model 3[c]	1.01 (1.01, 1.02)	<0.001	2.39 (1.49, 3.81)	<0.001
miR-122				
Univariate analysis	1.03 (1.02, 1.04)	<0.001	3.11 (2.15, 4.48)	<0.001
Multiple logistic regression model 1[a]	1.03 (1.02, 1.04)	<0.001	3.18 (2.19, 4.62)	<0.001
Multiple logistic regression model 2[b]	1.04 (1.02, 1.05)	<0.001	3.33 (2.27, 4.90)	<0.001
Multiple logistic regression model 3[c]	1.03 (1.01, 1.04)	<0.001	2.45 (1.54, 3.90)	<0.001

[a]The model included age, gender, smoking, drink and exercise.
[b]The model included age, gender, smoking, drink, exercise and BMI.
[c]The model included age, gender, smoking, drink, exercise and BMI, TC, TG, HDLC, LDLC, DBP and GLU.
OR: odds ratio, CI: confidence interval (OR means the times of greater risk of presenting CAD with a unit increase of each miRNA).
*: OR was calculated by using standardized miRNA levels.

($P<0.05$). By coronary angiography, 53 (32.9%) CAD cases had single-vessel disease, 76 (47.2%) had double-vessel disease and 32 (19.9%) had triple-vessel disease. The average LVEF (%) of the CAD patients was 63.41±5.76.

MiRNA Expression in the PBMCs of CAD Patients and Healthy Controls

The expression level of the seven selected miRNAs in the PBMCs of CAD patients and healthy controls were listed in **Table 2**. The expression levels of the miRNAs were logarithmic transformed for t-test. There are four miRNAs including miR-24, miR-33a, miR-103a, and miR-122 showed significant higher expression level among CAD patients than that among healthy controls ($P<0.001$, **Fig. 1**). However, no significant difference was found for expression level of miR-21, miR-29a, and miR-34a between the two groups ($P>0.05$). As the potential biomarker of CAD, the risk and clinical significance of miR-24, miR-33a, miR-103a, and miR-122 were evaluated in the further statistical analyses.

For the CAD patients, only the expression of miR-122 showed significant difference ($F=3.594$, $P=0.030$) among the three subgroups (single-, double-, and triple-vessel disease). The levels of miR-122 in patients with three-vessel disease were significantly higher than those with one- or two-vessel disease (66.06±50.18 vs. 44.38±28.67, $P=0.014$ and 66.06±50.18 vs. 46.47±39.62, $P=0.018$, respectively). However, there are no significant difference between patients with one-vessel and two-vessel disease ($P=0.765$).

The Correlation of miRNA Expression with Blood Lipids

The spearman correlation analysis showed that blood lipids are significantly associated with miR-24, miR-33a, miR-103a, and miR-122 in either CAD patients or health controls. As the results listed in **Table 3**, we found that miR-24 was positively correlated with TC ($P<0.05$) and TG ($P<0.05$), while miR-33a was negatively related to HDLC ($P<0.05$). Moreover, expression level of miR-103a and miR-122 were found positively related to TC ($P<0.05$). As an important risk factor of CAD, LDLC was significantly associated with miR-24, miR-103a, and miR-122 in CAD patients ($P<0.05$).

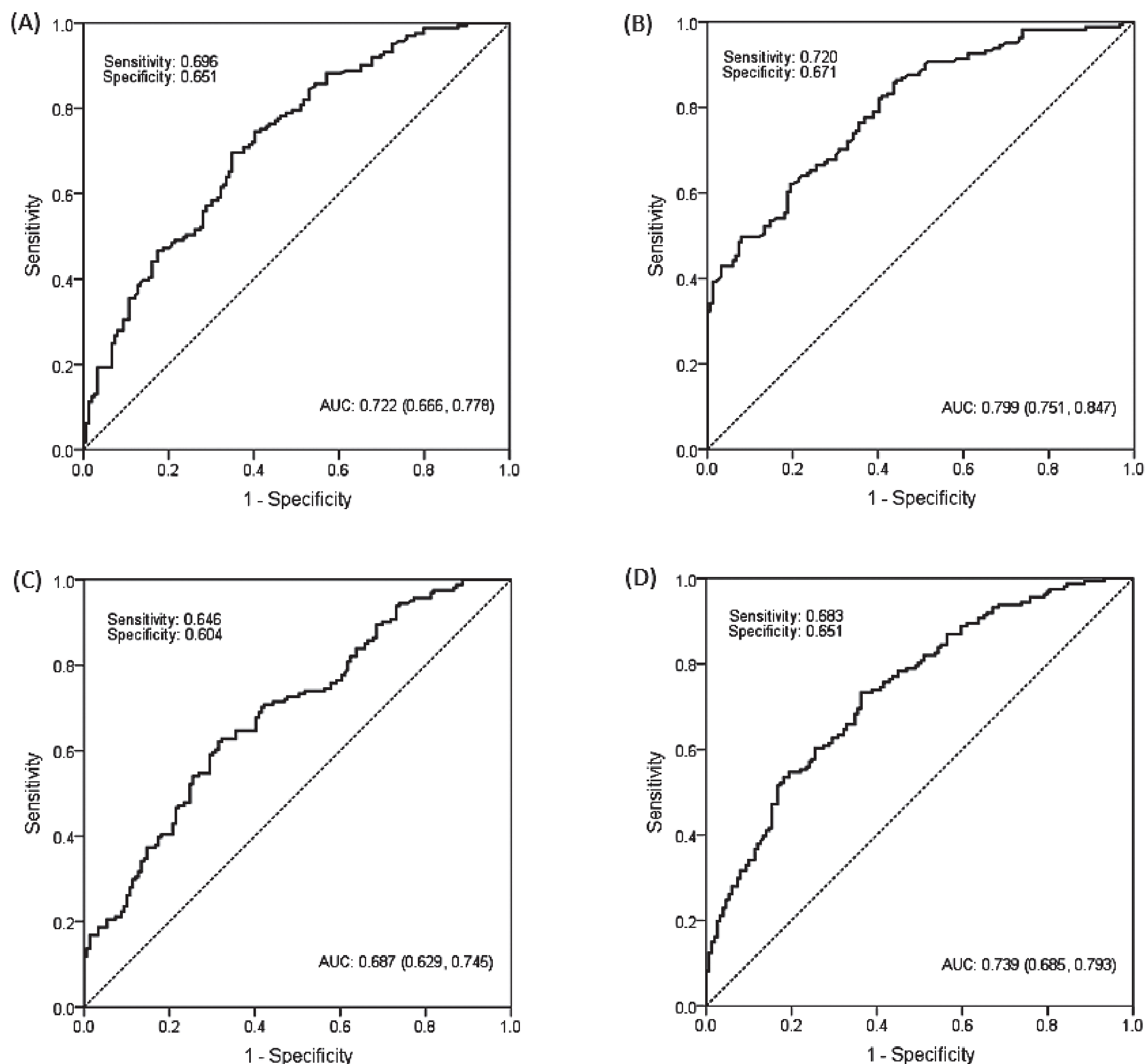

Fig. 2. Receiver operating characteristic (ROC) curve analysis for CAD diagnosis. Area under the curve (AUC) estimation for the microRNAs: (A) miR-24, (B) miR-33a, (C) miR-103a, and (D) miR-122.

Risk of miRNA Expression Contributed to the Presence of CAD

Univariate logistic regression revealed that expression levels of the four miRNA markers were positively associated with the presence of CAD ($P<0.05$) (**Table 4**). After corrected for possible confounding variables, these miRNAs remained significantly associated with CAD ($P<0.05$) (**Table 4**). With a unit increase of miRNA-33a level, there was 1.57 (95% CI 1.35−1.81) times of greater risk of presenting CAD after adjustment for age, gender, smoking, drink, exercise and BMI, TC, TG, HDLC, LDLC, DBP, and GLU. With a unit increase of miRNA-24 level, there was 1.32 (95% CI 1.07, 1.62) times of greater risk of presenting CAD. To compared the magnitude of the associations between the miRNAs and CAD, the ORs* were calculated using standardized miRNA levels. Compared with the other three miRNAs (miR-24, miR-103a, and miR-122), miR-33a showed the highest level of association with CAD. Secondly, miR-122

showed a higher level of risk for the presence of CAD when comparing with miR-24 and miR-103a.

The Diagnostic Accuracy of the miRNAs

The diagnostic accuracy of miR-24, miR-33a, miR-103a, and miR-122, measured by AUC, was 0.722 (cutoff value: 2.10), 0.799 (cutoff value: 9.91), 0.687 (cutoff value: 18.59), and 0.739 (cutoff value: 25.85), respectively ($P<0.05$). The corresponding sensitivity and specificity was presented in **Fig. 2**. As compared with the other three miRNAs, miR-33a is a more valuable biomarker for diagnosis for CAD.

Establishing the Predictive miRNA Panel

A stepwise logistic regression model to estimate the risk of being diagnosed with CAD was applied. All the four miRNAs turned out to be significant predictors ($P<0.05$). The predicted probability of being diagnosed with CAD from the logit model based on the four miRNA panel, logit (P=CAD) = −6.693 + 0.412 × miR-24 + 0.381 × miR-33a + 0.018 × miR-103a + 0.035 × miR-122 was used to construct the ROC curve. The diagnostic performance for the established miRNA panel was evaluated using ROC analysis. The AUC for the miRNA panel was 0.911 (95% CI 0.880−0.942, **Fig. 3**). The diagnostic performance for two pair and three pair of miRNA panel was also analyzed (**Table 5**), which may be useful for clinical usage with less number of miRNAs to predict CAD.

Discussion

The present study examined the expression level of seven lipometabolism-related miRNAs in PBMCs to establish their potential role as CAD markers. We found that miR-24, miR-33a, miR-103a, and miR-122 average expression level in PBMCs were significantly higher in CAD patients than in healthy controls. Further, the increased levels of these four miRNAs were associated with CAD presence, independent of other cardiovascular risk factors. Our study also revealed that miR-24, miR-33a, miR-103a, and miR-122 in PBMCs were potential markers for diagnosing CAD. The miRNA panel with the four miRNAs from the multivariate logistic regression model demonstrated higher accuracy in the diagnosis of CAD than the separated miRNA.

MiR-33a is an intronic miRNA located within intro 16 of human SREBF-2, a master switch in controlling genes involved in cholesterol uptake and synthesis[20]. In a series of parallel yet independent studies, miR-33a was identified as a key post-transcriptional regulator of cellular cholesterol homeostasis[21]. In addition to the cholesterol transport genes ABCA1,

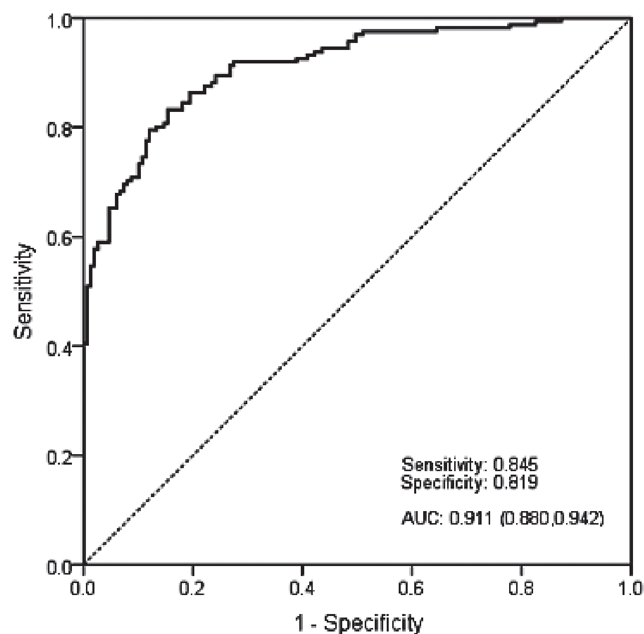

Fig. 3. ROC plot for the microRNA panel (miR-24, miR-33a, miR-103a, and miR-122) discriminating CAD.

ABCG1 and NPC1, Gerin I *et al*. showed that miR-33a binding sites are highly conserved in the 3′ UTR of *Cpt1a*, *Crot*, and *Hadhb* in various cell types[22]. Each of these genes plays a distinct role in the fatty acid oxidation pathway. We found that miR-33a expression in PBMCs negatively correlated to HDLC in CAD patients ($r=-0.221$, $P=0.005$) and health controls ($r=-0.161$, $P=0.049$). Compared with the other selected miRNAs, miR-33a showed the highest level of association with CAD (adjusted OR* = 7.84, 95% CI 4.06, 15.14) after adjustment for confounding variables. MiR-33a is also a valuable biomarker for diagnosis for CAD (AUC = 0.799). These results are consistent with previous reports on association between plasma miR-33a and CAD[6, 20], indicating that similar regulation may also exist in PBMCs.

It has been shown that miR-122 is over expressed in the liver of hyperlipidemia animals[23]. MiR-122 is located within exon 2 of the known noncoding RNA gene hcr (gi: 51212)[23]. Several genes involved in fatty acid synthesis and oxidation including SREBP, MTTP, KLF6 and CAT-1 were found to be regulated by miR-122[10, 24, 25], and silencing of miR-122 resulted in decreased plasma levels of both TC and TG[10, 24]. Gao W *et al*. found that plasma level of miR-122 was increased in patients with hyperlipidemia, and positively correlated with TC, TG, and LDLC[26]. In the present study, we also found positive correlation between miR-122 and TC in ether CAD patients and health controls, and positive correlation between miR-

Table 5. The diagnostic performance for two pair and three pair of miRNA panel

	AUC (95%CI)	logit (P = CAD)
miR-24, miR-33a	0.832 (0.788, 0.876)	− 5.067 + 0.430 × miR-24 + 0.390 × miR-33a
miR-24, miR-33a, miR-103a	0.855 (0.815, 0.895)	− 5.428 + 0.388 × miR-24 + 0.384 × miR-33a + 0.016 × miR-103a
miR-24, miR-33a, miR-122	0.882 (0.845, 0.918)	− 6.326 + 0.459 × miR-24 + 0.385 × miR-33a + 0.035 × miR-122

122 and LDLC (r = 0.207, P = 0.009) in CAD group. Our observation that expression levels of miR-122 in PBMCs was significantly associated with CAD (adjusted OR = 1.03, 95% CI 1.01 − 1.04) is consistent with Gao's finding that the increased plasma level of miR-122 may be novel risk factors for CAD[26].

Ng R *et al.* revealed an important role for the crosstalk between miR-24 and Insig1 in the control of hepatic lipid accumulation and hypertriglyceridemia *in vivo*[7], suggesting that miR-24-evoked, Insig1-dependent inhibition of SREBP processing represents a candidate pathway to cause fatty liver and possibly hypertriglyceridemia. MiR-24 has been identified as a specific miRNA in early and advanced atherosclerosis plaque by the co-inertia approach[27]. Our data showed that miR-24 positive related to TC and TG in both CAD patients and health controls indicating miR-24 may be a risk factor for CAD.

The role of miR-103 is implicated in multiple mRNA regulations that are involved in cellular acetyl-CoA and lipid metabolism[28]. MiR-103 promotes 3T3-L1 cell adipogenesis through AKT/mTOR signal pathway with its target being MEF2D[29]. Bork-Jensen J *et al.* found that miR-103 levels were positively and independently associated with BMI, and that, a trend towards a positive association with plasma triglyceride levels[30]. In our study, miR-103a was found contributing to the presence of CAD (adjusted OR = 1.01, 95% CI 1.00 − 1.02). However, significant correlation was only found between miR-103a and TC when data was stratified by status of disease.

The spectrum of pathologies indicates that numerous metabolic factors like glucose, lipoproteids as well as various inflammatory and anti-inflammatory mediators lead to monocyte activation in the blood circulation[31]. Circulating peripheral blood cells, particularly PBMCs, have been proposed as cardiovascular disease (CVD) biomarkers[11]. They are easily accessible and its extraction is not invasive which facilitates its clinical testing. In recent years, there are quite a few studies focus on assessment of serum or plasma miRNAs used as biomarkers to detect and monitor CAD based on the rational that miRNAs released into circulation during the disease. PBMCs are main actors in atherosclerosis[12]. Our study measured lipometabolism-related miRNAs in PBMCs in CAD patients. Previous data showed that expressions of representative target genes of each miRNAs were changed in PBMCs (**Supplementary Table 2**) when diseases presented, indicating that these miRNAs may involve the pathogenesis of CAD by regulating lipometabolism gene. The present study may enable us to have better accuracy to identify their clinical value compared with those studies focus on plasma/serum miRNAs. However, well-designed prospective studies with larger sample sizes are required to validate our findings.

Conclusion

We found that the increased expression level of miR-24, miR-33a, miR-103a, and miR-122 in PBMCs were associated with CAD presence. The miRNA panel with the four miRNAs demonstrated high accuracy in the diagnosis of CAD. Lipometabolism-related miRNAs in PBMCs may emerge as valuable biomarkes for preventive, diagnostic, and therapeutic approaches for CAD.

Acknowledgments

This study was supported by the National Natural Science Foundation (81573214), the Beijing Municipal Natural Science Foundation (7162020), the Scientific Research Project of Beijing Municipal Educational Committee (KM201510025006), and the National Science and Technology Support Program (2012BAI37B03).

Conflict of Interest

There was no conflict of interest in this study.

Reference

1) Hata J, Kiyohara Y: Epidemiology of stroke and coronary artery disease in Asia. Circ J, 2013; 77: 1923-1932

2) Barton M, Minotti R, Haas E: Inflammation and atherosclerosis. Circ Res, 2007; 101: 750-751
3) Moller DE, Kaufman KD: Metabolic syndrome: a clinical and molecular perspective. Annu Rev Med, 2005; 56: 45-62
4) NCEP: Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel Ⅲ) final report. Circulation, 2002; 106: 3143-3421
5) Chen K, Rajewsky N: The evolution of gene regulation by transcription factors and microRNAs. Nat Rev Genet, 2007; 8: 93-103
6) Rayner KJ, Suárez Y, Dávalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, Fernández-Hernando C: MiR-33 contributes to the regulation of cholesterol homeostasis. Science, 2010; 328: 1570-1573
7) Ng R, Wu H, Xiao H, Chen X, Willenbring H, Steer CJ, Song G: Inhibition of microRNA-24 expression in liver prevents hepatic lipid accumulation and hyperlipidemia. Hepatology, 2014; 60: 554-564
8) Xu Y, Zalzala M, Xu J, Li Y, Yin L, Zhang Y: A metabolic stress-inducible miR-34a-HNF4α pathway regulates lipid and lipoprotein metabolism. Nat Commun, 2015; 6: 7466
9) Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH, Stoffel M: MicroRNAs 103 and 107 regulate insulin sensitivity. Nature, 2011; 474: 649-653
10) Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF: miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab, 2006; 3: 87-98
11) Creemers EE, Tijsen AJ, Pinto YM: Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res, 2012; 110: 483-495
12) Libby P, Lichtman AH, Hansson GK: Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity, 2013; 38: 1092-1104
13) Krychtiuk KA, Kastl SP, Hofbauer SL, Wonnerth A, Goliasch G, Ozsvar-Kozma M, Katsaros KM, Maurer G, Huber K, Dostal E, Binder CJ, Pfaffenberger S, Oravec S, Wojta J, Speidl WS: Monocyte subset distribution in patients with stable atherosclerosis and elevated levels of lipoprotein(a). J Clin Lipidol, 2015; 9: 533-541
14) Vatandoost N, Amini M, Iraj B, Momenzadeh S, Salehi R: Dysregulated miR-103 and miR-143 expression in peripheral blood mononuclear cells from induced prediabetes and type 2 diabetes rats. Gene, 2015; 572: 95-100
15) Economou EK, Oikonomou E, Siasos G, Papageorgiou N, Tsalamandris S, Mourouzis K, Papaioanou S, Tousoulis D: The role of microRNAs in coronary artery disease: From pathophysiology to diagnosis and treatment. Atherosclerosis, 2015; 241: 624-633
16) Novák J, Bienertová-Vašků J, Kára T, Novák M: MicroRNAs involved in the lipid metabolism and their possible implications for atherosclerosis development and treatment. Mediators Inflamm, 2014; 2014: 275867
17) Fihn SD, Gardin JM, Abrams J, Berra K, Blankenship JC, Dallas AP, Douglas PS, Foody JM, Gerber TC, Hinderliter AL, King SB 3rd, Kligfield PD, Krumholz HM, Kwong RY, Lim MJ, Linderbaum JA, Mack MJ, Munger MA, Prager RL, Sabik JF, Shaw LJ, Sikkema JD, Smith CR Jr, Smith SC Jr, Spertus JA, Williams SV; American College of Cardiology Foundation; American Heart Association Task Force on Practice Guidelines; American College of Physicians; American Association for Thoracic Surgery; Preventive Cardiovascular Nurses Association; Society for Cardiovascular Angiography and Interventions; Society of Thoracic Surgeons: 2012 ACCF/AHA/ACP/AATS/PCNA/SCAI/STS Guideline for the diagnosis and management of patients with stable ischemic heart disease: a report of the American College of Cardiology Foundation/ American Heart Association Task Force on Practice Guidelines, and the American College of Physicians, American Association for Thoracic Surgery, Preventive Cardiovascular Nurses Association, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons.J Am Coll Cardiol. 2012; 60: e44-e164
18) Yan YX, Wu LJ, Zhang J, Wang S, Wang W, Dong J, He Y: Let-7 related genetic variation and risk of metabolic syndrome in a Chinese population. Endocr J, 2015; 62: 887-896
19) Yan YX, Dong J, Liu YQ, Zhang J, Song MS, He Y, Wang W: Association of suboptimal health status with psychosocial stress, plasma cortisol and mRNA expression of glucocorticoid receptor α/β in lymphocyte. Stress, 2015; 18: 29-34
20) Marquart TJ, Allen RM, Ory DS, Baldán A: miR-33 links SREBP-2 induction to repression of sterol transporters. Proc Natl Acad Sci U S A, 2010; 107: 12228-12232
21) Novák J, Bienertová-Vašků J, Kára T, Novák M: MicroRNAs in lipid metabolism. Curr Opin Lipidol, 2011; 22: 86-92
22) Gerin I, Clerbaux LA, Haumont O, Lanthier N, Das AK, Burant CF, Leclercq IA, MacDougald OA, Bommer GT: Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. J Biol Chem, 2010; 285: 33652-33661
23) Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, Xu C, Mason WS, Moloshok T, Bort R, Zaret KS, Taylor JM: miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. RNA Biol, 2004; 1: 106-113
24) Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, Mao H, Wei M, Clark KR, Mendell JR, Caligiuri MA, et al: Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest, 2012; 122: 2871-2883
25) Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF, Hsu MT, Wu JC, Huang HD, Shiao MS, Hsiao M, Tsou AP: MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest, 2012; 122: 2884-2897
26) Gao W, He HW, Wang ZM, Zhao H, Lian XQ, Wang YS, Zhu J, Yan JJ, Zhang DG, Yang ZJ, Wang LS: Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. Lipids Health Dis, 2012; 11: 55

27) Jovanović I, Zivković M, Jovanović J, Djurić T, Stanković A: The co-inertia approach in identification of specific microRNA in early and advanced atherosclerosis plaque. Med Hypotheses, 2014; 83: 11-15
28) Wilfred BR, Wang WX, Nelson PT: Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. Mol Genet Metab, 2007; 91: 209-217
29) Li M, Liu Z, Zhang Z, Liu G, Sun S, Sun C: miR-103 promotes 3T3-L1 cell adipogenesis through AKT/mTOR signal pathway with its target being MEF2D. Biol Chem, 2015; 396: 235-244
30) Bork-Jensen J, Thuesen AC, Bang-Bertelsen CH, Grunnet LG, Pociot F, Beck-Nielsen H, Ozanne SE, Poulsen P, Vaag A: Genetic versus Non-Genetic Regulation of miR-103, miR-143 and miR-483-3p. Expression in Adipose Tissue and Their Metabolic Implications-A Twin Study. Genes (Basel), 2014; 5: 508-517
31) Gratchev A1, Sobenin I, Orekhov A, Kzhyshkowska J. Monocytes as a diagnostic marker of cardiovascular diseases. Immunobiology. 2012; 217: 476-482
32) Llorente-Cortés V, de Gonzalo-Calvo D, Orbe J, Páramo JA, Badimon L. Signature of subclinical femoral artery atherosclerosis in peripheral blood mononuclear cells. Eur J Clin Invest. 2014; 44: 539-548
33) Mandas A, Abete C, Putzu PF, la Colla P, Dessì S, Pani A. Changes in cholesterol metabolism-related gene expression in peripheral blood mononuclear cells from Alzheimer patients. Lipids Health Dis. 2012; 11: 39
34) Nakamura T, Ebihara I, Nagaoka I, Takahashi T, Tomino Y, Koide H. Abnormal regulation of insulin-like growth factor gene expression in peripheral blood mononuclear cells from patients with IgA nephropathy. Am J Nephrol. 1992; 12: 292-302
35) Cerda A, Issa MH, Genvigir FD, Rohde CB, Cavalli SA, Bertolami MC, Faludi AA, Hirata MH, Hirata RD. Atorvastatin and hormone therapy influence expression of ABCA1, APOA1 and SCARB1 in mononuclear cells from hypercholesterolemic postmenopausal women. J Steroid Biochem Mol Biol. 2013; 138: 403-409
36) Crujeiras AB, Parra D, Goyenechea E, Martínez JA. Sirtuin gene expression in human mononuclear cells is modulated by caloric restriction. Eur J Clin Invest. 2008; 38: 672-678
37) Gharagozloo M, Javid EN, Rezaei A, Mousavizadeh K. Silymarin inhibits cell cycle progression and mTOR activity in activated human T cells: therapeutic implications for autoimmune diseases. Basic Clin Pharmacol Toxicol. 2013; 112: 251-256
38) Arosio A, Sala G, Rodriguez-Menendez V, Grana D, Gerardi F, Lunetta C, Ferrarese C, Tremolizzo L. MEF2D and MEF2C pathways disruption in sporadic and familial ALS patients. Mol Cell Neurosci. 2016; 74: 10-17

Supplementary Table 1. The miRNA-specific primer sequences

microRNA	Primer Sequence	Product (bp)
u6	5'-CAAGGATGACACGCAAATTCG-3'	121
hsa-miR-21	5'-TAGCTTATCAGACTGATGTTGA-3'	122
hsa-miR-24	5'-TGGCTCAGTTCAGCAGGAACAG-3'	122
hsa-miR-29a	5'-TAGCACCATCTGAAATCGGTTA-3'	122
hsa-miR-33a	5'-GTGCATTGTAGTTGCATTGCA-3'	121
hsa-miR-34a	5'-TGGCAGTGTCTTAGCTGGTTGT-3'	123
hsa-miR-103a	5'-AGCAGCATTGTACAGGGCTATGA-3'	123
hsa-miR-122	5'-TGGAGTGTGACAATGGTGTTTGT-3'	122

Supplementary Table 2.
Summary of the data indicating the expression of representative target genes of the selected microRNAs are actually changed in PBMCs

microRNA	Target gene symbol	Gene name	Description	Refs.
miR-21a	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	Adherence and phagocytosis of neutrophils and monocytes	[32]
miR-24a	SREBP	Sterol regulatory element binding protein	A transcription factor regarded as the main regulator of cholesterol homeostasis	[33]
miR-29a	IGFI	Insulin-like growth factor 1 (IGF-1)	Implication in the metabolism of carbohydrates and lipids	[34]
miR-33a	ABCA1, ABCG1	ATP binding cassette A1 (ABCA1), ATP binding cassette G1 (ABCG1)	Regulation HDL cholesterol transport	[35]
miR-34a	SIRT1	Sirtuin 1	Playing an important role in metabolic pathway regulation	[36]
miR-103a	MEF2D	Myocyte enhancer factor 2-D	Directly regulating PI3K/Akt/mTOR pathway	[37], [38]
miR-122	SREBP, KLF6	Krüppel-like factor 6 (KLF6)	Regulating cholesterol homeostasis	[33], [38]