

Novel Micro Non-coding RNAs (miRNA-221 and miRNA-197) As Biomarkers for Acute Myocardial Infarction Diagnosis

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ABSTRACT

Background: Acute myocardial infarction is one of the most causes of death all over the world that is known by decreased of blood supply to the myocardium due to obstruction in the arteries. Most recent studies aimed to early prediction of Acute myocardial infarction (AMI) to avoid its hazard. **Objectives:** we aimed to evaluate the predictive effect of non-coding RNAs expression especially the micro in AMI. **Methodology:** Using in silico data analysis to retrieve microRNAs related to AMI that result in selection of miRNA-197 and miRNA-221. We measured the expression of the serum non-coding RNAs in 25 healthy volunteers, 20 patients with chest pain due to non- cardiac causes and 65 patients with using quantitative real-time PCR. **Results:** the study data analysis showed significant down regulation in the expression of serum levels of miRNA-221 in the patients with AMI compared with healthy volunteers. Also, there is upregulation in expression of serum miRNA-197 in the AMI groups in comparison with the healthy control group. Performance characteristics analysis showed that the studied micro non-coding RNAs were potential biomarkers for prediction of AMI.

1. Introduction

Cardiovascular diseases (CVDs) are the world's leading cause of death and a major impediment to long-term human development. It is a type of diseases that involve disorders in the heart or blood vessels of the body [1]. CVD includes many

syndromes like angina and MI. Acute myocardial infarction (AMI), which is generally known as heart attack. Acute myocardial infarction results in irreversible damage to the heart muscle due to an absence of oxygen. An MI can impair diastolic and systolic function, making the patient vulnerable to arrhythmias. Furthermore, a MI can

result in a variety of serious complications. The key is to restore blood flow and reperfusion of the heart. The better the prognosis, the earlier the treatment (less than 6 hours from symptom onset) [2].

Currently, the clinical diagnosis of AMI frequently uses biomarkers from circulatory systems, especially the blood circulatory system, such as troponins (TnI/TnT), CK-MB, and myoglobin [3]. These biomarkers, which are all proteins, could only be partially diagnostic due to the patient's genetic structure, age, lifestyle, medications, and other factors. However, it has obvious limits because troponin levels take time to become detectable. Additionally, circumstances worsened by renal failure, lung illness, atrial fibrillation, or myocarditis make troponin less accurate [4].

So, in this study we aim to select from the micro non-coding RNAs that can be used as a novel genetic non-invasive biomarker for early detection of AMI and avoid the conflict caused by traditional cardiac biomarkers.

Only 2% of genomic transcripts are responsible for protein coding. The bulk of RNAs are classified as non-coding RNAs since they are not expressed. Non-coding RNAs, on the other hand, are crucial because they play a role in protein modification, promotion, and silencing. MicroRNAs (miRNAs), snoRNAs, siRNAs, snRNAs, piRNAs, and long non-coding RNAs are examples of noncoding RNAs (NcRNAs). Apart from their critical functions in cancer development, new data suggests that they are also closely linked to the pathophysiology of the human heart [5].

MicroRNAs constitute a large class of short RNAs (e.g., 20–24 nucleotides in length), which have key roles in cell development and differentiation by mediating the post-

transcriptional regulation of protein-coding genes [6]. These molecules exert regulating effects on gene expression by inhibiting translation and causing degradation of target messenger RNA (mRNA) [7]. The expression of miRNAs inside myocardium is the most exciting and advanced mechanism in the regulation of myocardial infarction, heart failure, and hypertrophy [8].

MiRNA-221, That is from the short RNA type located in chromosome Xp11.3. mir-221 is known as anti-angiogenic miRNA and is an oncogenic microRNA [9,10]. It targets CD117, which then prevents cell migration and proliferation in endothelial cells [11]. While MiRNA-197, It is a short RNA that is located on chromosome 1p13.3. There is no much data for this microRNA but a study shows the prognostic potential of miR-197 as a predictor of cardiovascular mortality was corroborated in a cohort of 873 patients with CAD within 4 years of follow-up [12].

2. Material and Methods

2.1. Specimens

The specimens included in this study were 110 human blood samples, where the collection procedures were permitted by the Research Ethical Committee of Faculty of Medicine, Ain Shams University, Egypt. The specimens were collected from January 2019 up to January 2020. The samples were divided into three groups: 25 of Healthy controls, 20 non-cardiac patients who were suffering from chest pain but after examination and some analytical tests, they were diagnosed as non-cardiac chest pain and 65 of acute myocardial infraction documented patients recruited from Ain Shams University Hospital with acute and ongoing chest pain for 8 hours. AMI was diagnosed on the foundation of

raised serum Troponin levels, CK-MB besides Clinical symptoms. Patients with history of hepatitis, end-stage renal failure, congenital heart disease, bleeding disorders, or malignant were have excluded disease from our study.

2.2 Biochemical parameters investigation

Assessment of creatinine [13], total cholesterol [14], HDL [15], LDL [16], and triglycerides [17] using specific kits from Bio-diagnostic Co., Egypt. Also, estimation of cardiac parameters: CK-MB [18] [DiaSys Diagnostic Systems, Germany], troponin [19] [Abcam, UK].

2.3. In silico data analysis

miRNA 221 and miRNA-197 were chosen based on in silico data, it was known that they were connected to cytokines, inflammation, fibrosis apoptosis, lipid metabolism and linked to AMI [20,21,22].

2.4. Detection of serum micro non-coding RNA

2.4.1 Purification of total RNA

We used QIAamp® RNA Blood For total RNA purification from human whole blood kit [QIAGEN, CA, USA] according to the manual. Nanodrop™ 2000 Spectrophotometer [Thermo Scientific™, USA] and Qubit® 3.0 Fluorometer [Invitrogen, Thermo Fisher Scientific, USA] were used for concentration and purity assessment of RNA samples [23].

2.4.2 Quantitative real time-PCR

RT-PCR was carried out using TaqMan® Small RNA Assays [AB Applied Biosystem, USA.] to construct cDNA then using RT2 SYBR Green ROX qPCR Master mix [Qiagen, Germany] and U6 sn RNA, TaqMan™ microRNA Control Assay 5X [Qiagen, Germany] as internal control using Applied biosystem 7500 fast real

time PCR system [Applied Biosystem, USA] [24]. The relative quantification of gene expression methods to calculate $RQ = 2^{-\Delta\Delta Ct}$ using Livak method [25] considered that it is negative expression if Ct value was ≥ 36 was used.

2.5. Statistical analysis

Analysis of data was performed with IBM SPSS Statistics Version 25 for Windows (SPSS Inc., IBM Corporation, NY, USA). In order to verify normal distribution of data the Shapiro-Wilk test was used to test normality hypothesis of all quantitative parameters for further choice of appropriate parametric and non-parametric tests. The ROC curve was used to evaluate the predictive values for different biomarkers. Moreover, chi-square test was used. All statistical tests were 2-tailed and a P -value ≤ 0.05 was considered statistically significant [26,27].

3. Results

3.1. Demographic parameters

Regarding age and sex distribution, there was no significant difference between the investigated groups, however there was a significant difference for BMI ($P=0.005$) (Table 1 & Figures 1 and 2).

3.2 Clinical and biochemical data of the study

In this study, no differences among the three studied groups in serum LDL and HDL levels ($P>0.05$), but there was marked differences in serum creatinine, total cholesterol, total Triglycerides, CK-MB and cardiac troponin ($P<0.05$) as shown in (Tables 2 and 3 & figure 3).

3.3. Relative expression of serum miRNA-197 among the Study Groups

According to one-way ANOVA, there was a highly significance difference between the different studied groups ($P \leq 0.001$). There was increase in expression levels of miRNA-197 in the noncardiac chest pain, and acute myocardial infarction groups by 245% and 2363.33% respectively. Moreover, the expression of miRNA-197 give a high statistical significance according to Kruskal-Wallis Range Test between the AMI group against other groups as shown in **Table 4 and figure 4** in details.

3.4. Relative expression of serum miRNA-221 among the Study Groups

There was a decreased significance difference between the different subjected groups ($P < 0.001$). Compared to the healthy control group, the expression levels of miRNA-221 was highly significantly decreased in the noncardiac chest pain, and acute myocardial infarction groups by -43.25% and -98.41% respectively. Furthermore, the expression of miRNA-221 give a high statistical significance using Kruskal-Wallis Range Test between the cardiac group against other groups (**Table 4 and Figure 5**).

3.5. ROC curves of different investigated serum cardiac parameters to give the evidence as a potential diagnostic biomarker for AMI

By generating the ROC curves, the control group to be compared with the AMI group was the healthy control and non-cardiac groups. The ROC curves of the CK-MB level, troponin concentration, and circulating expression levels of miRNA-197 and miRNA-221 to differentiate AMI

patients from healthy controls are shown in **Figure 6 and Table 5**. With an AUC of 0.749 (95% CI:0.649-0.849, $P=0.000$), CK-MB levels had the lowest diagnostic effectiveness of all the measures and can provide up to 73.7% sensitivity and 69.2% specificity at a cut off of 23.5 (**Figure 6A**). However, the AUC of troponin was 0.929 (95% CI:0.879-0.978, $P=0.000$) with a cut-off point of 1.45, which result in a sensitivity of 97.8% and a specificity of 81.5% (**Figure 6B**). Furthermore, miRNA-197 (**Figure 6C**) gives an area under curve (AUC) of 0.950 (95% CI:0.904-0.996, $P=0.000$) with an optimal cut-off point of 1.334 which associated with sensitivity of 82.2% and specificity of 98.5%. Moreover, the area under curve for miRNA-221 had the strongest diagnostic value for AMI was 0.955 (95% CI:0.915-0.995, $P=0.000$) with a finest cut-off of 2.56 that give sensitivity = 86.7 % and specificity = 95.4 % as shown in **Figure 6D**.

3.6. Positivity rate of serum investigated parameters based among the study groups

Using the chi-square test on our data, which wereperformed using the ROC curves and the selected cut-off values for each parameter was in **Table 6**. for healthy control people, they should give negative result with CK-MB and troponin and miRNA-197 while they give positive with expression of miRNA-221. All parameters give high statistical significance ($P < 0.001$). For the expression of miRNA-197, there were positivity of 100%, 60% and 98.46% for the healthy control, non-cardiac chest pain and the AMI groups respectively. Furthermore, in the expression of miRNA-221 the positivity of the healthy control, non-cardiac chest pain and the AMI groups

was 92%, 80% and 95.38% respectively.

4. Discussion

Cardiovascular diseases especially acute myocardial infarction (AMI) is the first leading cause of death all over the world [28]. Acute coronary syndrome, pathologically causes the development of so-called plaques, ulceration, and hemorrhage, followed by complete or incomplete occlusive thrombosis. Smoking, drinking alcohol, having diabetes, having dyslipidemia, and being older are just a few of the well-known risk factors [29].

Early and rapid detection, diagnosis, and early prevention of acute myocardial infarction are demanded to stop the progressive development of AMI especially its treatment and prognostic evaluation mainly depend upon the severity of the coronary lesions assessed by coronary angiography, an invasive and expensive test. Thus, leads to effective therapy and prevention of adverse progression of AMI to improve survival of patients [30].

While ECG is an important tool for diagnosis of AMI, it has limited sensitivity (50-60%) detection [31]. Also, unfortunately numerous false elevations of protein cardiac markers in skeletal muscle injury, cardiac trauma and end-stage of renal disease. Therefore, searching for novel biomarkers that are directly linked to AMI detection with high accuracy as well as high sensitivity at a biochemical and molecular levels has the most consideration point in current researches. Researchers are now focusing their efforts on the topic of RNA especially the non-coding. With the widespread and availability of use of microarray and next-generation RNA sequencing technologies since

the early 2000s, there is a growing body of evidence that noncoding RNAs as microRNAs, long non-coding RNAs and circular RNAs have a role in illness progression. Noncoding RNAs in the blood could be used as a marker for disease progression, prognosis and as potential therapeutic targets for numerous diseases, including cardiovascular diseases. These RNAs are more stable in the peripheral circulation and organ-specific than protein markers [32,33].

In our study, sex and age were found with no significance ($P > 0.05$) between different studied group. While Body mass index (BMI) has a statistical significance between groups ($P = 0.01$), so the obesity should be controlled to decrease incidence of AMI and this agree with Zhu *et al.* [34].

We assessed troponin-T and CK-MB since they are the most commonly used enzymatic cardiac tests for diagnosing and monitoring AMI. Skeletal and cardiac muscle contain a combination of three proteins called troponins. In particular, troponin T, one of the three subunits, can be extremely specific for cardiac muscle necrosis since its levels vary between skeletal muscle and cardiac muscle in contrast to troponin C, which is present in both types of muscles [35]. CK is an enzyme that is mostly found in skeletal and cardiac muscles. One of CK's three isoenzymes, CK-MB, is found in the heart muscle [36]. The early clearance of CK-MB over troponins facilitates the detection of reinfarction [37]. Our study demonstrates a very statistically significant difference ($P < 0.001$) between the subject groups for the cardiac markers CK-MB and troponin because these indicators' levels are higher in the AMI group than in the healthy group. These findings concur with Tucker and Gerhardt [38,37] and are in contrast to Voss *et al.* [39]. The

increased sensitivity of hs-cTnT, however, increases the possibility of false positives in a healthy population [40], so it is necessary to enhance and improve the current biomarkers. One such molecular biomarker is circulating non-coding RNA, which may help to increase the specificity of protein biomarkers.

As known that the human genome consists of 98.5% of junk DNA that don't code proteins [41]. Part of non-protein coding DNA is transcribed into what called non-coding RNAs which is a group of heterogeneous functional RNA [42]. Non-coding RNAs is said to be the architects of genomic complexity in eukaryotes [43]. Non-coding RNA are classified into two subclasses:

- Long non-coding RNA (lncRNA) which consists of ≥ 200 nucleotides.
- Short RNA which is < 200 nucleotides especially the micro-RNA (miRNA), its length ranges from 19-25 nucleotides.

It was suggested that these ncRNA are of great values in gene-expression patterns, diverse biological processes like enzyme-activity control, inhibition of transcription regulators, splicing pattern determination, and regulation of mRNA transcription [44]. Many studies have shading on the role of non-coding RNA (long or micro) especially the circulating ones as biomarker in most cases of coronary vascular diseases in particular AMI that consider our study [45]. Using in-silico data analysis to choose micro noncoding RNA. We aim to evaluate the diagnostic accuracy in early detection of AMI patients that give false results with cardiac enzymes tests for miRNA-197 and miRNA-221 for myocardial development marker.

For miRNA-197 that are situated on Chr1, one of the highly expressed

microRNAs in platelets and has a role in its activation moreover, it said that it to contributes with dyslipidemia in metabolic syndrome [46]. In our study, it was found upregulation in the expression of miRNA-197 in the group of acute myocardial infarction patients comparing with healthy and non-cardiac ones. This agreed with Liu *et al.* [47] that found increase in expression of circulating miRNA-197-5P in myocardial infarction and patient with heart failure under the age of 50 years. Also, another study that carried out on a large scale of population has declared that increase the level of expression of miRNA-197 lead to cardiovascular diseases with high risk and can be considered as a biomarker for diagnosis and prognosis for cardiac diseases [12].

Furthermore, miRNA-221 was found to be downregulated in the AMI group when compared with healthy and non-cardiac groups according to our analyzed data. This result was converse to the studies known: that first reported increase in expression level of miR-221-3p in AMI patients [48], to Xue *et al.* [49] that reported high levels of miRNA-21-3p in the human blood vessels with atherosclerosis and with the research that show highly significant elevation of circulating miRNA-221 in patients with cardiac syndrome [50]. The study of Jia *et al.* [51] was similar to our data as they decreasing in expression of miRNA-221 in coronary heart disease patients compared with control but our results is more preferable as the AUC of ROC curve was higher than them.

After data analysis using the different statistical tests, we can consider the studied non-coding RNAs (miRNA-197 and miRNA-221) as a new biomarker that can be used in the early detection and diagnosis of AMI patients especially with cases that

make conflict with CKMB and troponin tests.

Conclusions:

In this study, we reported the relative expression levels of miRNA-221 were significantly downregulated in the AMI group compared with the healthy volunteers while miRNA-197 expression levels showed significance upregulation. In conclusion, miRNA-197 and miRNA- expressions can be considered as novel non-invasive biomarkers for diagnosis of AMI.

Institutional Review Board

Statement

The study was approved by the Ain Shams Research Ethics Committee, Faculty of Medicine, Egypt, dated 13/5/2019, FWA 000017585 in accordance to the guidelines of the Declaration of Helsinki.

Informed Consent Statement

Informed consent was taken from all participants involved in the study.

Author Contributions

All authors had input to the study's conception and design. Study design, methodology, data analysis, drafting, and supervision was done by S.H.A.A., S.M.H., and A.A.G. S.H.A.A., S.M.A.M. and A.R.M.S. shared in bioinformatic analysis, statistical analysis, and drafting. The manuscript's first draught was written by S.M.A.M. All authors read and agreed to the final manuscript.

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Contrary Interests

The writers have not disclosed any financial or professional conflicts of interest.

Availability of Data: Data are accessible upon request.

Availability of the code: Not applicable

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Table (1): Distribution of gender among the healthy control, non-cardiac, and acute myocardial infarction groups:

Groups	N	Gender		Chi-square	P-value
		Males	Females		
		Count (%)	Count (%)		
Healthy Control	25	20 (80%)	5 (20%)	0.247	0.884
Non-cardiac	20	15 (75%)	5 (25%)		
Acute myocardial infarction	65	52 (80%)	13 (20%)		

Table (2): Median values of serum creatinine of the healthy control, non-cardiac, and acute myocardial infarction groups:

Parameters	Groups	N	Median	P-value
Creatinine (mg/dL)	Healthy Control	25	1.1 ^a	0.009**
	Non-cardiac	20	1.1 ^{ab}	
	Acute myocardial infarction	65	1.3 ^c	

- All data are presented as median.
- Kruskal-Wallis Range Test, Level of significance is $P < 0.05$
- ** P - value is highly significant.

Table (3): Median values of serum CK-MB, serum troponin of the healthy control, non-cardiac chest pain, and acute myocardial infarction groups:

Parameters	Groups	N	Median	P-value
CK-MB (U/L)	Healthy Control	25	8 ^a	0.000**
	Non-cardiac chest pain	20	28.5 ^b	
	AMI	65	33 ^b	
Troponin (Pg/mL)	Healthy Control	25	0.2 ^a	0.000**
	Non-cardiac	20	0.75 ^a	
	AMI	65	39 ^b	

- All data are presented as median.
- Kruskal-Wallis Range Test, Level of significance is $P < 0.05$.
- ** P - value is highly significant.

Table (4): Median values of the serum miRNA-197 and miRNA-221 of the healthy control, non-cardiac, and acute myocardial infarction groups:

Parameters	Groups	N	Median	P-value
miRNA-197	Healthy Control	25	0.3000 ^a	0.000**
	Non-cardiac chest pain	20	1.0350 ^a	
	AMI	65	7.3900 ^b	
miRNA-221	Healthy Control	25	12.6000 ^a	0.000**
	Non-cardiac chest pain	20	7.1500 ^a	
	AMI	65	0.2000 ^b	

- All data are presented as median.
- Kruskal-Wallis Range Test, Level of significance is $P < 0.05$.
- ** P - value is highly significant.

Table (5): Performance characteristics of cardiac biomarkers collected from ROC curve analysis:

Parameters	Sensitivity (%)	Specificity (%)	PPV (Positive Predictive Value) (%)	NPV (Negative Predictive Value) (%)	Accuracy (%)
CK-MB	73.7 %	69.2 %	78.9 %	62.3 %	70.9 %
Troponin	97.8 %	81.5 %	98.1 %	78.6 %	88.2 %
miRNA-197	82.2 %	98.5 %	88.8 %	97.3 %	91.8 %
miRNA-221	86.7 %	95.4 %	86.7 %	95.4 %	91.8 %

Table (6): Positivity rate of serum investigated cardiac parameters based on the data and cut-off point from ROC curves analysis among the study groups:

Groups		Cardiac parameters			
		CK-MB	Troponin	miRNA-197	miRNA-221
		N (%)	N (%)	N (%)	N (%)
Control (N=25)	Positive	1 (4%)	0 (0%)	0 (0%)	23 (92%)
	Negative	24 (96%)	25 (100%)	25 (100%)	2 (8%)
Non-cardiac chest pain (N=20)	Positive	11 (55%)	1 (5%)	8 (40%)	16 (80%)
	Negative	9 (45%)	19 (95%)	12 (60%)	4 (20%)
Acute myocardial infarction (N=65)	Positive	45 (69.23%)	53 (81.54%)	64 (98.46%)	3 (4.62%)
	Negative	20 (30.77%)	12 (18.46%)	1 (1.54%)	62 (95.38%)
X2		30.871	67.047	84.417	76.524
P - value		0.000**	0.000**	0.000**	0.000**

• ** P-value \leq 0.001, highly significance.

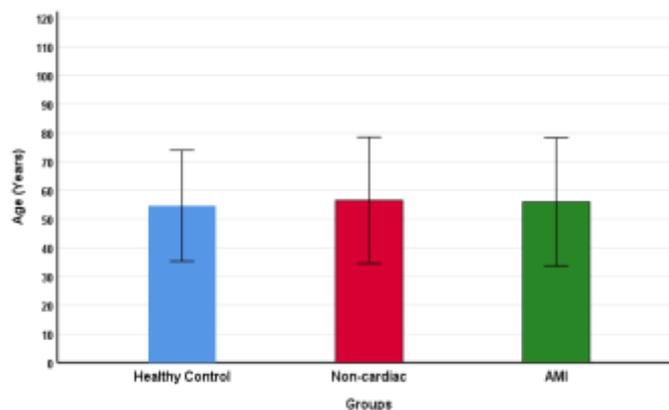


Figure (1): Mean values \pm S.D. of age among healthy control, non-cardiac chest pain and AMI groups.

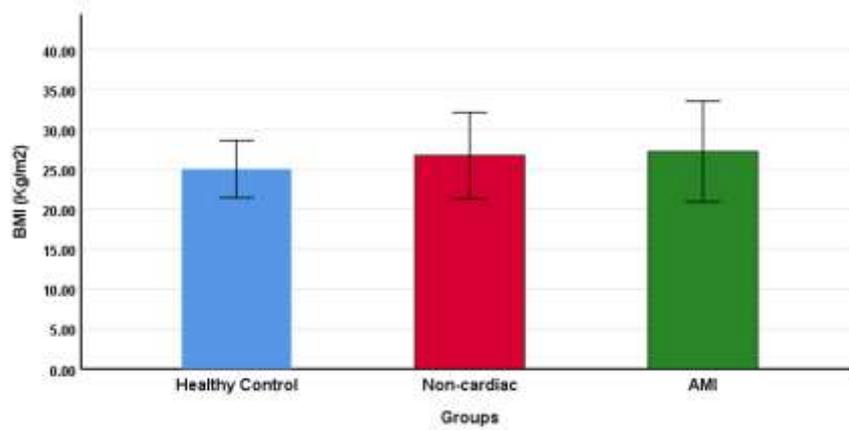


Figure (2): Mean values \pm S.D. of BMI for the healthy control, non-cardiac chest pain and AMI groups.

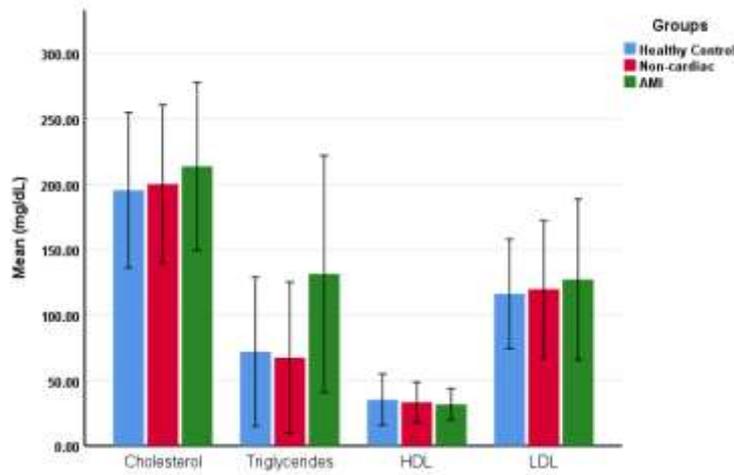


Figure (3): Mean values \pm S.D. (mg/dL) of serum cholesterol, serum triglycerides, serum HDL and serum LDL of the healthy control, non-cardiac chest pain, and acute myocardial infarction groups.

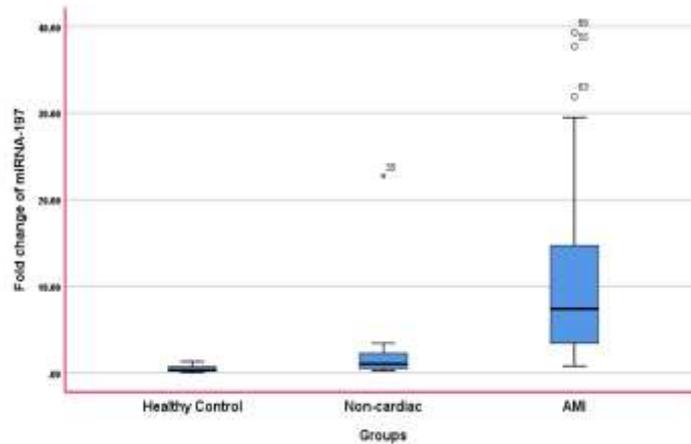


Figure (4): Boxplot for expression of the fold change of serum miRNA-197 of the Healthy control, non-cardiac chest pain, AMI groups.

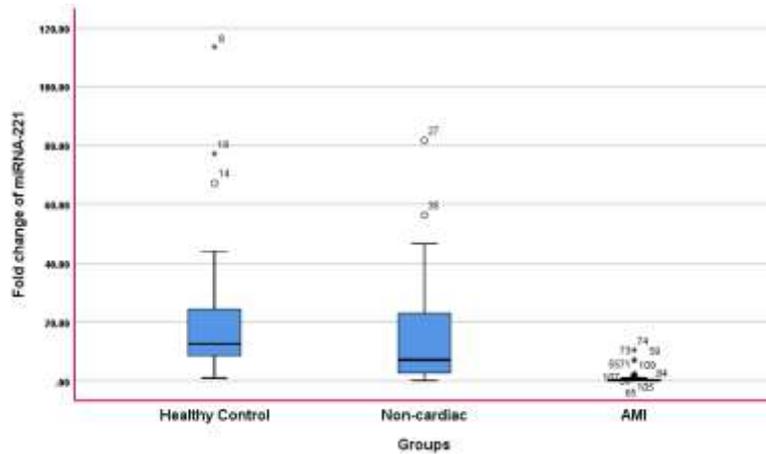


Figure (5): Boxplot represents serum miRNA-221 expression based on fold change among the Healthy control, non-cardiac chest pain, AMI groups.

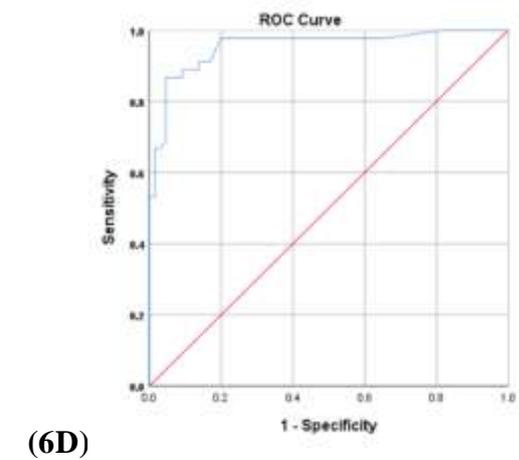
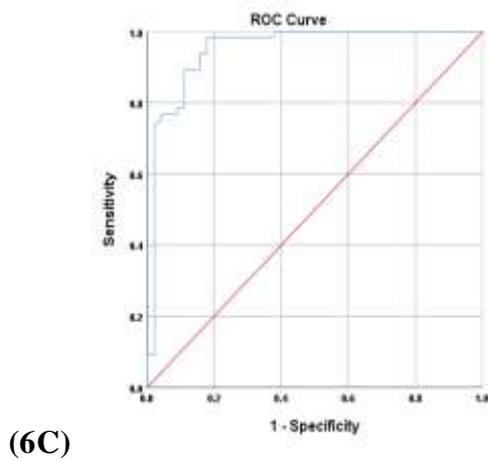
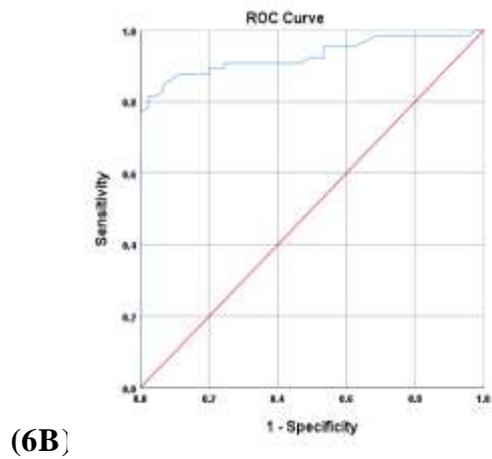
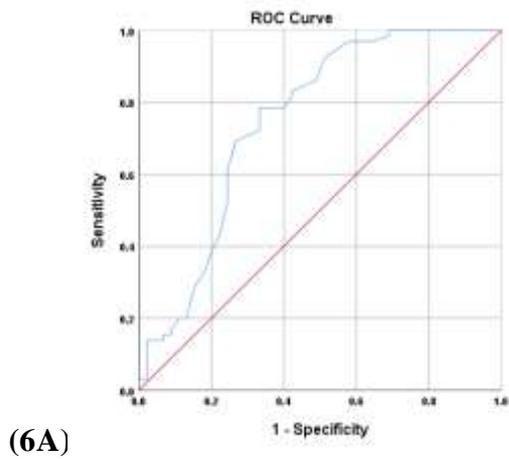


Figure (6): ROC curves of the serum (A) CK-MB, (B) Troponin, (C) miRNA-197 and (D) miRNA-221 expression to discriminate AMI patients from control.