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ORIGINAL ARTICLE

The Value of Concomitant Measurement of miR 323b-5p and miR 486-5p with Ankle Brachial Index in Diagnosis of Peripheral Arterial Disease.

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ABSTRACT

Background: Peripheral artery disease (PAD) is an advanced atherosclerotic disease-causing augmented risks of hospitalization and death. So, several studies tried to detect novel biomarkers for PAD diagnosis. The study is aimed to explore the role of plasma miRNA 323b-5p and miRNA 486-5p levels to diagnose PAD.
Methods: The research was performed in Medical Biochemistry & Cardiology Departments – Zagazig Faculty of Medicine. It included 100 volunteers categorized into 2 groups; Group (1): included 50 healthy volunteers served as control group with ankle brachial index (ABI) > 0.9. Group (2): included 50 PAD patients with ABI < 0.9. Plasma 323b-5p and miR 486-5p expression level were measured via real time PCR. Color Doppler was performed to calculate ABI, which was correlated with miRNA levels.
Results: There was a highly significant difference between groups as regards miR 323b-5p. Furthermore, miR 486-5p expression level showed significant variance between groups. Regarding miRNA 323b-5p expression level, cutoff value was 2.55, sensitivity was 89.3% and specificity was 72.7%. Concerning miR 486-5p expression level, cutoff value was 1.92, sensitivity was 82.1% and specificity was 81.8%. As regards combined miR 323b-5p and miR 486-5p ROC curve, it was 96.4% sensitivity and 95.5% specificity. Each miRNA showed a negative correlation with ABI.
Conclusion: miRNA 323B-5P and miRNA 486-5P can be a novel and potent biomarker for PAD diagnosis, particularly for risky patients

Keywords: ABI, PAD, Real time-PCR, miRNA323b-5p, miRNA 486-5p.

INTRODUCTION

Peripheral artery disease (PAD) is an advanced atherosclerotic disorder that affected nearly 150 million individuals universally in 2016. By means of advanced aging of the worldwide people, the occurrence of PAD has risen twice in geriatrics from 2003 to 2012 [1], causing augmented hazards of hospitalization and death. Critical limb ischemia (CLI) is the greatest advanced phase of PAD, and could cause claudication, tissue ulcers, putrefaction, and death. Furthermore, research shown that nearly 3-4 % of symptomatic PAD cases deteriorate to have CLI within 1-year [2] and greater than 41% of those CLI cases will undergo leg amputations, two thirds of these cases have DM. So, it is essential to diagnose, forecast and manage CLI at a primary phase in PAD cases to reduce the limb amputation and death rates. Presently, numerous techniques are used to identify CLI, as (ABI) and color Doppler investigation. [3, 4]. But these checks are only partly valuable in diabetic cases with PAD. For instance, investigators have revealed that the hazard factors linked with a decreased ABI can differ and are unlike in cases having or not having diabetes [5]; therefore, the diagnostic worth of ABI is imperfect. Consequently, analysis of new indicators for early diagnosis, and more fruitful treatment, would help risky cases with PAD [6].

MicroRNAs (miRNAs) are minor, non-coding RNA fragments that control gene expression and intermediate compound life processes. It was stated that numerous pathologic situations, counting CVD , DM , tumors and PAD [7]., are related with different miRNAs. miRNAs are considered to enhance cognition, the mechanism of several diseases, and as possible indicators for diagnosis [8].

Preceding research has revealed that the level of numerous miRNAs, such as miR-27b and miR-130a, is markedly significant between lower limb ischemic cases and control group. Though, the miRNA outline of diabetic and other risky cases with PAD is still blurred, particularly when CLI is existing. Consequently, in our study we examined whether the level of miRNAs was significantly

different between PAD patients and healthy controls and assessed the diagnostic worth of miRNAs for PAD diagnosis in high-risk patients. [9]. We chose miRNA 323b-5p and miRNA 486-5p as their measurements together could have a pivotal role in diagnosis of PAD and hence help in early management with avoidance of complications.

The aim of the work is to investigate the role of plasma miRNA 323b-5p and miRNA 486-5p levels **to** diagnose PAD.

METHODS

Research Subjects: Between May 2023 and July 2023, this case-control study was done at the Medical Biochemistry Department and Cardiology Department. Zagazig Faculty of Medicine. Fifty (50) PAD cases were collected for the study. They had one or more risk factors for PAD e.g.: diabetes mellitus (DM), hypertension. The cases were identified by their complaint of intermittent leg claudication and leg pain increased with walking. As well, tissue doppler examination for cases showed ABI < 0.9. Fifty healthy individuals matching in age and sex contributed as a control group. The control group had no risk factors for atherosclerosis and showed ABI > 0.9. A written informed consent was obtained from all participants. The study was performed according to Declaration of Helsinki for studies involving humans. The study was approved by the Zagazig university IRB with approval number #:10729-28-5-2023.

The following was administered to each participant: Color Doppler ultrasound on brachial artery and dorsalis pedis artery to calculate ankle brachial index (ABI). ECG to observe ischemic changes after a detailed history taking that included age, gender, history of DM, family history of hyperlipidemia and hypertension. We carefully measured and documented the levels of fasting blood sugar and lipid profile.

Methods:

Acquisition of venous blood samples

Three ml of venous blood samples were withdrawn on EDTA for real-time PCR investigation of plasma miR 323b-5p and miR 486-5p levels. MiRNA extraction from plasma was via using miRNeasy kits from Qiagen, Germany. All steps were conducted in an environment free of RNA contamination.

Synthesis of cDNA

Then miRNA reverse transcription was performed by miScript IIRT kit Qiagen, Germany. The cDNA was transferred to a –20°C freezer.

Real time PCR for miRNA levels

The amplification was done in a 20 µL combination including 5µL of the cDNA, 100 pmol/mL of each primer miR 323b-5p (Qiagen, MS00021105) , miR 486-5p (Qiagen, MS00004284) ,10 µL 2x QuantiTect SYBR Green PCR Master Mix (Qiagen) and 4 µL distilled water. RNU6 (Qiagen, MS0003374) was used as internal control. The amplification was conducted via Real time Cycler (Stratagene Mx3005P) qPCR System along with the next protocol; initial start steps 95 ºC for fifteen minutes then forty rounds of 95 ºC for 15 second, 55 ºC for thirty second and lastly 70 ºC for 30 sec. The amplitude of discrepancy of the miRNA level identified in cases compared to controls was estimated by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis:

Statistical Package for Social Science version 16 was used to manage the data after it was collected and tabulated (SPSS Inc., Chicago, IL). They were expressed as mean ± standard deviation (SD); statistical difference among the study groups were evaluated by one-way analysis of variance (ANOVA) followed by the Turkey post-hoc test for inter-group comparisons. Statistical significance was defined as a p-value of less than 0.05.

RESULTS

Table (1) showed demographic data among the study groups. The mean for age in control group was 48.94 ± 4.44 while in PAD cases group, the mean of age was 49.04 ± 4.11 with no significant variance between the two groups. Concerning gender, there was no significant variance between the two studied groups.

Regarding number of smokers, diabetics, hypertensive and +ve family history, the results showed a significant increase in PAD patient group compared to control group.

Table (1) also showed lipid profile and fasting blood sugar (FBS) level among the study groups. Fasting blood sugar showed highly statistically significant difference between the two groups. FBS in control group ranged from 70 to 110 with mean ± SD = 92.28 ± 12.78 while in PAD cases group the fasting blood sugar ranged from 102 to 160 with mean ± SD = 120.68 ± 14.4

Regarding TC, TG, LDL-C and HDL-C, the results showed greatly significant variance between the two groups (Table 1). Total cholesterol l (TC) means ± SD in control group was 153.96 ± 9.4 mg /dl while in PAD cases group, it was 248.26 ± 15.26 mg /dl. Triglyceride (TG) in control group mean ± SD was 138.38 ± 6.94 mg / dl while in PAD cases group, it was 311.04 ± 23.08 mg /dl. LDL-C in control group mean ± SD was 55.41 ± 7.82 mg /dl while in PAD cases, it was 134.66 ± 11.89 mg /dl. HDL-C in control group

mean ± SD was 57.24 ± 5.45 mg /dl while in PAD cases group, it was 34.94 ± 4.09 mg /dl.

Table (2) showed plasma miR 323b-5p and miR 486-5p expression levels among the study groups. miRNA 323b-5p expression level in control group was 1.02 ± 0.09 while in PAD cases group, it was 2.59 ± 0.26. MiR 486-5p expression level in control group was 0.95 ± 0.06 while in PAD cases group, it was 1.91 ± 0.11. There was high statistically significant difference between the two groups as regards miR 323b-5p. Furthermore, miR 486-5p expression level showed upregulation in PAD cases group.

Figure (1) showed ankle brachial index (ABI) among the study groups. ABI in control group ranged from 0.95 to 1.3 while in PAD cases group, the ABI ranged from 0.4 to 0.85 with highly statistical remarkable difference between the two groups.

Figure (2) showed Pearson's correlation coefficients (r) between ankle brachial index (ABI) and Plasma miR 323b-5p and miR 486-5p levels. There was a strong negative relationship between the ABI and miRNA 323b-5p. As well, there was a strong negative relationship between miR 486-5p expression level and ABI.

Table (3) and figure (3) showed sensitivity, specificity and cut of value of miRNA 323b-5p, miRNA 486-5p and combined miRNA 323b-5p and miRNA 486-5p to detect ABI < 0.9 in patient group only. Regarding miRNA 323b-5p expression level, AUC was 0.852, cutoff value was 2.55, sensitivity was 89.3% and specificity was 72.7%. Concerning miR 486-5p expression level, AUC was 0.876, cutoff value was 1.92, sensitivity was 82.1% and specificity was 81.8%. Regarding combined miR 323b-5p and miR 486-5p, AUC was 0.994, cutoff value was 4.47, sensitivity was 96.4% and specificity was 95.5%.

Table (1) : Demographic data , lipid profile and fasting blood sugar level among the study groups

	Control group (n = 50)	PAD cases group (n = 50)	Test of Sig.	p
Age (Mean ± SD).	48.94 ± 4.44	49.04 ± 4.11	t = -0.117	0.907
Gender -Male	23 (46%)	25 (50%)	X2 = 0.16	0.689
Gender -Female	27 (54%)	25 (50%)	X2 = 0.16	0.689
TC (mg/dl)			t = -18.527	<0.001
Mean ± SD.	153.96 ± 9.4	202.78 ± 16.09		
TG (mg/dl)			t = -37.241	<0.001
Mean ± SD.	155.95 ± 18.29	311.04 ± 23.08		
LDL-C (mg/dl)			t = -31.191	<0.001
Mean ± SD.	55.41 ± 7.82	106.78 ± 8.63		
HDL-C (mg/dl)			t = 18.718	<0.001
Mean ± SD.	57.24 ± 5.45	38.68 ± 4.41		
FBS (mg/dl)			t = -10.43	<0.001
Mean ± SD.	92.28 ± 12.78	120.68 ± 14.4		

t: *Independent T test* ***SD:*** *standard deviation* ***IQR:*** *interquartile range*

p*: p value for comparing between the studied groups*

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.001: Highly significant

Table (2) : Plasma miR 323b-5p and miR 486-5p expression levels among the study groups

	Control group (n = 50)	**PAD cases group (n = 50)**	**Test of Sig.**	**p**
miRNA 323b-5p expression level			**t = -40.842**	**<0.001**
Mean ± SD.	**1.02 ± 0.09**	**2.59 ± 0.26**		
Median (IQR)	**1 (0.92 - 1.1)**	**2.6 (2.4 - 2.78)**		
miR 486-5p expression level				

	Control group (n = 50)	PAD cases group (n = 50)	Test of Sig.	p
Mean ± SD.	**0.95 ± 0.06**	**1.91 ± 0.11**	**t=-54.967**	**<0.001**
Median (IQR)	**0.95 (0.92 - 0.97)**	**1.94 (1.9 - 1.98)**		

Table (3) : Sensitivity, specificity and cut of value of miRNA 323b-5p , miRNA 486-5p and combined miR 323b-5p and miR 486-5p to detect ABI < 0.9 in patient group only

	Diagnostic parameters			
	AUC	Cutoff value	Sensitivity	Specificity
miRNA 323b-5p	**0.852**	**2.55**	**89.3%**	**72.7%**
miRNA 486-5p	**0.876**	**1.92**	**82.1%**	**81.8%**
Combined miR 323b-5p and miR 486-5p	**0.994**	**4.47**	**96.4%**	**95.5%**

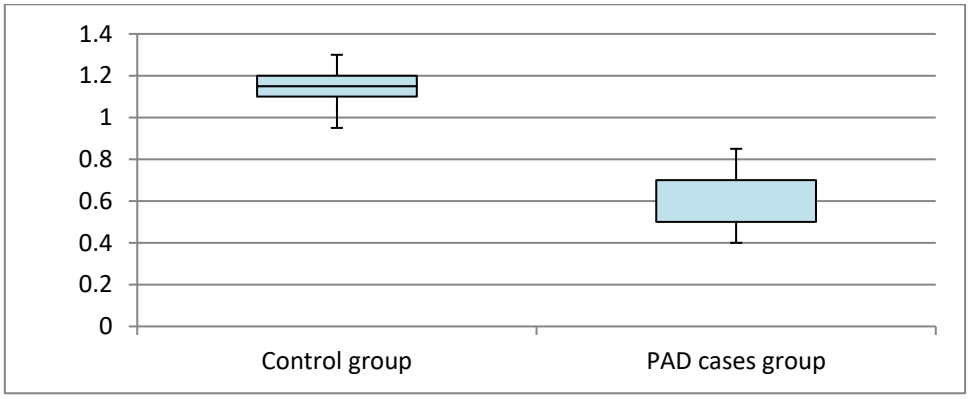

Figure (1): Box-plot showing difference between the study groups regarding Ankle Brachial Index.

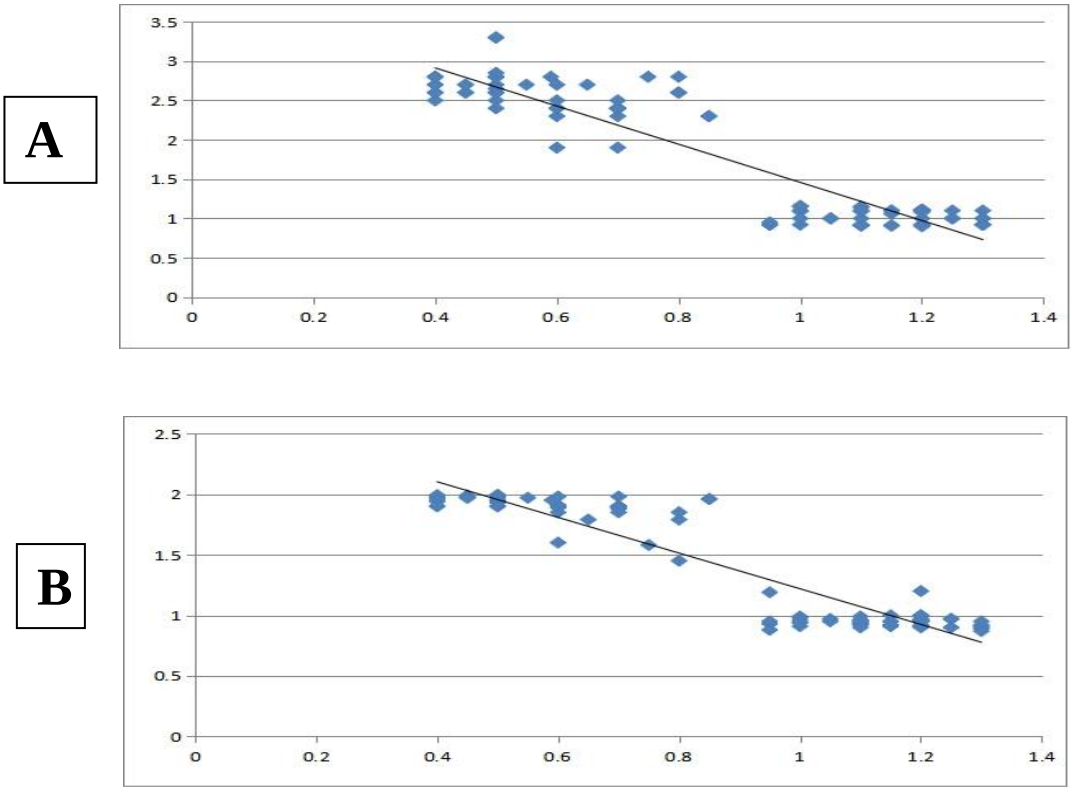

Figure (2): Scatter plot graph showing negative correlation between Ankle Brachial Index (ABI) and (A)Plasma miR 323b-5p expression level.(B) Plasma miRNA 486-5p level.

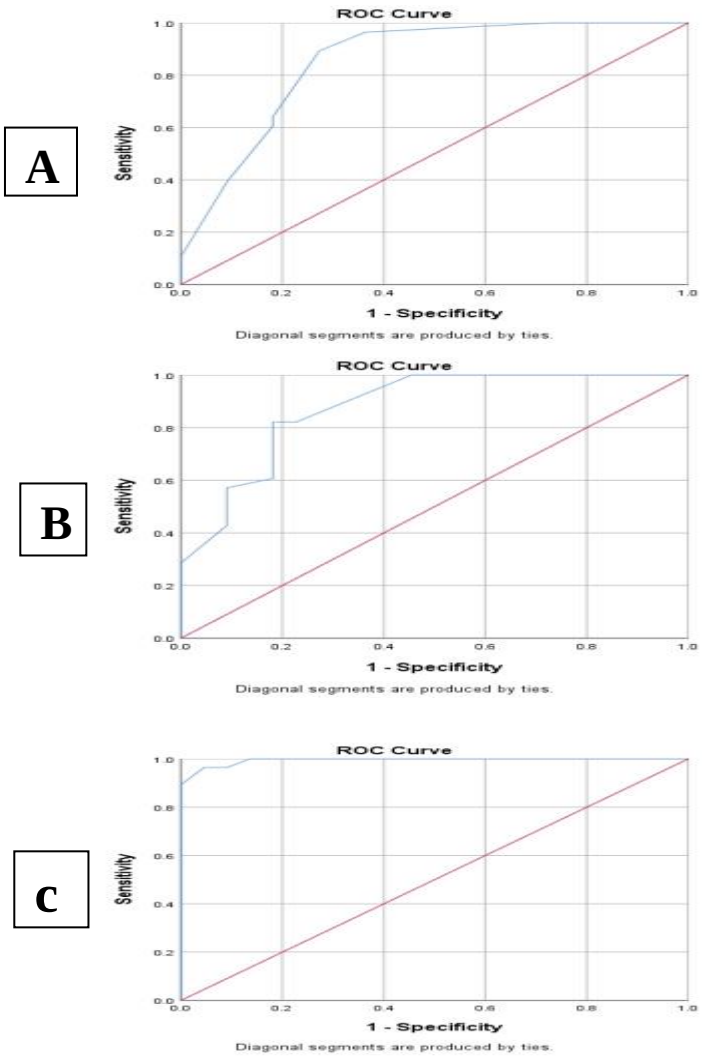

Figure (3): ROC curve for miRNA 323b-5p expression level in (A) , miRNA 486-5p in (B) and combined miRNA 323b-5p and miRNA 486-5p in (C) to diagnose PAD

DISCUSSION

PAD is a principal etiology of death globally, it is linked with extensive vessel atherosclerosis, including the coronary arteries [10]. It is well-thought-out to be a clinical indicator of general atherosclerosis [11].

Smoking and diabetes are main hazard factors for PAD, as well as arterial hypertension and hyperlipidemia [12]. The mechanism of PAD is complicated and has not so far been entirely clarified [13]. Despite major advances in surgical vascular procedures, there is presently no treatment that can efficiently treat and enhance the prognosis of PAD cases [14]. Thus, PAD still possess a high death and morbidity. Thus, recognizing the mechanism causing PAD advance is essential for suggesting new treatments.

We controlled for age and sex with no substantial variation between the two groups to reduce the bias for the research.

In the present study, there was significant alteration between the groups regarding fasting blood sugar with PAD patients had higher FBS (120.68 ± 14.4) than controls (92.28 ± 12.78).

DM is known to be implicated as one of the main risk factors of PAD. This can be attributed to the process of atherosclerosis. It starts at the subclinical level and progresses to severe form of PAD. Besides diabetes, it is well known that two thirds of PAD cases have other risk factors, such as HTN, hyperlipidemia and smoking [15], and this is in accordance with our results. Regarding smoking, diabetes mellitus, HTN and family history, our results reveled a significant difference between the two studied groups.

The ABI, the relation of ankle-to-brachial artery measurement of systolic pressure, is a subtle and specific screening method for PAD. The ankle measurement is obtained at the dorsalis pedis artery. It is the primary-line noninvasive, cheap and technique for evaluating lower limb vessels [16].

ABI is recommended by American Heart Association for diagnosis of PAD. Particularly in high risky patients like diabetic or hypertensive

[17]. An ABI lesser than 0.9 is revealing PAD and is linked with 4 times rise in death. An ABI greater than 1.3 is suggestive of poorly squeezable vessels subsequent from vessel calcification, that is as well linked with a higher risk of death and amputation [18].

Our study revealed that ABI in control group ranged from 0.95 to 1.3 with mean ± SD = 1.14 ± 0.1 while in PAD cases group the ABI ranged from 0.4 to 0.85 with mean ± SD = 0.57 ± 0.13 with extremely remarkable variance (p= <.001) between the two groups.

Critical limb ischemia (CLI) is a severe form of PAD. It is presented clinically by chronic ischemic pain during rest, tissue ulcers or putrefaction in one or both limbs. This pain starts immediately after sleeping, thus awakening the patient. [16].

Studies have revealed that calcification or decreased vessel wall elasticity could disturb the accurateness of ABI values causing false readings, particularly in CLI patients. Takahara et al. (2012) stated that there were no considerable variances in the ABI measurement of CLI cases including diabetics and non-diabetics. [19].

Consequently, because of the limits of present diagnostic techniques for CLI in and its dangerous complications, detection of new biomarkers is essential.

Lately, circulatory miRNAs have been revealed to aid as new biomarkers for cardiovascular disorders (CVD) [20]. Earlier studies have showed the value of miRNAs in the instruction of angiogenic rules of progenitor cells in the CVD. Variations of the miRNA values in the serum are supposed to be related to the occurrence of numerous CVD, signifying that they signify potential targets for treatment [21].

In the present research, we explored the plasma expression level of both miR 323b-5p and miR 486-5p and our results revealed that PAD patients had greater level of miR 323b-5p and miR 486-5p (2.59 ± 0.26 and 1.91 ± 0.11 respectively) than control group (1.02 ± 0.09 and 0.95 ± 0.06 respectively) with greatly significant variance (p= <.001) between the two groups.

Supporting the result of the present study, Khan, (2023) reported that miR 323b-5p values were higher in diabetic patients with CLI when compared to its levels for diabetic cases without CLI [22].

Moreover, Cheng et al. (2018) proposed a moderately high indicative accuracy of miR-323b-5p values for the diagnosis of CLI in diabetic cases, with a sensitivity of 62.67% and a specificity of 80.65% [15].

A contradictory result was documented by Syed et al. (2020) who investigated microRNA levels of CLI patients. It did not show a substantial change of miRNA-4739 or miRNA-323b-5p in CLI patients in comparison to controls [23]. The discrepancy in findings may be attributed to variance in methods.

Cheng et al. (2018) stated that investigation for miR-323b-5p presented valuable enhancement in the courses of B cell diversity, cell multiplication and apoptotic schedule, signifying the existence of shared mechanisms concerning miRNAs involved in atherosclerosis [15].

Horswell et al. (2013) revealed that miR-323b-5p values were elevated in familial dyslipidemia cases [24]. He also reported that this miRNA can enhance lipid gathering in adipocytes cells by decreasing CDKN2B (cyclin-dependent kinase 4 2B) values, signifying that CDKN2B may share in adipocytes metabolism. Consequently, CDKN2B RNA can be a gene targeted by miR-323b-5p [15].

As regards the second miRNA involved in our study, our findings were consistent with Zhang et al. (2015) who exhibited that the miR-486 values as well as miR-150 were greater in AMI patients when compared to healthy volunteers. (ROC) curve analyses support their diagnostic value in AMI cases [25].

MiR-486 is present obviously in muscle. Papers showed that its level was decreased in numerous muscular diseases, such as Duchenne's muscular dystrophy [26]. Augmented levels of miR-486 can cause muscle hypertrophy. Thus, higher expression values of miR-486 in AMI patients might be attributed to cardiac hypertrophy.

Luo et al. (2020) recognized that miR-486-5p autonomously connected with acute lung injury (ALI). miR-486-5p can intermediate the development of ALI by enhancing the expression of inflammation mediators. Furthermore, miR-486-5p suppression can decrease the inflammatory reply in ALI mice, thus suggesting its role as enhancer of inflammation [27].

So, one proposed mechanism for explanation of the result of the present study is the role of miR-486-5p in inflammation and oxidative stress that can induce endothelial dysfunction and promote the progress of atherosclerosis.

In contrast, Zhu et al. (2022) described that coronary artery stenosis (CAS) cases showed suggestively reduced serum miR-486-5p values when compared to control group and can recognize asymptomatic CAS. It also presented a negative relation with the grade of carotid stenosis. [28].

The explanation of this disparity in results may be first, the sample size was relatively small. Second, it may be caused by ethnic variances which is a significant factor that can influence gene studies of this kind. Third, PAD is a complicated disease, concerning possible relations among genes as well as environment.

CONCLUSION

Our research analyzed the changes in plasma levels of miR 323b-5p and miR 486-5p in PAD patients. Both miRNA levels were increased in PAD cases when compared to control. Also, both miRNAs were negatively correlated with ABI. The ROC curve of combined miRNA 323B-5P and miRNA 486-5P showed excellent sensitivity and specificity, so miRNA 323B-5P and miRNA 486-5P can be a novel and potent biomarker for PAD diagnosis, particularly for risky patients.

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