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### RESEARCH ARTICLE

#### DETERMINATION LEVELS OF miRNA 499A-5P IN PLASMA OF PATIENTS WITH ACUTE MYOCARDIAL INFARCTION.

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#### Abstract

**Background:** Acute myocardial infarction (AMI) is the major complication of the coronary artery disease. The successful treatment of AMI patients contributes to the early and accurate diagnosis. Several cardiac biomarkers are used in the diagnosis of MI but the ideal biomarker still lacking. Currently, the micro-RNAs are under evaluation which may be a novel biomarker for MI diagnosis.

**Objective:** The aim of this study is detection levels of the miRNA499a-5p in plasma of patients with AMI at the time of admission to the emergency unit (symptoms less than 12 hours) and detection utility of the miRNA499a-5p in the early diagnosis of AMI, and whether miRNA499 detect the infarct size or differentiate among PCI group.

**Method:** Seventy patients with AMI, and 20 healthy individuals were subjected to study of plasma miRNA499a-5p levels by qRT-PCR, with the titer of serum cardiac troponin-I.

**Results:** The miRNA499a-5p levels were increased above the cutoff value (P-value is 0.000, O.R= 11.9) in 38.6% of AMI patients group at first hour of admission. Pearson correlation showed that elevated levels of plasma miRNA499 in patients group were highly significant (PV= 0.004) correlated with cardiac troponin -I, while it non significantly correlated with the positive and negative troponin results (accuracy: 54.3%, OR: 183 with acceptable ROC: 0.667). The elevated results of miRNA499 were linked with 21.4% in the period less than 5 hours of onset chest pain (accuracy: 50% and OR: 0.36 and ROC: 0.564), and showed that elevated miRNA499 levels recognized 45.2% of patients with ST-wave, while it was with 33.3 % of NST-patients (accuracy: 57.1% and OR: 1.64 and ROC: 0.462), and discriminated 27.3% of patients were subjected to percutaneous coronary intervention (PCI) (accuracy: 45.7% and OR: 1.82 and ROC: 0.465).

**Conclusions:** The plasma miRNA499-5p was elevated in AMI patients at the time of admission and positively correlated with the troponin but it unlike troponin-I and cannot detect the infarct size, with little diagnostic utility in the first 5 hours after chest pain onset. Elevated levels of miRNA499-5p showed low ability in the differentiation among ECG groups and among PCI groups.

suggest that miRNA499-5p is a useful confirmatory biomarker for MI diagnosis and it may has a significant value in the deleterious stages of injury post MI .

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### ..... **Introduction:-**

Myocardial infarction (MI) is the major pathological complication of the coronary artery disease (CAD) and the leading cause of mortality and disability in the world. Injury and necrosis of the heart muscle are the main consequences of the progressive ischemic events resulting from obstruction of the coronary arteries.[1] As early diagnosis in the first hours of the chest pain onset has important role in reducing further myocardium damage and reduces mortality among MI patients[2]. Various intracellular molecules emerged from infarcted area to the blood stream and used as useful biomarkers for the diagnosis of AMI in the past decades [3]. Most of these biomarkers have limited diagnostic specificity and sensitivity, and detects other pathophysiological conditions and not distinguish between mechanisms of myocardial injury or differentiate between MI severity,thus, the MI management field still needs for the ideal biomarker that meet the emergency requirements [4,5]. Extensive development in the molecular techniques facilitated isolation and identification of a subclass of non-coding RNA (approximately 22 nucleotides in length) known as micro RNA (miRNA) has ability to repress RNA translation. These miRNAs are transcribed from DNA template by the activity of RNA polymerase II (Pol II) which result in formation of a copy of 70 to 80 nucleotides with hairpin structure known as pri- miRNA [6]. The pri- miRNA is recognized by specific nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8 ) catalysis RNase III domain of a nuclear protein called Drosha to form the Microprocessor complex that liberates the hairpins from pri- miRNAs processing it into smaller size known as pre-miRNA [6,7] which , then exported out of the nucleus by aid of a protein exportin-5 [8]. In the cytoplasm, the mature pre-miRNA is incorporated in a complex known as RNA Induced Silencing Complex (RISC) contained Dicer enzyme and many associated proteins to form the miRISC configuration [9]. Dicer separates the duplex pre-miRNA into two strands ; the first is functional, named guide strand(miRNA) which contained the seed region that coinciding to the specific sequence in the target mRNA, and the second is passenger strand (miRNA\*) which have lower stability and normally degraded [10] . The passenger strand is coupled into the miRISC and result in formation of RISC loading complex (RLC) which enabling the miRNA for targeting the specific mRNA in order inhibiting its activity by several mechanisms either through direct degradation or translation repression, or via gene silencing. [11, 12, 13, 14]. Recent studies have demonstrated that certain miRNA isoforms have tissue specificity and can be detected in the plasma, and their levels are altered in individuals with various pathological conditions [15,16]. The concentrations of circulating miRNAs were not influenced by age and sex in several previous studies cohort [17]. It has been shown in several previous studies that myocardial related miRNAs have important role in the regulation of cardiac angiogenesis, fibrosis , remodeling and cardiomyocyte hypertrophy upon MI [18,19] that enabling the authors in initiating the mirRNA data base associated heart diseases [20]. The principle expression of miRNA-499 in conditions of cardiac damage involving injury, cardiac dysfunction, hypertrophy and the cardiomyopathy in both human and murine hearts have been identified by the whole genome analysis [21]. It has been shown in previous studies that certain miRNAs exclusively increased in the blood circulation shortly after onset of a MI symptoms and correlated with troponins. Among these miRNAs, miRNA-499 considered the best candidate for AMI diagnosis [22,23]. The aim of this study is detection the diagnostic accuracy of miRNA499a-5p in the AMI to be a novel cardiac biomarker that may aid in the early diagnosis of patients with AMI and whether miRNA 499 discriminate among the percutaneous coronary intervention (PCI) group or among the ECG waves( ST/ non ST).

### **Materials,sampling and methods:-**

Seventy AMI patients (42 males and 28 females), and twenty healthy individuals (10 males and 10 females) were enrolled in this study during the period between May and December 2017. Those patients were chosen from a group of newly admitted patients who successfully diagnosed as AMI by the medical staff in the the emergency unit of Ebin Al-Nafees Hospital according to their clinical symptoms, ECG finding and available laboratory tests. Blood samples were collected from MI patients at the 1<sup>st</sup> hour of their admission before receiving any medication. The history of chest pain onset of the MI patients, electro-cardiogram (ECG) findings, and those MI patients who subjected to PCI procedure were recorded, and all AMI patients group had onset of chest pain more than 12 hours were excluded from this study. Among all MI patients only 31 patients expressed ST-wave in their ECGs, and only 11 patients were subjected to the PCI procedure. The blood samples of both patients and control groups were separated in to sera and plasma, and subjected immediately for detection of serum cardiac troponin-I by using of

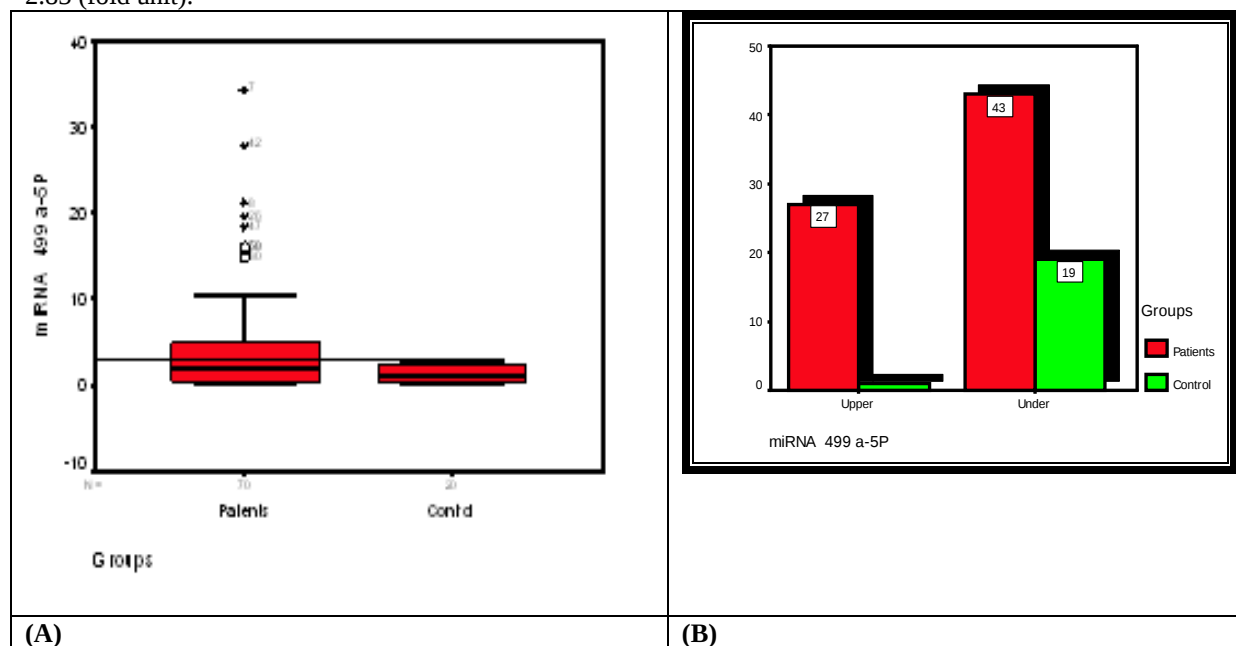
(one step LABNOVATION quantitative kit, china, with sensitivity (troponin-I < 0.5 ng/ml with linearity range 0.5-50 ng/ ml), while the plasma samples were immediately was transferred to labeled RNase/DNase-free tubes and stored at -80°C until the time of miRNA isolation and quantification. Extraction of total RNA was performed according to the manufacturer's instruction of ( Qiagen; Shanghai, China) isolation kit,. DNase enzyme was added to eliminate contamination and keep without enrichment for small RNAs ( Qiagen). The concentration and quality of the extracted RNA samples were determined using a BioPhotometer . Generation of the cDNA was performed by using of Reverse transcriptas with the specific miScript primer (Qiagen) which the used as a template for generation of miRNA 499. Elongation of the 3' ends of all miRNAs with a poly (A) tai was performed by using of E coli poly (A) polymerase. The Caenorhabditis elegans miRNA 39 (Qiagen) was used as internal control (15). A specific primer set (Qiagen) were used for generation 0f miRNA499 . The levels of miRNA499a5-p were estimated by using of TaqMan-based miRNA quantitative real-time polymerase chain reactions (qRT-PCRs) specific kits with SYBR-green according to the Master Mix protocol in the manufacturer's instructions of Qiagen . The reaction was carried out using the 7900HT Sequence Detection System. The given threshold cycle (Ct) and the relative expression values of the microRNAs were calculated by using of the calculation formula:  $(2^{-\Delta\Delta Ct})$ . (24)

#### Statistical Analysis:-

The results of the statistical assessment were carried out by application of the statistical package (SPSS) ver. (14.0). The cutoff value of miRNA 499 was created from the point in case control by using of Stem-Leaf technique in order discriminate the abnormal values expressed above the cutoff point. Other screening tests, such as: Sensitivity, Specificity, Accuracy Rate, and Prevalence Rate. Receiver Operation Characteristic Curve (ROC) statistics (95% Confidence Interval of Arc) with the odds ratio (OR) were used in order to evaluate the degree of utility of miRNA499 in comparison with the reference methods in the diagnosis of MI.

#### Results:-

THE FOLLOWING FIGURE (1:A) ILLUSTRATED CUTOFF POINTS THAT ARE BASED ON THE APPLYING OF THE SUGGESTED TECHNIQUE FOR THE MIRNA 499A-5P, AND AT THE SAME TIME THEY ARE REFLECTED THE LOWER BOUNDED OF ALTERNATIVE STATUS OF THAT METHODS. The statistical analysis showed that cutoff value of mirna499a-5p was 2.83 (fold unit).



**Figure [1]:-** (A) Illustrated redistribution of the of the plasma miRNA499a-5P of the patients group according to the cutoff points. (B) The graphical redistribution of miRNA 499 a-5P of both patients group and control group according to the cutoff point

THE RESULTS IN THE TABLE (1) AND FIGURE (1:B) SHOWED HIGHLY SIGNIFICANT ELEVATED LEVELS OF PLSMA MIRNA 499 IN 38.6% THE PATIENT GROUP ABOVE THE CUTOFF VALUE [pV= 0.000 , OD=11.93] .

**Table (1):-** Redistribution (Under/Upper) a cutoff point's outcomes in light of miRNAs 499 in the studied groups

| Biomarkers | Patients % upper cutoff | Control% upper cutoff | significance | P-value | C.S. <sup>(*)</sup><br>Odds Ratio |
|------------|-------------------------|-----------------------|--------------|---------|-----------------------------------|
| miRNA 499  | 38.6%                   | 5%                    | H.S          | 0.000   | 11.93                             |

<sup>(\*)</sup> S: Sig. at P<0.05; [ C.C.: Testing based on Contingency Coefficient test

The following table (2) showed no significant correlation between elevated levels of miRNA499a-5p and the main risk factors of cardiovascular disease in the patients group [PV< 0.05].

**Table (2):-** Pearson Correlation Coefficients among Studied parameters in the patients group with significant levels.

| Group    | Parameters        | Pearson Correlation And Sig. Levels | mi R N A 4 9 9 a 5 p |
|----------|-------------------|-------------------------------------|----------------------|
| Patients | Age               | Pearson Correlation                 | -0.088               |
|          |                   | Sig. (2-tailed)                     | 0.471                |
|          | BMI               | Pearson Correlation                 | -0.131               |
|          |                   | Sig. (2-tailed)                     | 0.280                |
|          | Blood Pressure    | Pearson Correlation                 | 0.220                |
|          |                   | Sig. (2-tailed)                     | 0.067                |
|          | Cholesterol       | Pearson Correlation                 | 0.047                |
|          |                   | Sig. (2-tailed)                     | 0.697                |
|          | Diabetes mellitus | Pearson Correlation                 | 0.230                |
|          |                   | Sig. (2-tailed)                     | 0.066                |

<sup>(\*)</sup> HS: Highly Sig. at P<0.01; Sig. at P<0.05; Non Sig. at P>0.05

The following table (3) of pearson correlation showed a highly significant correlation (PV= 0.004) between plasma miRNA499a and troponin-I in the patients group.

**Table (3):-** Pearson Correlation Coefficients among Studied parameters in the patients group with significant levels

| Parameters          | Pearson correlation and sig. levels | miRNA499a-5p |
|---------------------|-------------------------------------|--------------|
| Troponine-I (titer) | Pearson correlation                 | 0.341        |
|                     | Sig. (2-tailed)                     | 0.004        |

The following table (4) illustrated the miRNA499a-5p outcomes as suggested technique's through redistribution (under/upper) a cutoff points in the diagnosis of MI in contrast applying Troponine-I , time of symptoms onset , ECG and PCI method, within patients group, as well as other statistical screening tests expressed as [sensitivity , specificity , accuracy, PV, O.R with 95% C.I respectively], and the ROC analysis with its associated curve are listed in the table (5) and figure[2] which reflects the utility of miRNA499a as diagnostic method in comparison with the cardiac troponin-I as reference methods , and its reliability in the diagnosis of MI in pre and post 5 hours intervals , in the differentiation between ECG waves and among the PCI groups .

**Table (4):-** Redistribution suggested technique's outcomes (Under/Upper) a cut off points and ROC Curve of miRNA499 in light of Troponin-I (titer), time, symptoms onset , ECG and PCI in patients group.

| methods                  | Reaction         | miRNA 499a5p% upper cutoff value | Total AMI patients | Sens. % | Spec. % | Accuracy % | Sign. P.V   | OR%  |
|--------------------------|------------------|----------------------------------|--------------------|---------|---------|------------|-------------|------|
| Troponin-I (Titer)       | Positive> 1ng/ml | 40%                              | 40                 | 66.6    | 47.8    | 54.3       | NS<br>O.O76 | 1.83 |
|                          | Negative         | 26.7%                            | 30                 |         |         |            |             |      |
| Time of chest Pain Onset | < 5 hours        | 21.4%                            | 14                 | 11.1    | 74.4    | 50.0       | NS<br>O.14  | 0.36 |
|                          | > 5 hours        | 42.9%                            | 56                 |         |         |            |             |      |
| ECG                      | STElevation      | 45.2%                            | 31                 | 51.9    | 60.5    | 57.1       | NS<br>O. 31 | 1.64 |
|                          | NST Elevation    | 33.3%                            | 39                 |         |         |            |             |      |

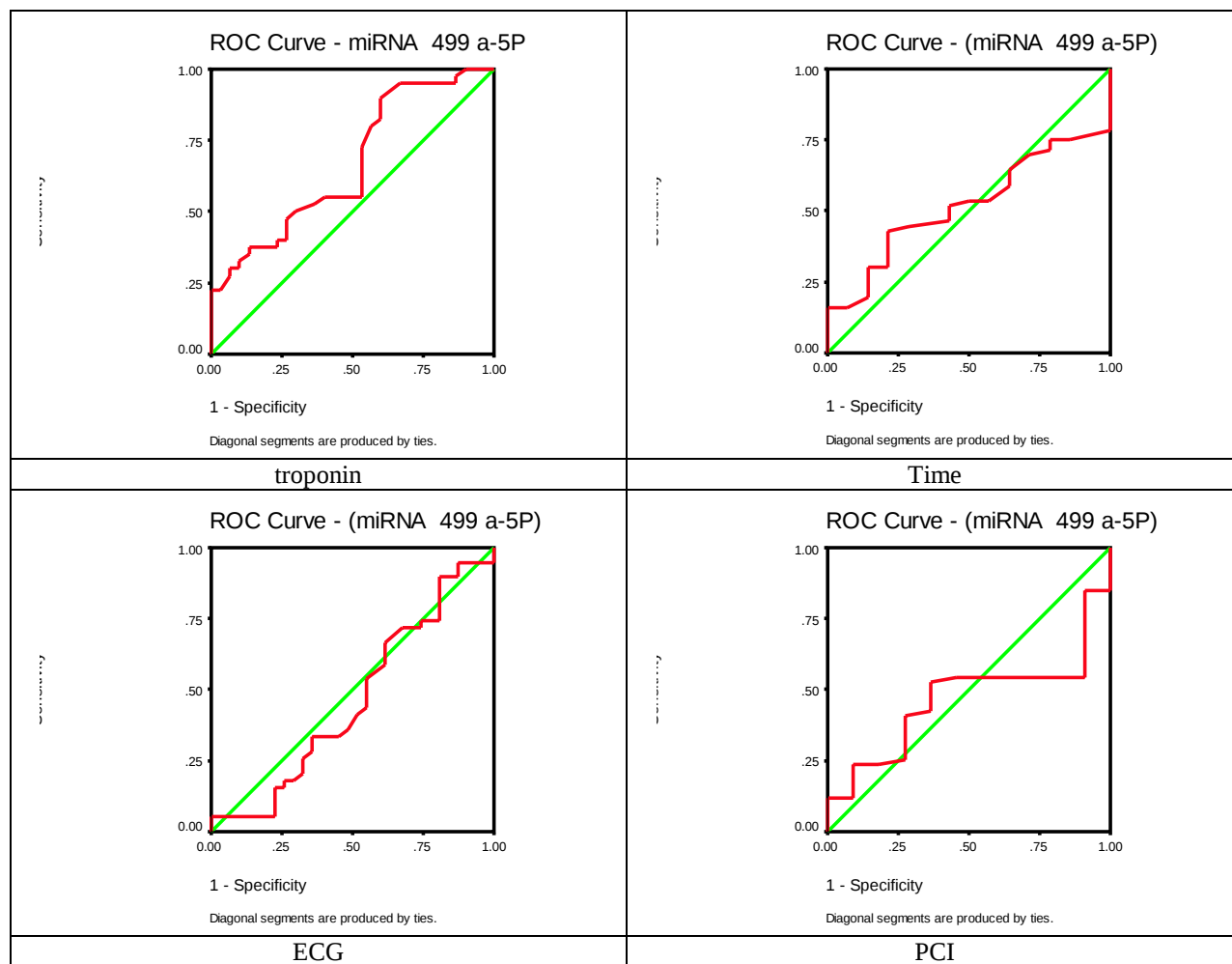
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|-----|----------|-------|----|------|------|------|----|------|
| PCI | Positive | 27.3% | 11 | 88.9 | 18.6 | 45.7 | NS | 1.82 |
|     | Negative | 40.7% | 59 |      |      |      |    |      |

\*= upper cut off .<sup>(\*)</sup> Odds Ratio (Upper / Under), with 95% Confidence Interval; and Screening Tests.

**Table (5):-** Statistics of ROC Curve for miRNA outcomes in contrast of Troponine-I (Titer) , time of symptoms onset ,ECG and PCI.

| ROC Curve miRNA 499 with | Area  | Std. Error | Asymptotic Sig. | Asymptotic 95% Confidence Interval |             |
|--------------------------|-------|------------|-----------------|------------------------------------|-------------|
|                          |       |            |                 | Lower Bound                        | Upper Bound |
| Troponin-I               | 0.667 | 0.065      | 0.018*          | 0.539                              | 0.795       |
| Time symp.onset          | 0.52  | 0.073      | 0.820           | 0.377                              | 0.663       |
| ECG                      | 0.462 | 0.071      | 0.591           | 0.322                              | 0.602       |
| PCI                      | 0.465 | 0.077      | 0.711           | 0.313                              | 0.616       |

<sup>(\*)</sup> S: Sig. at P<0.05; Non Sig. at P>0.05; The positive actual state is Pos.; Sig. in degrade effect



**Figure (2):-** ROC Curve for miRNA499 outcomes in contrast of Troponine-I , time of symptoms onset , ECG and PCI.

The results in the table (4) revealed that the elevated levels of miRNA499a in the plasma of the patients group were associated with 40% of positive troponin-I and with 26.7% of negative troponin-I [66.6, 47.8, 54.3, 0.076, 1.83 respectively], and ROC showed area involved 0.667 of the elevated miRNA499a results. The data in this table also showed that elevated levels of miRNA499a were associated with 21.4% of MI patients at first 5 hours of symptoms onset ,while it was with 42.9% of the patients in period more than 5 hours [11.1, 74.4, 50.0, 0.14, 0.36 respectively], and ROC area involved 0.564 of the elevated miRNA499a results. The elevated results of miRNA499a in this table recognized 45.2% of MI patients who had ST- waves in their ECGs while it was recognize 33.3% of the MI patients who had Non

ST-waves [51.9, 60.5, 57.1, 0.31, 1.69 respectively] with ROC area of 0.462. In the same table, the data showed that the elevated levels of miRNA499a discriminated 27.3% of MI patients who underwent PCI procedure and 40.7 % of MI patients who had not subjected to PCI [88.9, 18.6, 45.7, 0.31, 1.82 respectively] with ROC area involved 0.465% of elevated miRNA499a results.

### Discussion:-

The successful management of patients with MI contributes to the accurate and early diagnosis in the first hours of chest pain. Our findings showed that there was a detectable of miRNA499a-5p levels in the plasma of AMI patients at the first hour of admission. This result is likely to be acceptable in sight several previous studies [17, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35] demonstrated that plasma miRNA 499a-5p was elevated in MI patients and considered it a useful biomarker of MI diagnosis while other studies [36, 37, 38, 39, 40, 41] were not found miRNA499 a biomarker for MI diagnosis and reported that miRNA499 a is not exclusively expressed in the heart muscle indicating for its low specificity. However, only few studies found a significant but not linear association between miRNA499s and the common risk factors of heart diseases [17]. The studies [25, 31, 32, 33] found that miRNA499 was positively correlated with cardiac troponin. Although, the patients group was poorly differentiated by miRNA499a according to the positive and negative troponin-I in table (4), miRNA499a significantly correlated with the troponin-I by pearson estimator, and ROC revealed some acceptance for miRNA499a as a new cardiac biomarker but unlike the diagnostic activity of the troponin. While other study by Ying-Qing Li found that miRNA499a was not significantly correlated with troponin[34]. The present results showed that most elevated levels of miRNA499a-5p were in the period after 5 hours of chest pain onset, suggest that miRNA499a is expressed in the early stages of myocardial injury and become more detectable in the deleterious stages of injury. Same results was observed in a study by Adachi detected miRNA 499a-5p in the heart tissue by using of the microarray technique [30], and in the study by Devaux .. et al [ 35] were reported that miRNA499 increased in MI patients with peak 6 – 12 hours. The over expression of the miRNAs is relay on the type and time of stage of the myocardial injury in which the concentration of miRNA may detect the fate of the myocardium [42]. The early stages of AMI such as ischemia, hypoxia and edema are belong the acute coronary syndrome and associated with presence of related miRNAs, while the miRNAs of MI are often associated with myocardial injury and necrosis, and most these miRNAs shares between the two diseases or among their stages, or potentiates the deleterious stages of left ventricular hypertrophy post MI [43]. The miRNA 499 is one of the main miRNAs involved in stem and progenitor cells differentiation and also responsible for differentiation of fibroblast [44]. However, the progressive events in an injured myocardium still functioning under stress may worsen the injured area and accelerates more fiber letdown. It has been documented that plasma miRNA 499a might leak out of the necrotic myocardium into the circulation during the early stages of AMI and its levels become detectable in the blood of MI patients as the AMI progresses [28]. The miRNA expression, kinetics and release are affected by the stage and the duration of the injury in an injured area surrounded by ischemic tissue. It is not known the entire conditions of the miRNAs which released in a random manner from the injured myocardium to the circulation. These mechanisms are differ from that of troponins the cytosolic structural proteins which are released directly and in a detectable amount from the myocardium in to circulation in a response to a tiny event of myocardial injury. The second is related to the method of detection which assays only the mature form of miRNAs that may not reflect the true expression of miRNAs, in turn, as a method detecting protein like troponin is more applicable and standardized than molecular technique. Moreover, the standard cardiac biomarker for the true comparison other than troponins is not found.

The data in table (4) showed that miRNA499a cannot differentiate between the ECG waves (ST- and NST), and has low ability in recognizing the positive PCI group that may refer to a weak association between expression of miRNA499a and the obstructive events in the coronary arteries and its branches. Our findings were agreement with study by Ying-Qing Li..etal demonstrated that miRNA499 is a good biomarker for MI diagnosis but not significantly differentiate between the ST and NST waves of ECG and among PCI patients [34], while Devaux .. et al found such association with the NST wave and with the positive PCI group [ 35]. The suggestion is the miRNA 499a-5p is a useful biomarker in the diagnosis of AMI and at this time it can be used as a confirmatory biomarker with other cardiac biomarkers, and it may be a successful biomarker detects the deleterious stages of injury following MI.

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