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Original Article

Biochemistry Section

Diagnostic Value of Circulating MicroRNAs for Middle Aged Coronary Artery Disease Patients: A Case-control Study

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ABSTRACT

Introduction: Coronary Artery Disease (CAD) remains a major problem worldwide. New and useful biomarkers for early diagnosis are necessary. MicroRNAs (miR) are short, non-coding RNAs that post transcriptionally regulate gene expression through degradation and translational repression of mRNAs.

Aim: The current case-control study was designed to assess strength and relevance of diagnostic miR-126, 122 and Vascular Endothelial Growth Factor (VEGF) level in the diagnoses of angiographically proven CAD cases.

Materials and Methods: Circulating levels of miR-126 and miR-122 and VEGF levels were measured in serum from 100 middle aged 46-58 years patients with CAD and 100 patients without CAD through quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Results: Circulating miR-122 level was significantly higher in CAD cases compared to control (1.60±1.06 and 0.93±0.43, p=0.001), however miR-126 was significantly lowered in CAD cases compared to control (0.82±0.51 and 1.01±0.47, p=0.02). Circulating VEGF level was significantly higher in CAD cases compared to control (182.97±156.49 and 105.49±103.88, p=0.02). Circulating miR-122, 126 and VEGF level did not show any association with demographic and clinical parameters. Area Under the Curve (AUC) for circulating miR-122, 126 and VEGF were 0.700, 0.644 and 0.649 with sensitivity and specificity of 66.67%, 56.41%, 61.18% and 70%, 60% and 64%, respectively. The combined diagnostic efficacy of miR-122 and 126 showed higher sensitivity and specificity.

Conclusion: Circulating miR-122 and 126 might be novel, non-invasive biomarkers for early diagnosis of CAD. Further exposition of the role of miR-122, 126 and VEGF in the progression of CAD will add to the understanding of the disease process leading to a new diagnostic approach. However, further studies on larger patient cohorts are required to validate the findings.

Keywords: Biomarker, Coronary disease, Real time PCR

INTRODUCTION

The Coronary Artery Disease (CAD) remains a major public health problem, with a leading cause of death more than any other disease. In 2015, 17.7 million people died from Cardiovascular Disease (CVD) worldwide that represent the 31% of all global deaths out of which 7.4 million people died due to CAD [1,2]. In India, CAD is responsible for 25% of all death. The burden of CAD in the rural population is 3-5% compared to 7-10% in urban population [3]. The major established risk factors such as hypertension, smoking, dyslipidemia, obesity, and diabetes varies widely between different countries. In a comparative study, Indians have higher C-reactive protein, Plasminogen Activator Inhibitor (PAI-1) and homocysteine level which cannot be explained by conventional risk factor [3]. Diagnosis of CAD is made by invasive Coronary Angiogram (CAG) technique as well as Electrocardiogram (ECG) and Exercise Tolerance Testing (ETT) have also been widely used. Diagnosis of CAD requires careful examination and medical history. The current mainstay of the diagnosis of coronary heart disease is the echocardiogram, Computerised Tomography (CT), angiography and cardiac catheterisation. Many individuals continue to succumb to CAD, despite advances in risk factor management at an epidemiological level [4]. Recently, it has been identified that changes in circulating microRNAs (miRNA) have a potential in identifying cardiac dysfunctions and shown a major interest as a biomarker for CAD diagnosis [5]. MicroRNAs are small (19-25nt), highly specific, endogenous, single-stranded, non-coding Ribonucleic acid (RNA). It regulates the wide variety of biological processes such as endothelial dysfunction, inflammation, apoptosis, angiogenesis, atherosclerosis, and neointimal hyperplasia or restenosis [6-11]. Significantly, dysregulated miRNAs have been reported in patients with acute coronary syndrome, unstable angina, Acute Myocardial Infarction (AMI), heart failure and stroke [12-17]. In stable CAD, expression of miR-135a and miR-31 was found to be up-regulated as compared to healthy subjects [15,16]. Both microRNA-126 and -122 have already been incriminated in the field of coronary vascular disease. However; still there is a lack of use of circulating miRNAs as a biomarker for the diagnosis of CAD. Vascular Endothelial Growth Factor (VEGF) is another angiogenic factor related with endothelial function. Studies demonstrated distinct expression of VEGF and its receptors in atherosclerotic lesions in coronary arteries and VEGF may have some role in progression of coronary atherosclerosis [18]. Endothelial enriched miRNA, miR126 has been reported to play important role in modulating vascular development and angiogenesis [19,20]. The study have hypothesised that the serum miRNA levels and angiogenic markers such as VEGF could predict the presence of CAD in middle aged (46-58 years) Indian population. Hence, the current study was designed to assess the level of circulating miR-126, 122 and VEGF in serum of middle aged angiographically proven CAD cases and healthy controls.

MATERIALS AND METHODS

Study Population

Study subjects were recruited between December 2018 to December 2019 from Lari Cardiology Centre, Department of Cardiology, King George Medical University, Lucknow, UP, India. CAGs were evaluated by a consultant through visual estimation of luminal narrowing in multiple segments based on the American Heart Association/American College of Cardiology (AHA/ACC) classification [21]. On the basis of this data, significant CAD was defined as atleast one major epicardial vessel with >50% stenosis. Patients with neither

detectable coronary stenosis nor atherosclerotic vascular disease were considered as healthy controls. Patients were interviewed to collect medical history and lifestyle habits. Patients aged 40-60 years were interviewed to collect medical history and lifestyle habits. Risk factors were determined by physician. Hyperlipidemia was defined as Total Cholesterol (TC) level of ≥5.72 mmol/L and/or Triglyceride (TG) level of ≥1.70 mmol/L, or if the patient was being treated with lipid-lowering medication. Hypertension was defined as resting systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or in the presence of active antihypertensive treatment. Diabetes was defined as fasting blood glucose ≥7.0 mmol/L or a diagnosis of diabetes needing diet or anti-diabetic therapy. Individuals who formerly or currently smoked ≥10 cigarettes per day for at least two years were defined as smokers [20]. A written informed consent was taken from the patients. The research related to human use has been complied in accordance the tenets of the Helsinki Declaration, and has been approved by the Institutional Ethical Committee (Number: 81th ECM II-A/P2) of King George Medical University, Lucknow.

Inclusion criteria: All the patients who fulfilled the inclusion criteria were recruited for the study.

Exclusion criteria: Subjects were excluded from the study if they were affected by a hepatic failure, renal failure, abnormal liver function, hepatitis, cardiomyopathy, congenital heart disease, bleeding disorders, previous thoracic irradiation therapy, and malignant diseases in view of principal investigator.

Sample Collection and Serum Isolation

A 3 mL of peripheral blood was collected from cases and controls in plain, Ethylenediamine Tetraacetic Acid (EDTA) and Fluoride vials (NOVAC, Polymed, Poly Medicure LTD., India). Serum was separated by centrifugation at 1900 g for 10 minutes, followed by a 10 minutes high-speed centrifugation at 16,000 g and stored at -80°C until further processing. All samples were processed within one hour of the collection to avoid potential contamination of leukocytes.

Biochemical Examination

All biochemical parameters were measured by fully automated biochemical analyser (ARCHITECT i2000SR, Abbott Diagnostic & Selectra ProXL, ELITech Group).

RNA Isolation from Serum

Total RNA was extracted using a Trizol-based miRNA isolation protocol (Invitrogen, Carlsbad, CA, USA) (1). RNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc. Wilmington, USA), and the RNA samples were stored at -80°C for future use.

cDNA synthesis and quantitative real-time PCR: Total RNA extracted from serum were initially reverse transcribed using (Multiscribe) MuLV reverse transcriptase kit (Cat. no. K1622, Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was amplified with specific primer sets: miR-122 (hsa-miR-122-5p, Cat. no. 4427975), miR126 (hsa-miR-126-5p, Cat no. 4427975) and RNU6 (Cat no. 4427975). Data were normalised for RNU6 (housekeeping gene), the relative expression levels of miRNAs were calculated using the 2ΔΔCt method and fold-changes were calculated for each miRNA [22,23].

ELISA for VEGF: Serum VEGF level was determined by Enzyme-linked Immunosorbent Assay (ELISA) using RayBio Human VEGF ELISA kit and the reading were recorded by iMarkTM microplate absorbance reader (BIOS) at 450 nm. The level of VEGF concentration in cases was determined by comparing the Optical Density (OD) of the samples with the standard curve.

STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) V.20.0 (SPSS, Chicago, Illinois, USA). Data for age, gender, HbA1c, TC, TG, High-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C), Very Low-density lipoprotein cholesterol (VLDL-C), Folate II, Vitamin B12, Small dense LDL (sdLDL), Vit.D 25-OH, Thyroid-stimulating Hormone (TSH) and total Homocysteine (HCY) were presented as Mean±Standard Deviation (SD), median and 25th-75th quartile. The significance of the comparison was determined by the student t-test and Mann-Whitney U test. The association of levels of miR-122 and 126 in more than two groups was compared by non-parametric Kruskal-Wallis one-way ANOVA. Data for circulating miRNA levels were presented as Mean±SD and significance of the comparison was determined by Mann-Whitney test. The diagnostic value of miRNA expression was determined using the area under the Receiver Operating Characteristic (ROC) curve. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Basic characteristics of the study population

In the present study, 100 CAD patients and 100 healthy controls between December 2018 to December 2019 were included. The baseline characteristics of all the subjects enrolled in this study are listed in [Table/Fig-1]. Laboratory data including biochemical

Characteristics		No. of patients cases (%)	No. of patients control (%)
Age (Years)	≤45	26 (26.0)	87 (87.0)
	>45	74 (74.0)	13 (13.0)
Sex	Male	73 (73.0)	66 (66.0)
	Female	27 (27.0)	34 (34.0)
Occupation	Business	15 (15.0)	23 (23.0)
	Farmer	21 (21.0)	03 (3.00)
	Government employee	16 (16.0)	06 (6.00)
	House wife	19 (19.0)	13 (13.0)
	Labour	12 (12.0)	00 (00.0)
	Other	17 (17.0)	55 (55.0)
Educational status	Basic	42 (42.0)	40 (40.0)
	High school	20 (20.0)	10 (10.0)
	Graduate	19 (19.0)	36 (36.0)
	Illiterate	19 (19.0)	14 (14.0)
Nature of work	Hard	27 (27.0)	03 (3.33)
	Moderate	51 (51.0)	70 (70.0)
	Sedentary	22 (22.0)	27 (27.0)
Exercise	Regular	11 (11.0)	37 (37.0)
	Occasionally	08 (8.00)	17 (17.0)
	Sedentary	12 (12.0)	03 (3.00)
	None	69 (69.0)	43 (43.0)
Smoking	Current	16 (16.0)	10 (10.0)
	Ex-smoker	24 (24.0)	07 (7.0)
	Never	60 (60.0)	83 (83.0)
Alcohol	Current drinker	09 (9.00)	00 (0.0)
	Occasionally	08 (8.00)	90 (90.0)
	Never	83 (83.0)	10 (10.0)
Hypertension	Yes	62 (62.0)	24 (24.0)
	No	38 (38.0)	76 (76.0)
Diet	Vegetarian	43 (43.0)	27 (27.0)
	Non-vegetarian	50 (50.0)	40 (40.0)
	Veg+Non-vegetarian	07 (7.00)	33 (33.0)
Chest pain	High	09 (9.0)	00 (0.0)
	Intermediate	20 (20.0)	00 (0.0)
	Low	56 (56.0)	06 (6.00)
	No pain	15 (15.0)	94 (94.0)

[Table/Fig-1]: Basic characteristic of cases and controls.

parameters are listed in [Table/Fig-2]. There was a significant difference in age, HbA1c, HDL, LDL and total cholesterol level between CAD cases and controls (p=0.001, 0.0004, 0.0015, 0.0004 and 0.0002, respectively). The sdLDL was significantly higher in CAD cases compared to controls (p=0.001). However, there was no significant difference in weight, BMI, VLDL and TG between CAD cases and controls. In CAD cases, the level of Folate II, Vit.D and Vit.B12 was significantly different as compared to normal controls (p=0.001, 0.0083 and 0.0016, respectively) and described in [Table/Fig-2].

Variable	CAD cases (Mean±SD) Median (Q1-Q3)	Normal controls (Mean±SD) Median (Q1-Q3)	p-value*
Age (Years)	52.07±9.94, 52 (46-58.7)	36.13±8.12, 35 (32-40)	<0.0001
Height (Inch)	5.3±0.39, 5.4 (5.0-5.6)	5.4±0.46, 5.4 (5.2-5.7)	0.259
Weight (Kg)	67.2±13.4, 64.5 (55.2-77.2)	62.2±11.8, 60.5 (55-70)	0.06
BMI (kg/m^2)	25.9±4.8, 25.8 (22.7-28.0)	25.2±4.7, 24.3 (21.4-29.1)	0.496
HbA1c (%)	6.8±2.0, 6.1 (5.6-6.9)	5.4±1.0, 5.3 (4.8-5.7)	0.0004
HDL (mg/dL)	44.9±15.9, 40.7 (34.3-50.9)	55.4±12.3, 49.3 (42.0-54.7)	0.0015
LDL (mg/dL)	56.3±37.4, 51 (30-74)	87.6±44.4, 76.5 (55.2-110.0)	0.0004
VLDL (mg/dL)	39.5±31.4, 28.5 (20.2-42.5)	38.3±33.9, 27.5 (19.2-38.7)	0.862
TG (mg/dL)	145.7±72.6, 137.4 (99.8-165.7)	157.2±91.4, 139.5 (94.7-172.2)	0.243
TC (mg/dL)	132.7±46.2, 95.3 (98.6-156.8)	172.2±48.8, 168 (130.5-207.4)	0.0002
sdLDL (mmol/l)	24.7±15.7, 22.1 (12.3-34.1)	7.2±4.2, 6.7 (5.3-9.0)	0.0001
Folate (nmol/L)	14.6±10.6, 10.2 (6.9-19.5)	7.8±5.0, 6.5 (4.4-8.5)	0.001
Vit.D (nmol/L)	19.2±9.8, 16.5 (10.3-28.8)	31.8±38.5, 17.8 (12.4-32.7)	0.0083
Vit. B12 (pg/mL)	472.2±417.0, 274.0 (146.9-759.5)	215.6±189.6, 145.0 (99.1-265.0)	0.0016
TSH (ulU/mL)	3.2±2.5, 2.7 (1.3-4.0)	2.4±1.2, 2.1 (1.7-2.1)	0.096
Total HCY (umol/L)	21.1±10.6, 21.0 (12.8-28.5)	25.0±22.2, 17.7 (13.1-30.1)	0.163

[Table/Fig-2]: Laboratory data from the study subjects.
*p-value of <0.05 was considered as significant; BMI: Body mass index; HDL: High density lipoproteins; LDL: Low density lipoproteins; VLDL: Vey low density lipoproteins; TG: Triglycerides; TC: Total cholesterol; sdLDL: Small dense low density lipoproteins; TSH: Thyroid stimulating hormone; HCY: Homocysteine; SD: Standard deviation

The expression levels of circulating miR-122 and miR-126 and VEGF in CAD and healthy controls

The fold change in miR expression level is summarised in [Table/Fig-3]. The level of serum miR-122 in CAD cases was significantly higher as compared to controls (1.60±1.06 vs. 0.93±0.43, p=0.001). However, the circulating miR-126 was significantly higher in controls compared to CAD cases (1.01±0.47 vs. 0.82±0.51, p=0.02). The circulating serum VEGF level was significantly higher in CAD cases compared to control (182.97±156.49 vs. 105.49±103.88, p=0.02).

MicroRNAs	CAD patients (Mean±SD)	Healthy controls (Mean±SD)	p-value*
miR-122	1.60±1.06	0.93±0.43	0.001
miR-126	0.82±0.51	1.01±0.47	0.02
VEGF	182.97±156.49	105.49±103.88	0.02

[Table/Fig-3]: Fold change in Circulating MicroRNA levels and VEGF in coronary Artery disease (CAD) cases and controls
*p-value of <0.05 was considered as significant; SD: Standard deviation; VEGF: Vascular endothelial growth factor: miR; MicroRNA

Association of circulating miR-122, miR-126 and VEGF with demographic and biochemical parameters

The association of circulating miR-122, 126 and VEGF levels with demographic and clinicopathological characteristic of CAD patients is summarised in [Table/Fig-4]. The study did not found any association of miR-122 and miR-126 with demographic and clinicopathological characteristics. The miR-122 and 126 levels were higher in cases that were presented with high and intermediate chest pain as compared to low and no chest pain (p=0.433, 0.343), current and ex-smoker as compared to non-smoker (p=0.082, 0.983). The level of miR-122 was higher in cases with the total triglyceride level of <30, HDL with range of 40-60 and LDL with ≤130, however the difference was not statistically significant (p=0.255, 0.679 and 0.07, respectively). The study did not found any association of VEGF level with demographic and clinical characteristics of the cases.

Diagnostic role of serum miR-122 and miR-126 and VEGF in CAD cases

ROC curves were drawn for distinguishing CAD patients from controls. AUC for miR-122 was 0.700. At a cut-off point of ≥1.17, miR-122 discriminated CAD cases from controls with sensitivity, specificity and diagnostic accuracy of 66.67%, 70% and 67.59%, respectively. The AUC for miR-126 was 0.644. At a cut-off point of ≤0.894, miR-126 discriminated CAD cases from controls with sensitivity, specificity and diagnostic accuracy of 56.41%, 60.00% and 57.41% each respectively. The AUC for VEGF was 0.649. At a cut-off point of >106.1, VEGF discriminated CAD cases from controls with sensitivity, specificity and diagnostic accuracy of 61.18%, 64.00% and 61.82%, respectively [Table/Fig-5].

Combined diagnostics of miR-122 and miR-126 and VEGF in CAD cases

Compared with individual diagnostics of miR-122 and miR-126, combined diagnostics using one or more test positivity as a positive test, and all two tests negative as negative interpretation, the combination of miR-122 and miR-126 shall provide efficacy in distinguishing CAD cases from controls. Combination of miR-122 and miR-126 with VEGF provides higher sensitivity and specificity of 98.72 and 96.00%, respectively with diagnostic accuracy of 98.06 [Table/Fig-5].

DISCUSSION

In developed and developing countries, CAD is a major cause of heart attack. It is estimated that an approx. of 7.8 billion deaths will result from a heart attack [24]. Circulating miRNA played a crucial role in various pathophysiological processes. As consequences of pathological changes in a different form of CVD, altered level of miRNA has been reported [25,26].

In different pathophysiological conditions, circulating miRNA has been identified as an important biomarker [27]. Compared to proteomic biomarker identification, circulating miRNA has been reported as a more efficient biomarker. It has been demonstrated that there is an emerging role of miRNA in cardiovascular disease and cardiac arrhythmia, however, most of these biomarkers showed reduced sensitivity, specificity, or unsuitability for early diagnosis [28-30].

FichtIscherer S et al., 2010, reported that miR-133 and miR-208a were up-regulated in CAD cases compared to controls [31]. Study of D'Alessandra Y et al., reported the positive correlation of miR-1, miR-122, miR-126, miR-133a, miR-133b, and miR-199a in patients with stable and unstable angina, whereas miR-337-5p and miR-145 found up-regulated in stable or unstable angina patients compared to controls [32]. In CAD patients; miR-133a, miR-208a, miR-1, miR-122, miR-133b, miR-337-5p, miR-433 and miR-485-3p were found to be significantly up-regulated, whereas miR-126, miR-17, miR-92a, miR-145, miR-155, and miR-199a levels were markedly down-regulated in CAD [33].

Characteristics		miR-122 Mean±SD	miR-126 Mean±SD	VEGF Mean±SD	p-value* 122	p-value* 126	p-value* VEGF
Age (Year)	≤45	1.73±1.14	0.75±0.31	178.57±146.95	0.360	0.879	0.740
	>45	1.56±1.04	0.84±0.57	169.54±160.55			
Sex	Male	1.50±1.07	0.80±0.47	183.34±156.97	0.097	0.990	0.05
	Female	1.90±1.00	0.88±0.64	117.80±147.34			
Occupation	Business	1.55±1.45	0.66±0.33	212.38±164.04	0.842	0.259	0.05
	Farmer	1.44±0.98	1.07±0.68	202.23±141.43			
	Govt. Employee	1.99±1.02	0.72±0.30	174.40±206.49			
	House wife	1.68±0.98	0.76±0.31	123.89±147.82			
	Labour	1.58±1.02	0.65±0.25	202.23±140.01			
	Other	1.30±1.06	0.94±0.80	68.13±46.94			
Educational status	Basic	1.58±0.87	0.83±0.32	156.92±150.53	0.612	0.380	0.254
	High school	1.39±1.31	0.90±0.74	195.28±116.14			
	Graduate	1.71±1.09	0.71±0.28	163.38±205.16			
	Illiterate	1.79±1.19	0.83±0.77	175.13±153.81			
Nature of work	Hard	1.64±1.27	0.77±0.35	182.40±115.32	0.958	0.636	0.426
	Moderate	1.60±1.11	0.84±0.49	182.10±176.50			
	Sedentary	1.56±0.98	0.83±0.72	155.30±163.91			
Exercise	Regular	1.92±0.85	0.72±0.42	72.25±91.95	0.584	0.683	0.482
	Occasionally	1.55±0.94	0.66±0.26	116.29±85.84			
	Sedentary	1.69±0.63	0.93±0.89	147.64±125.32			
	None	1.55±1.17	0.83±0.46	187.41±167.18			
Smoking	Current	1.62±1.32	0.74±0.26	166.21±161.22	0.082	0.983	0.509
	Ex-smoker	1.98±0.90	0.90±0.70	184.25±131.97			
	Never	1.45±1.03	0.81±0.48	166.29±170.85			
Alcohol	Current	1.43±0.80	0.83±0.38	126.34±120.26	0.898	0.702	0.325
	Ex-drinker	1.68±1.13	0.72±0.44	185.89±117.25			
	Never	1.61±1.09	0.83±0.54	173.12±171.22			
Hypertension	Yes	1.59±1.02	0.77±0.48	187.28±145.90	0.193	0.991	0.347
	No	1.63±1.44	0.89±0.56	165.31±161.44			
Diet	Vegetarian	1.33±0.99	0.94±0.68	190.60±172.00	0.401	0.04	0.429
	Non-vegetarian	1.71±1.00	0.72±0.33	139.71±126.37			
	Veg.+Non-vegetarian	2.64±1.37	0.73±0.27	129.77±112.36			
Chest pain	High	1.47±0.68	0.78±0.36	231.41±186.73	0.433	0.343	0.145
	Intermediate	1.99±1.40	0.95±0.70	145.68±141.38			
	Low	1.39±1.34	0.74±0.28	177.05±147.47			
	No pain	1.19±0.63	0.61±0.29	146.49±164.57			
BMI (kg/m^2)	≤25	1.65±1.04	0.79±0.26	171.78±162.34	0.628	0.416	0.721
	>25	1.58±1.08	0.83±0.61	171.77±146.19			
HbA1c (%)	<8	1.62±1.03	0.57±0.23	127.51±115.11	0.937	0.051	0.270
	6-8	1.58±1.18	0.87±0.59	172.40±178.46			
	<6	1.61±1.00	0.87±0.51	185.21±141.78			
Total cholesterol (mg/dL)	<130	1.61±1.17	0.73±0.31	167.21±141.70	0.446	0.462	0.413
	130-200	1.66±0.94	0.95±0.69	167.65±166.39			
	>200	1.06±0.93	0.66±0.26	242.49±156.33			
Total triglyceride (mg/dL)	<30	2.60±0.59	0.61±0.32	164.43±123.57	0.255	0.516	0.643
	30-200	1.55±1.06	0.76±0.32	173.80±145.31			
	>200	1.72±1.09	1.17±1.04	187.01±93.13			
HDL (mg/dL)	<40	1.62±1.20	0.74±0.31	167.35±134.54	0.697	0.179	0.228
	40-60	1.66±1.01	0.88±0.69	167.90±117.18			
	>60	1.37±0.75	0.97±0.34	226.78±116.60			
LDL (mg/dL)	≤130	1.63±1.06	0.82±0.52	165.11±155.43	0.07	0.357	0.07
	>130	0.54±0.68	0.69±0.43	278.42±148.32			
sdLDL (mmol/l)	≤30	1.58±0.99	0.84±0.59	167.42±137.64	0.168	0.235	0.352
	>30	1.84±1.16	0.75±0.32	191.96±124.87			

Folate II (nmol/l)	0-44	1.62±1.07	0.85±0.53	177.55±157.78	0.603	0.092	0.09
	>44	1.43±1.01	0.57±0.28	54.85±51.60			
Vit.D (nmol/l)	≤25	1.65±0.97	0.83±0.58	164.67±147.64	0.160	0.866	0.357
	26-50	1.49±1.26	0.79±0.35	182.96±138.87			
Vit. B12 (pg/mL)	0-300	1.61±1.11	0.83±0.48	177.00±165.17	0.685	0.522	0.733
	>300	1.63±1.00	0.82±0.56	164.30±145.21			
TSH (ulU/mL)	0-5	1.58±0.90	0.84±0.54	177.95±154.27	0.741	0.615	0.087
	>5	1.77±1.82	0.69±0.26	119.63±90.43			
Total HCY (umol/L)	0-20	1.64±1.06	0.80±0.50	187.93±157.38	0.670	0.602	0.348
	>20	1.56±1.12	0.77±0.33	154.43±125.57			

[Table/Fig-4]: Association of circulating miR-122, miR-126 and VEGF with demographic, clinical and laboratory parameters.
SD: Standard deviation; VEGF: Vascular endothelial growth factor: miR; MicroRNA; BMI: Body mass index; HDL: High density lipoproteins; LDL: Low density lipoproteins; sdLDL: Small dense low density lipoproteins; TSH: Thyroid stimulating hormone; HCY: Homocysteine

Diagnostic feature	Cut-off value	AUC	p-value*	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	Diagnostic accuracy
miR-122 vs. Control	≥1.17	0.700	0.001	66.67 (50.08-76.94)	70.00 (50.60-85.27)	85.25 (76.59-91.07)	44.68 (35.32-54.44)	67.59
miR-126 vs. Control	≤0.894	0.644	0.02	56.41 (44.70-67.61)	60.00 (40.60-77.34)	78.57 (69.41-85.56)	43.48 (26.46-43.79)	57.41
VEGF	>106.1	0.649	0.02	61.18 (49.99-71.56)	64.00 (42.52-82.03)	82.25 (76.93-90.92)	32.65 (24.58-41.90)	61.82
miR-122+ miR-126 vs. Controls				88.46 (79.22-94.59)	90.00 (73.47-97.89)	95.83 (88.69-98.54	75.00 (61.60-84.87)	88.89
miR-122+ miR-126+VEGF vs. Controls				98.72 (93.06-99.97)	96.00 (79.65-99.90)	98.72 (91.86-99.81)	96.00 (77.36-99.41)	98.06

[Table/Fig-5]: Receiver operator characteristic curve (ROC) analysis of miR-122, miR-126 and VEGF for predicting coronary artery disease.
*p-value of <0.05 was considered as significant; AUC: Area under curve; PPV: Positive predictive value; NPV: Negative predictive value; VEGF: Vascular endothelial growth factor; miR: MicroRNA; CI: Confidence interval

In this study group, the miR-122 and 126 levels were higher in cases with an LDL level of ≤130, however, the difference was not statistically different (p=0.07, 0.357). The level of miR-126 was also lowered in CAD cases with a total cholesterol level of >200 mg/dL. These findings entail a link between the deregulation of serum miR-126 and elevated levels of LDL cholesterol in CAD patients. Sun X et al., found a significantly decreased level of miR-126 in patients with CAD and high Low-Density Lipoprotein (LDL) cholesterol. In contrast, the miR-126 level increased significantly when LDL cholesterol was high in patients who had risk factors for CAD but did not have angiographically significant CAD [34].

Subjects with similar clinical features were recorded to ensure that the study populations were comparable. Statistical analysis showed that these factors do not affect circulating miR-122 and miR-126 levels in present study population. The present study findings suggest that serum circulating miRNAs might be feasible biomarkers for the diagnosis of CAD patients. The serum miR-122 level was significantly up-regulated in CAD cases compared to healthy controls (p=<0.0001) while the miR-126 level was significantly down-regulated in cases compared to controls (p=0.02). It is reported that low Vitamin D level negatively affects cardiac function. In present study samples, vitamin D levels were significantly lower in CAD cases compared to controls (p=0.0083), levels of miR-122 and 126 were higher in cases with lower Vitamin D level (≤25 vs. 26-50 ng/mL). Observational studies of dietary Vitamin D showed that both measured 25(OH)D and estimated 25(OH)D were inversely associated with risk of incident hypertension in both men and women [15]. Interestingly; the miR-122 and 126 levels were higher in cases with a lower level of folate. Vitamin B12 was significantly higher in CAD cases compared to controls (p=0.0016). The miR-122 and 126 levels were higher in cases with >300 pg/mL. The present study finding of Vitamin B12 is consistent with a finding of Hung J et al., reporting that the lower Vit.B12 concentration does not increase the risk of fatal cardiovascular disease in a general population [35]. However, in present study group, the total homocysteine level was lowered in CAD cases compared to controls (p=0.163) and miR-122 and 126 levels were higher in cases with homocysteine level with 0-20 umol/L. However; the effect of folate and vitamin B12 on homocysteine concentrations and risk of cardiovascular disease in the general population is still not clear so far [36].

The AUC values for miR-122 and 126 were 0.700, 0.644, respectively when compared to non-CAD patients. The combined sensitivity and specificity of miR-122 with miR-126 when either test positive was taken as positive for CAD cases showed a maximum sensitivity and specificity of 88.46% and 90.00 with a diagnostic accuracy of 88.89 to discriminate CAD cases from controls. The combination of miR-126 and miR122 with VEGF gives much higher sensitivity and specificity with a diagnostic accuracy of 98.06 compared to the combination of miR-122 and 126.

In present study, the VEGF level in CAD cases was significantly higher (182.97±156.49) compared to normal control (105.49 ±103.88) (p=0.02). The finding of present study is supported by the study of Blann AD et al., [37]. Lin TH et al., reported higher VEGF level in CAD cases compared to control [18]. However, any association of VEGF level with demographic and clinical characteristics of cases was not found [Table/Fig-4]. In a study of Amr KS et al., and Sasahira T et al., reported the down-regulation of miR-126 with increased activity of VEGF [38,39].

Limitation(s)

The major limitation of the present study was the small sample size.

CONCLUSION(S)

To conclude, serum miR-122 and 126 and level of circulating VEGF might be a valuable novel non-invasive biomarker for early diagnosis of CAD patients, further leading to therapeutic attenuation of CAD patients by targeting these miRNAs i.e., 122 and 126 in addition to the anti-VEGF therapy may be a promising candidate for the treatment of CAD subjects.

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REFERENCES

[1] Braza-Boils A, Mari-Alexandre J, Molina P, Domingo D, Abellan Y, Sancho J, et al. P62 Role of microRNAs associated with non-alcoholic fatty liver disease in sudden cardiac death from coronary artery disease. Cardiovasc Res. 2014;103(1):S-10-S10.
[2] Libby P, Theroux P. Pathophysiology of coronary artery disease. Circulation. 2005;111(25):3481-88.
[3] Prabhakaran D, Jeemon P, Roy A. Cardiovascular diseases in India: Current epidemiology and future directions. Circulation. 2016;133(16):1605-20.
[4] Hochholzer W, Morrow DA, Giugliano RP. Novel biomarkers in cardiovascular disease: Update 2010. American Heart Journal. 2010;160(4):583-94.
[5] Santulli G. Epidemiology of cardiovascular disease in the 21st century: Updated numbers and updated facts. J Cardiovasc Dis. 2013;1(1):01-02.
[6] Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA. 2005;102(39):13944-49.
[7] Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature. 2005;436(7048):214-20.
[8] Peng Y, Song L, Zhao M, Harmelink C, Debenedittis P, Cui X, et al. Critical roles of miRNA-mediated regulation of TGFβ signalling during mouse cardiogenesis. Cardiovasc Res. 2014;103(2):258-67.
[9] Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. *Nature*. 2011;469(7330):336-42.
[10] Thum T, Catalucci D, Bauersachs J. MicroRNAs: Novel regulators in cardiac development and disease. Cardiovasc Res. 2008;79(4):562-70.
[11] Dangwal S, Bang C, Thum T. Novel techniques and targets in cardiovascular microRNA research. *Cardiovasc Res.* 2011;93(4):545-54.
[12] Zeller T, Keller T, Ojeda F, Reichlin T, Twerenbold R, Tzikas S, et al. Assessment of microRNAs in patients with unstable angina pectoris. Eur Heart J. 2014;35(31):2106-14.
[13] Widera C, Gupta SK, Lorenzen JM, et al. Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. J Mol Cell Cardiol. 2011;51(5):872-75.
[14] Wang GK, Zhu JQ, Zhang JT, Bang C, Bauersachs J, Bethmann K, et al. Circulating microRNA: A novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J. 2010;31(6):659-66.
[15] Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O, et al. Serum levels of microRNAs in patients with heart failure. Eur J Heart Fail. 2012;14(2):147-54.
[16] Tsai PC, Liao YC, Wang YS, Lin HF, Lin RT, Juo SHH, et al. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. J Vasc Res. 2013;50(4):346-54.
[17] Waters PS, Dwyer RM, Brougham C, Glynn CL, Wall D, Hyland P, et al. Impact of tumour epithelial subtype on circulating microRNAs in breast cancer patients. PloS one. 2014;9(3):e90605.
[18] Lin TH, Su HM, Wang CL, Voon WC, Shin SJ, Lai WT, et al. Vascular endothelial growth factor polymorphisms and extent of coronary atherosclerosis in Chinese population with advanced coronary artery disease. Am J Hypertens. 2010;23(9):960-66.
[19] Li HY, Zhao X, Liu YZ, Meng Z, Wang D, Yang F, et al. Plasma MicroRNA-126-5p is associated with the complexity and severity of coronary artery disease in patients with stable angina pectoris. Cell Physiol Biochem. 2016;39(3):837-46.
[20] Long G, Wang F, Duan Q, Chen F, Yang S, Gong W, et al. Human circulating MicroRNA-1 and MicroRNA-126 as potential novel indicators for acute myocardial infarction. Int J Biol Sci. 2012;8(6):811-18.
[21] King SB, Smith SC, Hirshfeld JW, Jacobs AK, Morrison DA, Williams DO, et al. 2007 focused update of the ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines: 2007 writing group to review new evidence and update the ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention, writing on behalf of the 2005 writing committee. Circulation. 2008;117(2):261-95.
[22] Bandrés E, Cubedo E, Agirre X, Malumbres R, Zárate R, Ramirez N, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer. 2006;5(1):29.
[23] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods. 2001;25(4):402-08.
[24] Okrainec K, Banerjee DK, Eisenberg MJ. Coronary artery disease in the developing world. Am Heart J. 2004;148(1):07-15.
[25] McManus DD, Ambros V. Circulating MicroRNAs in cardiovascular disease. Circulation. 2011;124(18):1908-10.
[26] Li S, Zhu J, Zhang W, Chen Y, Zhang K, Laurentiu M, et al. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection clinical perspective. Circulation. 2011;124(2):175-84.
[27] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA. 2008;105(30):10513-18.
[28] Santulli G, Iaccarino G, De Luca N, Trimarco B, Condorelli G. Atrial fibrillation and microRNAs. Front Physiol. 2014;5:15.
[29] Wronska A, Kurkowska Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta physiol. 2015;213(1):60-83.
[30] Charan Reddy KV. Regulatory noncoding RNAs in cardiovascular disease: Shedding light on 'Dark Matter'. J Cardiovasc Dis. 2015;3(1):301-07.
[31] Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating microRNAs in patients with coronary artery disease novelty and significance. Circ Res. 2010;107(5):677-84.
[32] D'Alessandra Y, Carena MC, Spazzafumo L, Martinelli F, Bassetti B, Devanna P, et al. Diagnostic potential of plasmatic MicroRNA signatures in stable and unstable angina. PloS one. 2013;8(11):e80345.
[33] Sayed AS, Xia K, Li F, Deng X, Salma U, Li T, et al. The diagnostic value of circulating microRNAs for middle-aged (40–60-year-old) coronary artery disease patients. Clinics. 2015;70(4):257-63.
[34] Sun X, Zhang M, Sanagawa A, Mori C, Ito S, Iwaki S, et al. Circulating microRNA-126 in patients with coronary artery disease: correlation with LDL cholesterol. Thromb J. 2012;10(1):16.
[35] Hung J, Beilby JP, Knuiman MW, Divitini M. Folate and vitamin B-12 and risk of fatal cardiovascular disease: Cohort study from Busselton, Western Australia. BMJ. 2003;326(7381):131.
[36] Dawson-Hughes B, Mithal A, Bonjour JP, Boonen S, Burckhardt P, Fuleihan G E-H, et al. IOF position statement: Vitamin D recommendations for older adults. Osteoporos Int. 2010;21(7):1151-54.
[37] Blann AD, Belgore FM, McCollum CN, Silverman S, Lip PL, Lip GYH, et al. Vascular endothelial growth factor and its receptor, Flt-1, in the plasma of patients with coronary or peripheral atherosclerosis, or Type II diabetes. Clin Sci. 2002;102(2):187-94.
[38] Amr KS, Abdelmawgoud H, Ali ZY, Shehata S, Raslan HM. Potential value of circulating microRNA-126 and microRNA-210 as biomarkers for type 2 diabetes with coronary artery disease. Br J Biomed Sci. 2018;75(2):82-87.
[39] Sasahira T, Kurihara M, Bhawal UK, Ueda N, Shimomoto T, Yamamoto K, et al. Down regulation of miR-126 induces angiogenesis and lymph angiogenesis by activation of VEGF-A in oral cancer. Br J Cancer. 2012;107(4):700-06.

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