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Diagnostic Potential of miRNAs and their Correlation with High Sensitivity Troponin-I Levels in ACS- NSTEMI Patients

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Abstract

Background: High-sensitivity cardiac troponins (hs-cTns) are used as biomarkers for Acute Myocardial Infarction (AMI). However, hs-cTn levels are not specific and increase in other diseases that are not associated with AMI. Studies have shown that circulating micro RNAs (miRNAs) vary between different types of Acute Coronary Syndrome (ACS) patients. The main objectives of our study are: 1) To measure circulating miRNA-208a (miR-208a) and miRNA-499 (miR-499) in suspected AMI cases in Acute Coronary Syndrome-Non-ST-segment Elevated Myocardial Infarction (ACS-NSTEMI) patients, 2) To determine the time of release of these miRNAs and compare with hs-cTnI, and 3) To evaluate prognostic significance of miR-208a and miR-499 in early prediction of AMI.

Methods: Serum miRNAs were isolated from suspected AMI patients (n=60) and non-ACSNSTEMI chest pain patients (n=10). These AMI patients were divided into 4 groups, very early (≤ 1 hr), early (≥ 1 hr - ≤ 4 hrs), late (≥ 4 hrs - ≤ 8 hrs) and very late (≥ 8 hrs - ≤ 12 hrs) based on the time gap between onset of chest pain and patient presented to hospital Emergency Department (ED). Chemiluminescence Microparticle Immunoassay (CMIA) and Quantitative Real-Time PCR (qRT-PCR) methods were used to determine the expression levels of hs-cTnI and miRNAs respectively.

Results: Plasma levels of hs-cTnI, miR-208a and miR-499 were higher in suspected AMI patients as compared to non-ACS-NSTEMI chest pain patients. Receiver Operating characteristic Curve (ROC) analysis indicated no association between hs-cTnI and miRNA levels between patients presented to ED very early (≤ 1 hr) or early (≥ 1 - ≤ 4 hrs), after onset of chest pain. A positive correlation between miRNAs and hs-cTnI levels in patients presented to ED late (≥ 4-3 ≤ 8 hrs) and very late (≥ 8 - ≤ 12 hrs) after chest pain was observed. Both miRNAs yielded the highest Area Under Curve (AUC) value of 1.000 (95% CI 1.000-1.000) with sensitivity and specificity of 100% in patients presented to ED very early (≤ 1 hr) or early (≥ 1 - ≤ 4 hrs) after onset of chest pain.

Conclusion: Circulating miR-208a and miR-499 are released into the circulation prior to hs-cTnI and appear to be better biomarker for early identification of suspected AMI cases in ACSNSTEMI patients.

Introduction

Acute myocardial infarction (AMI) is one of the leading causes of death in the world [1]. At the onset of AMI, several pathological conditions such as myocardial injury, hypoxia and necrosis take place in the heart, followed by the release of several degraded products including troponins and microRNAs (miRNAs) into the circulation [2,3]. Earlier diagnosis of AMI in patients with acute chest pain was based on the clinical and Electrocardiographic (ECG) findings, which are often misleading [4]. In the past two decades, significant improvements in the diagnosis of AMI have been made on biomarker based approaches [5,6]. Among conventional blood-based biomarkers available till date, cardiac Troponins (cTns) are considered as the 'gold standard' but are not specific enough for an early diagnosis of cardiac damage [7].

The diagnostic performance of cTns has been improved by the introduction of high-sensitivity troponin-I/T (hs-cTnI & hs-cTnT)

assays [2]. However, hs-cTns have several short comings like slow release from damaged myocardium, requirement of repetitive measurements and longer hospital stay. Another important limitation is that hs-cTns levels are also up-regulated in patients with end-stage renal disease [8]. This causes confusion regarding their use in clinical settings [9]. More so, in the setting of atypical symptoms in acute coronary syndrome-non-ST-elevation myocardial infarction (ACS-NSTEMI) patients, the diagnosis and subsequent management of AMI is not straightforward. In daily clinical practice, this scenario pertains to a majority of patients presenting in the emergency department (ED) with symptoms not suggestive of an AMI, making the early diagnosis a challenge. This situation normally occurs when rising cardiac biomarkers are detected without ST segment elevation on the ECG. Therefore, it is necessary to continue the search for new predictive molecular biomarkers with greater specificity for an early identification of suspected AMI cases among ACS-NSTEMI patients. microRNAs (miRNAs) represent a new class of novel molecular biomarkers for the diagnosis of AMI. These are small (19-22 nucleotides), single-stranded, non-coding regulatory RNA molecules, potentially associated with human diseases [10]. Recent studies showed that miRNAs are present in high abundance, release faster into

circulation, remarkably stable and readily detectable in the serum or plasma and differ under pathological conditions [11-13]. Heart muscle derived miRNAs (eg: miRNA-208 and miRNA-499) known to be up-regulated in patients with AMI and Unstable Angina Pectoris (UAP) [14].

miR-208 is predominantly expressed in the myocardium and the gene resides within an intron of α-Myosin Heavy Chain (α-MHC) [2]. miR-208 is involved in cardiomyocyte hypertrophy, fibrosis and regulation of other cardiac gene functions [15]. miR-208 was reported to be absent in plasma of normal healthy human subjects, but sharply rose in AMI patients [2,14]. In contrast, D'Alessandra et al. [16] have showed detectable levels of miRNA-208 only in a sub-set of AMI patients. Nabialek group [17] has reported that miR-208 levels were unchanged in AMI patients, questioning the specificity of this miRNA as predictive biomarker for AMI.

miR-499 is a newly discovered member of miRNA encoded by myosin gene family located in an intronic region of the Myh7b gene. miRNA-499 is expressed in myocardium and released into the circulation when myocardial cells get injured [18]. The circulating levels of miRNA-499 reported to be increased in patients with AMI [19,20]. Reichlin et al. [21] have reported that cardiac troponins improve the early diagnosis of AMI in recent onset of chest pain patients. In a comparative study on the diagnostic performance of miRNA-499 with traditional biomarkers (SMB, cTnI, cTnT, CK-MB, and LDH) revealed that miRNA-499 was present in plasma earlier than these conventional biomarkers of AMI as compared to healthy controls [22]. The present study was undertaken to evaluate the clinical effectiveness, release time and diagnostic potential of miR-208a and miR-499 in ruling out suspected, but not confirmed AMI cases among ACSNSTEMI patients.

Material and Methods

Human ethical committee approval

The study protocol was approved by the Institutional Human Ethics Committees (IHEC) of Lilavati hospital and Research Centre, Bandra, Mumbai, India. Written informed consents were obtained from all subjects before initiation of the study.

Baseline patients characteristics

Patients were interviewed and clinical examination was carried out to collect detailed history for the presence of cardiovascular risk factors (hypertension, smoking status, diabetes, dyslipidemia etc.). Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) were measured with a standard mercury sphygmomanometer. Serum biochemical parameters such as glucose, triglycerides, creatinine, lipid profiles (total cholesterol, HDL, LDL) were determined as per the standard protocols.

Patient's selection and grouping

This was a case-control study conducted with ACS-NSTEMI patients presenting to ED of Lilavati Hospital and Research Centre (LHRC) between March-2014 to February-2017. ACSNSTEMI patients presented to ED and satisfying the inclusion and exclusion criteria based on the reported guidelines of MI were included in the study [23]. ACS-NSTEMI patient showed clinically proven ST depression, lesion in a major coronary artery requiring PCI, a severe or sub-occlusive

lesion in the left anterior descending, circumflex or right coronary artery. In these patients, clinical and ECG findings are inconclusive and are suspected (not confirmed) to have AMI. A total of 228 ACS patients have been recruited. Of these patients, 160 patients were STEMI (70.18%) and 68 were NSTEMI (29.82%) (Figure 1). From 228 ACS patients, 68 patients were identified as suspected AMI patients. Of these 68 suspected AMI patients, 8 patient's samples were excluded from the study (due to poor quality and integrity of RNA). Of the remaining 60 suspected AMI patient samples with good RNA quality, 68.33% were males and 31.66% were females. Age and gender-matched control cohort (6 males and 4 females) having chest pain visited our hospital during the same period was also included for comparison of the data. These patients showed normal ECG and no history of CVD. In these patients, clear alternative cause of chest pain was identified as due to pleuritis, pneumonia or musculoskeletal pain.
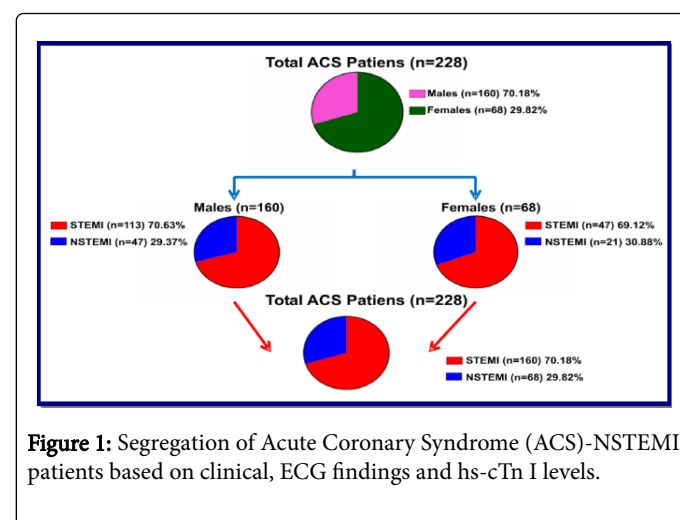

Figure 1: Segregation of Acute Coronary Syndrome (ACS)-NSTEMI patients based on clinical, ECG findings and hs-cTn I levels.

The suspected AMI patients were divided into following 4 groups based on the time gap between the onset of chest pain and patients presented to hospital followed by blood collection. Such a time lapsed study was not done earlier to identify suspected AMI cases among ACS-NSTEMI patients.

Group-1: Patients presented to ED very early i.e., within 1 hr (≤ 1 hr)

Group-2: Patients presented to ED early i.e., between 1-4 hrs (≥ 1 hr - ≤ 4 hrs)

Group-3: Patients presented to ED late i.e., between 4-8 hrs (≥ 4 hrs - ≤ 8 hrs) and

Group-4: Patients presented to ED very late i.e., between 8-12 hrs (≥ 8 hrs - ≤ 12 hrs).

Blood collection and separation of plasma

Venous blood (~ 5 ml from each patient) was collected into Ethylene-Diamine-Tetra-Acetic acid (EDTA) coated Vacutainer (Becton Dickinson, NJ, USA) from all the patients. Plasma was separated by centrifugation at 1,000 × g for 10 minutes at 4°C to remove blood cells. Further centrifugation of the supernatant was done at 14,000 × g for 10 minutes at 4°C to remove additional cellular nucleic acids attached to cell debris. The upper plasma was transferred into RNase/DNase-free tubes. Plasma aliquots (~ 200 μl) were either

stored at -80°C or processed immediately for the determination of hs-cTnI and miRNA levels.

Plasma total RNA extraction and RNA Quality Control (QC)

We used commercially available miRNA kit, mirVana PARIS (Ambion, USA) for the extraction of small RNAs following the manufacturer's protocol. Briefly, 200 µl of plasma sample was mixed with equal volume of 2x denaturing solution and vortexed for 15 seconds. The plasma samples were incubated at RT for 5 minutes to allow complete inactivation of plasma RNases. The final purified RNA was eluted in 30 µl of nuclease-free water (Ambion, USA). The total RNA concentration and its quality were evaluated on a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, USA). A260/A280 ratio was measured and only preparations with absorbance ratio of 1.8 to 2.0 were considered. The mean plasma concentrations of RNA in suspected AMI patients and non-ACS chest pain control patients were 49.53 ± 7.93 µg/µl and 51.30 ± 6.08 µg/µl . The A260/280 ratio was 1.945 ± 0.112 and 1.97 ± 0.098 respectively, indicating RNA preparations were devoid of protein, DNA and phenol contaminants.

Reverse transcription (cDNA synthesis)

miR-208a and miR-499 were reverse-transcribed using TaqMan^{TM} miRNA reverse transcription assay kit (Applied Biosystem, CA, USA) according to the manufacturer's protocol. Briefly, total RNA (10 ng) was used to synthesize miRNA-specific cDNA. RT reaction mixture (15 µl) consists of 5 µl of RNA, 10 µl of Master Mix (4.16 µl of nuclease-free H_2O, 3 µl of TaqMan^{TM} miRNA RT specific primer, 1.5 µl of 1x RT buffer, 0.19 µl of 20 U/µl RNase inhibitor, 0.15 µl of 100 mM dNTP mix, 1 µl of 50 U/ µl MultiScribe reverse transcriptase enzyme). The mixture was incubated at 16°C for 30 minutes, at 42°C for 30 minutes and at 85°C for 5 minutes and then was held at 4°C. The generated cDNAs were stored at -20°C until analysis.

Normalization of qPCR data

Based on the published literature, we selected hsa-miR-16-5p (miR-16) as candidate controls reference gene for normalization of miRNA-208a and miR-499 [24,25]. In qPCR assays, TaqMan^{TM} Universal Master Mix II without AmpEase UNG were used to quantify miR-16, miR-208a (Gene ID: 000511) and miR-499 (Gene ID: 002427) according to the manufacturer's protocols (Applied Biosystems, CA, USA). Briefly, qRT-PCR amplification was performed in duplicates using Bio-Rad CFX96C-1000 Touch Thermal Cycler. Individual qRT-PCR assays were performed in a final volume of 20 µl reaction mixture contained 1 µl of miRNA-specific cDNA, 8 µl of nuclease-free water, 1 µl of gene specific primer/probe mix from the TaqMan^{TM}miRNA assay, and 10 µl of 2x TaqMan^{TM} universal PCR master mix (Applied Biosystems, CA, USA). This RT reaction mixture was used for quantification of miR-16 and miR-208a and miR-499 at 95°C for 10 min, followed by 95°C for 15s (40 cycles) and 60°C for 1 min (40 cycles). The values were normalized by subtracting the mean Ct value of the miR-16 from the mean Ct value of the target genes (Ct mean target gene-Ct mean of miR-16), thereafter the difference of each normalized target gene was obtained (ΔCt miRNAs-ΔCt miR-16), and the relative fold difference was calculated. All cycle threshold (Ct)-values were generated in relation to endogenous reference genes. The Ct values are derived from the relative fluorescence against starting RNA. The miRNA expression was normalized to miR-16 and calculated by the equation ΔΔ-Ct method and data presented as relative fold-change [26]. The Ct value was defined as the cycle number at which the fluorescence exceeded the threshold.

Determination of hs-cTnI levels by Chemiluminescence Microparticle Immunoassay

ARCHITECT STAT based Chemiluminescenc Microparticle Immunoassay (CMIA), (Abbott laboratories, Illinois, USA) was used for the quantitative determination of cardiac troponin-I in human plasma samples on the ARCHITECTi System with STAT protocol capability. This system consists of on fully automated Abotti2000 immuno analyzer. Precision of the quantitative data was analysed using CLSI EP5-A2 software.

Statistical analysis

Statistical analysis was performed with IBM-SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 software packages. The data on miR-208a, miR-499 and hs-cTnI were analyzed by applying Kolmogorov-Smirnov test to verify whether they follow normal distribution of variables. For multiple comparisons, we did apply one-way ANOVA coupled with Bonferroni post-hoc test. Two tailed Kruskal-Wallis H test was performed to detect difference in miRNA gene expression at different time points after onset of chest pain. ΔCt threshold cycle method was used to evaluate the expression of miR-208a and miR-499 [26]. Receiver operating characteristic curves (ROC) were determined using Area under the Curve (AUC) and 95% confidence interval (95% CI). The diagnostic accuracy (specificity and sensitivity) of miRNAs was done as described earlier [21]. Normally distributed data are presented as mean ± Standard Deviation (SD). All reactions were run in triplicate. p-value<0.05 was considered significant and are indicated by asterisk (*p<0.05; ** =p<0.01; *** =p<0.001; ns: not significant).

Results

Baseline demographics and clinical characteristics of the study participants

A total of 228 ACS patients visited ED of Lilavati Hospital and Research Centre (a tertiary care hospital), Bandra, Mumbai, India were recruited in this study (Figure 1). Data was collected on several clinical parameters such as diabetes, previous stroke, status of smoking, alcohol consumption, etc. (Table 1). Personal history of hypertension was recorded based on selfreported use of anti-hypertensive drugs. The physical examination results revealed that Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP), glucose and lipid profiles between ACS-NSTEMI patients and control subjects did not change significantly regardless of their clinical presentation (Table 2).

Parameters	Non-ACS-NSTEMI Control patients (n=10)	ACS-NSTEMI patients (n=60)
SBP (mm/Hg)	122.50 ± 2.32	129.38 ± 8.19
DBP (mm/Hg)	81.80 ± 2.74	79.87 ± 6.40
Glucose (mg/dL)	91.50 ± 9.18	105.68± 33.94
Triglycerides (mg/dL)	97.61 ± 9.34	114.50 ± 12.76
Total Cholesterol (mg/dL)	169.10 ± 13.78	192.68 ± 43.32

LDL cholesterol (mg/dL)	96.90 ± 8.53	115.28 ± 15.16
HDL cholesterol (mg/dL)	44.60 ± 1.20	38.61 ± 6.69
LDL/HDL ratio	2.17	2.99
Total Cholesterol/HDL ratio	3.79	4.99
Creatinine (mg/dL)	0.90 ± 0.07	0.93 ± 0.095

Table 1: Baseline parameters of control subjects and ACS-NSTEMI patients with chest pain irrespective of the time of admission to Emergency Department (ED). Values are the mean ± SD. Student "t' test was used to compare differences between control subjects and patients.

Parameters	Non-ACSNSTEMI Control patients (n=10)	Suspected AMI patients (n=60)
Age (years)	61.37 ± 2.78	61.23 ± 6.24
Male/Female (n/n)		41/19
Age (years)	-	-
Male	61.84 ± 2.80	60.31 ± 6.28
Female	60.90 ± 3.39	63.21 ± 5.85
Current smokers (CS) n (%)	1 (10.0)	08 (13.33)
Hypertension (H), n (%)	0	09 (15.0)
Diabetes (D), n (%)	0	14 (23.33)
Hyperlipidemia (HL), n (%)	0	07 (11.67)
H, CS, n (%)	0	04 (6.67.0)
H, D, n (%)	0	06 (10.0)
D, CS, n (%)	0	03 (5.0)
H, D, CS, HL, n(%)	0	01 (1.67)
ACS-NSTEMI (No AMI risk factors, n (%)	-	08(13.33)

Table 2: AMI risk factors in non-ACS chest pain control patients (n=10) and in suspected AMI cases (n=60) in ACS-NESTMI patients with chest pain irrespective of the time of admission to Emergency Department (ED). Values are the mean ± SD.

Grouping of suspected AMI patients based on age

The mean age of suspected AMI patients (n=60) and control subjects (n=10) was 61.23 ± 6.24 years and 61.37 ± 2.78 years respectively (Table 2). Our results indicated that 58.33% of patients with acute chest pain presented to ED were from the age group of 60-69 years, followed by 50-59 years (30.0%) (Figure 2).

Presentation of suspected AMI patients to ED after onset of chest pain symptoms

The time lapse between onset of chest pain and patient arrival to ED was different in our study groups. Of the total 60 suspected AMI cases,

20% presented to ED within 1 hr (≤ 1) after onset of chest pain, whereas delayed arrival to ED (≥ 1 hr) was recorded in 80% patients. Of these delayed patients, 61.61%, 13.33% and 5.0% presented to ED early (≥ 1 - ≤ 4 hrs), late (≥ 4 - ≤ 8 hrs) and very late (≥ 8 - ≤ 12 hrs) respectively after onset of chest pain (Figure 3).
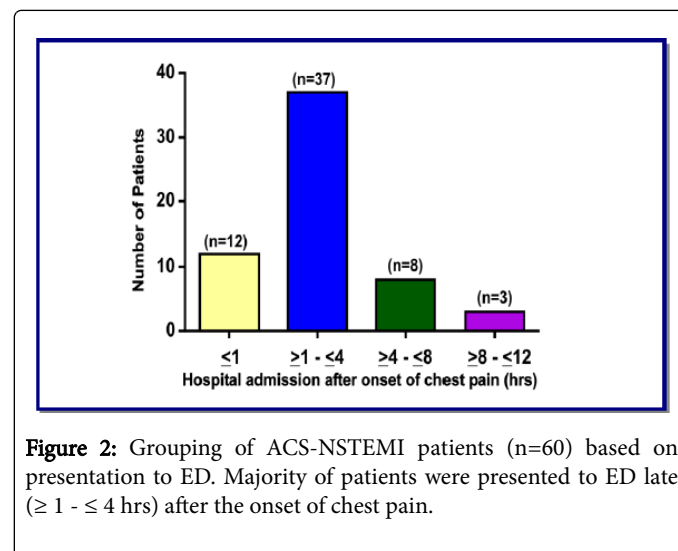

Figure 2: Grouping of ACS-NSTEMI patients (n=60) based on presentation to ED. Majority of patients were presented to ED late (≥ 1 - ≤ 4 hrs) after the onset of chest pain.
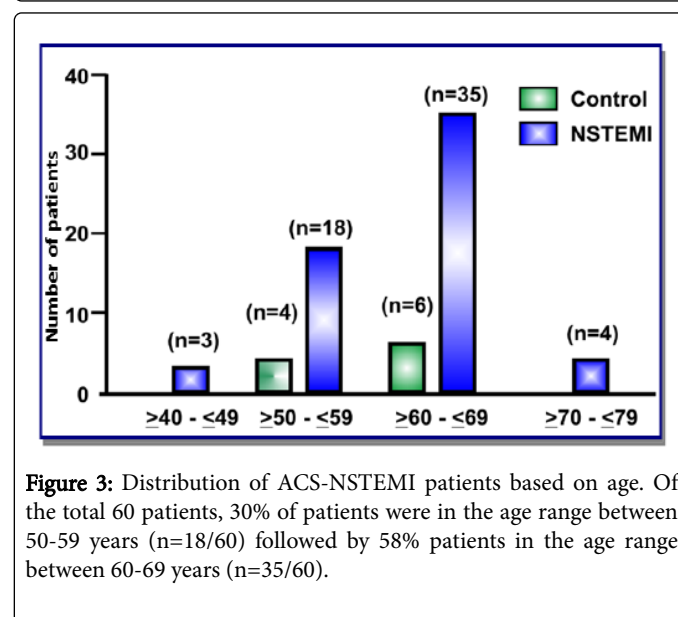

Figure 3: Distribution of ACS-NSTEMI patients based on age. Of the total 60 patients, 30% of patients were in the age range between 50-59 years (n=18/60) followed by 58% patients in the age range between 60-69 years (n=35/60).

Age or gender dependent correlation was not observed in hs-cTnI and miRNA levels

There was no significant effect of age on the expression of hs-cTnI, miR-208a and miR-499. Also the levels of these three markers did not vary between male and female suspected AMI patients. In male patients, the mean levels of hs-cTnI and relative fold changes of miR-208a and miR-499 were 25.19 ± 17.13 pg/ml, 6.27 ± 1.59 and 9.966 ± 2.29 respectively. In female patients, the mean levels of hs-cTnI, and relative fold changes in miR-208a and miR-499 were 29.09 ± 16.07 pg/ml, 6.60 ± 1.57 and 9.81 ± 2.69 respectively.

hs-cTnI levels were up-regulated in suspected AMI patients

Percentages of suspected AMI patients positive for these three markers were determined according to the time lapse between onset of chest pain and blood sampling on ED presentation. There were 12 suspected AMI cases among 228 ACS-NSTEMI patients who presented to ED within 1 hr after onset of chest pain. In all these patients, the mean levels of miR-208a and miR-499 were present in measurable quantity, whereas hs-cTnI present only in 25% of patients (3/12). In remaining 9 patients, hs-cTnI levels ($\geq$ 0.014 µg/L cut-off) (corresponding to the 99th percentile was recommended by the manufacturer) were detectable albeit at significantly low concentration (3.24 ± 1.06 pg/ml) and the values are at par with non-ACS control cohort group (3.32 ± 0.798 pg/ml).

The mean levels of hs-cTnI in remaining 48 suspected AMI patients presented to ED early hrs ($\geq$ 1 - $\leq$ 4 hrs) (n=37), late ($\geq$ 4 - $\leq$ 8 hrs) (n=08) and very late ($\geq$ 8 - $\leq$ 12 hrs) (n=03) after onset of chest pain were 23.32 ± 2.71 pg/ml, 58.19 ± 5.78 pg/ml and 51.67 ± 5.90 pg/ml respectively (Figure 4a). In terms of fold change, we observed 7.03, 17.54 and 15.56 fold increase in patients presented to ED early, late and very late hours after onset of chest pain respectively (Figure 4b). The peak rise of hs-cTnI levels in patients presented to ED late hours after onset of chest pain was gradually decreased and reached to baseline level at 12 hrs post onset of chest pain.

Expression analysis of miR-208a and miR-499 in suspected AMI cases

(a) miR-208a: The expression of miR-208a was analyzed using the comparative CT method in which each gene was first normalized using miR-16. When normalized the data using miR-16 as an internal controls, the relative expression of miR-208a was remained significantly up-regulated and the levels found to be 5.26 ± 0.516 in suspected AMI patients presented to the ED within 1 hr ($\leq$ 1) (n=12) after onset of chest pain, whereas the fold change was 7.73 ± 0.721, 4.24 ± 0.16 and 3.93 ± 0.07 in patients presented to the ED early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) respectively after onset of chest pain (Figures 5a and 5b). These results indicate that significant rise of miR-208a levels in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs) after onset of chest pain, thereafter the levels gradually decreased, and reached to baseline level after onset of chest at 12 hrs pain.

(b) miR-499 levels: When normalized the data using miR-16 as internal controls, the relative expression levels of miR-499 was found to be 9.77 ± 1.53, 11.17 ± 1.37, 6.66 ± 0.060 and 4.54 ± 0.10 in suspected AMI patients presented to ED very early ($\leq$ 1 hr), early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) after onset of chest pain respectively (Figures 6a and 6b). These results revealed peak increase of miR-499 was observed in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs) after onset of chest pain; thereafter the levels were gradually decreased and reached to baseline levels at 12 hrs after onset of chest pain.

The above results suggests that miRNAs showed good correlation with hs-cTn-I levels in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) after onset of chest pain. Relative fold increase in miRNAs and hs-cTnI was peaked early ($\geq$ 1 - $\leq$ 4 hrs) and late 4-8 hrs respectively after the onset of chest pain (Figure 7). However, no such positive correlation was observed in patients presented to ED within 1 hr after onset of chest pain.
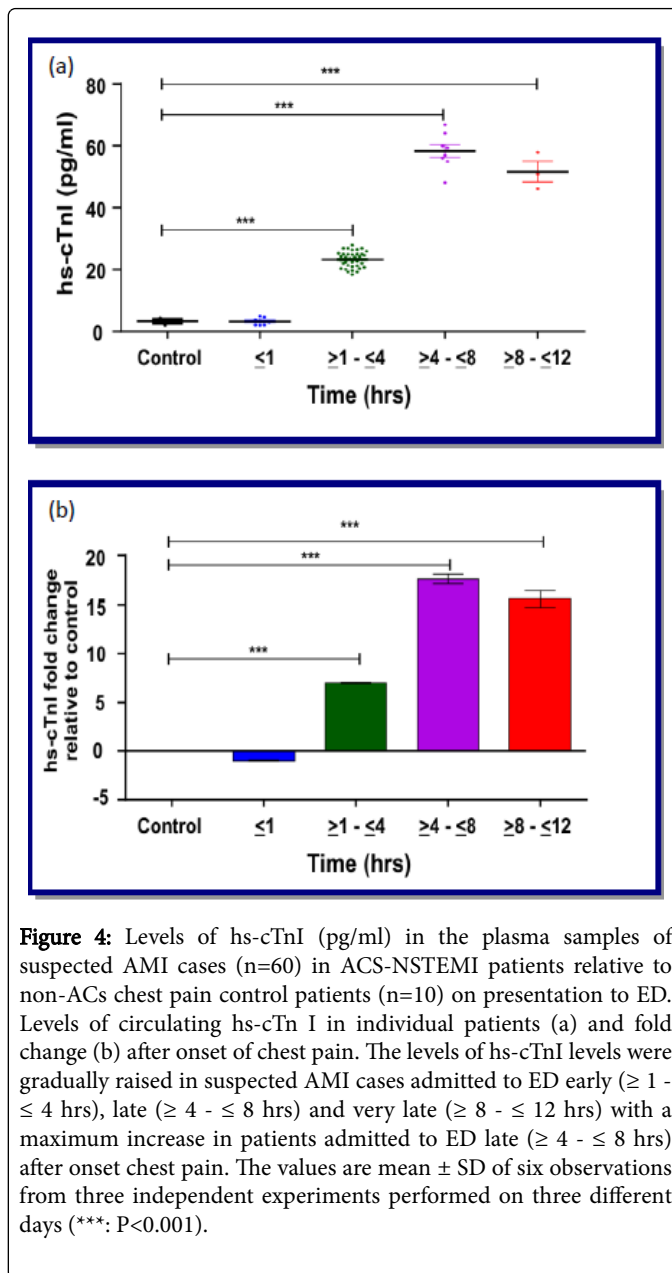

Figure 4: Levels of hs-cTnI (pg/ml) in the plasma samples of suspected AMI cases (n=60) in ACS-NSTEMI patients relative to non-ACs chest pain control patients (n=10) on presentation to ED. Levels of circulating hs-cTn I in individual patients (a) and fold change (b) after onset of chest pain. The levels of hs-cTnI levels were gradually raised in suspected AMI cases admitted to ED early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) with a maximum increase in patients admitted to ED late ($\geq$ 4 - $\leq$ 8 hrs) after onset chest pain. The values are mean ± SD of six observations from three independent experiments performed on three different days (***: P<0.001).

Circulating miR-208a, miR-499 and hs-cTnI levels as predictors of AMI

ROC curve and AUC can be considered diagnostic method for as the evaluation of accuracy of miRNAs. To evaluate the predictive value of up-regulated miRNA for suspected AMI cases, we calculated ROC curve for each of the two miRNAs with the AUC value. Overall, both miRNA were detectable in 100% of suspected AMI patients very early ($\leq$ 1 hr), early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) after onset of chest pain. In contrast, an opposite trend was observed for hs-cTnI levels.
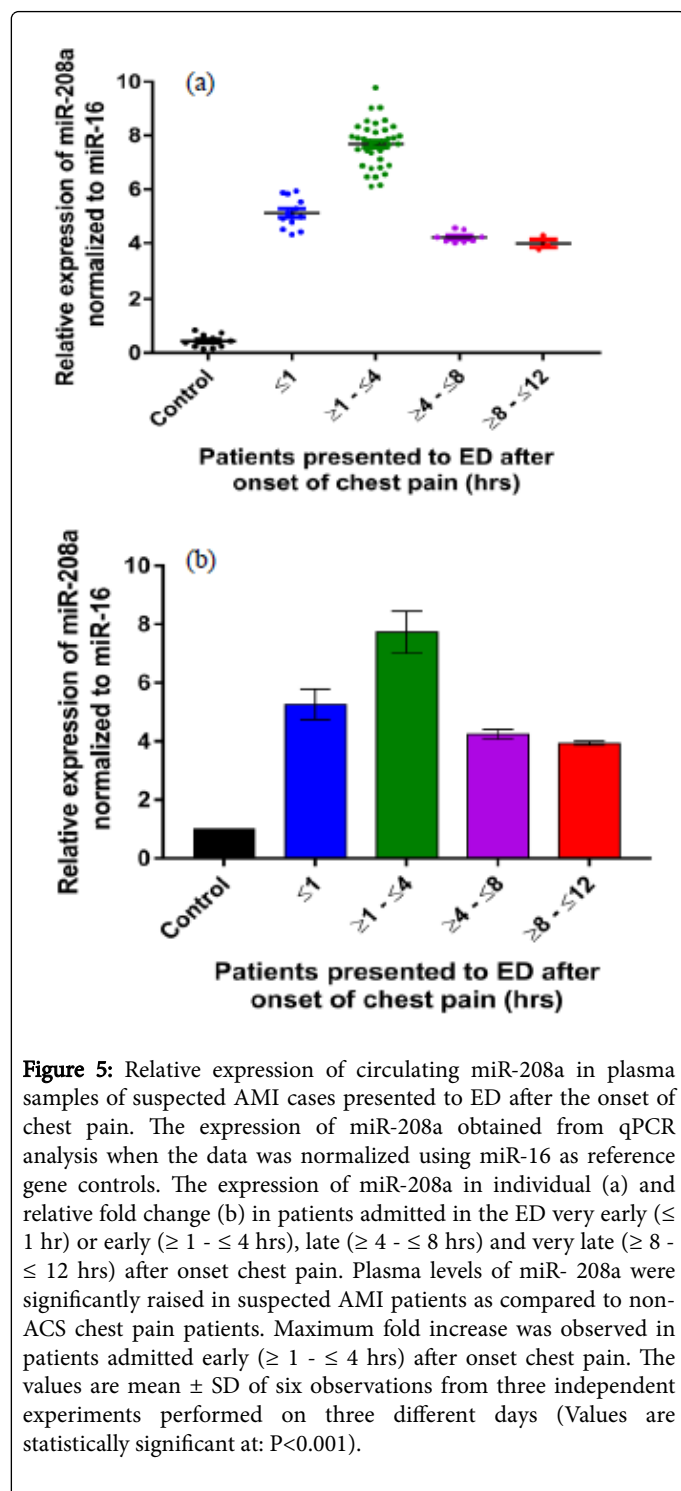
Figure 5: Relative expression of circulating miR-208a in plasma samples of suspected AMI cases presented to ED after the onset of chest pain. The expression of miR-208a obtained from qPCR analysis when the data was normalized using miR-16 as reference gene controls. The expression of miR-208a in individual (a) and relative fold change (b) in patients admitted in the ED very early ($\leq$ 1 hr) or early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) after onset chest pain. Plasma levels of miR- 208a were significantly raised in suspected AMI patients as compared to non-ACS chest pain patients. Maximum fold increase was observed in patients admitted early ($\geq$ 1 - $\leq$ 4 hrs) after onset chest pain. The values are mean $\pm$ SD of six observations from three independent experiments performed on three different days (Values are statistically significant at: P<0.001).

We obtained the following AUC values: for hs-cTnI, 0.52 (95% CI, 0.548-0.504; P<0.001), for miR-208a, 0.96 (95% CI, 0.95-0.97; P<0.001) and for miR-499, 0.92 (95% CI, 0.94-0.92; P<0.001) within 1hr after onset of chest pain (Figures 8 and 9), suggesting negative correlation exist between miRNAs and hs-cTnI at onset of chest pain. The AUC values for hs-cTnI, 0.567 (95% CI, 0.318-, 0.815; P<0.001), for both miR-208a and miR-499, 1.000 (95% CI, 1.000-1.000, P<0.001) in patients presented to ED within 1 hr after onset of chest pain. AUC

was only 57% (95% CI, 0.318-0.815) in patients presented to ED very late ($\geq$ 8 - $\leq$ 12 hrs) after onset of chest pain (Data not shown).
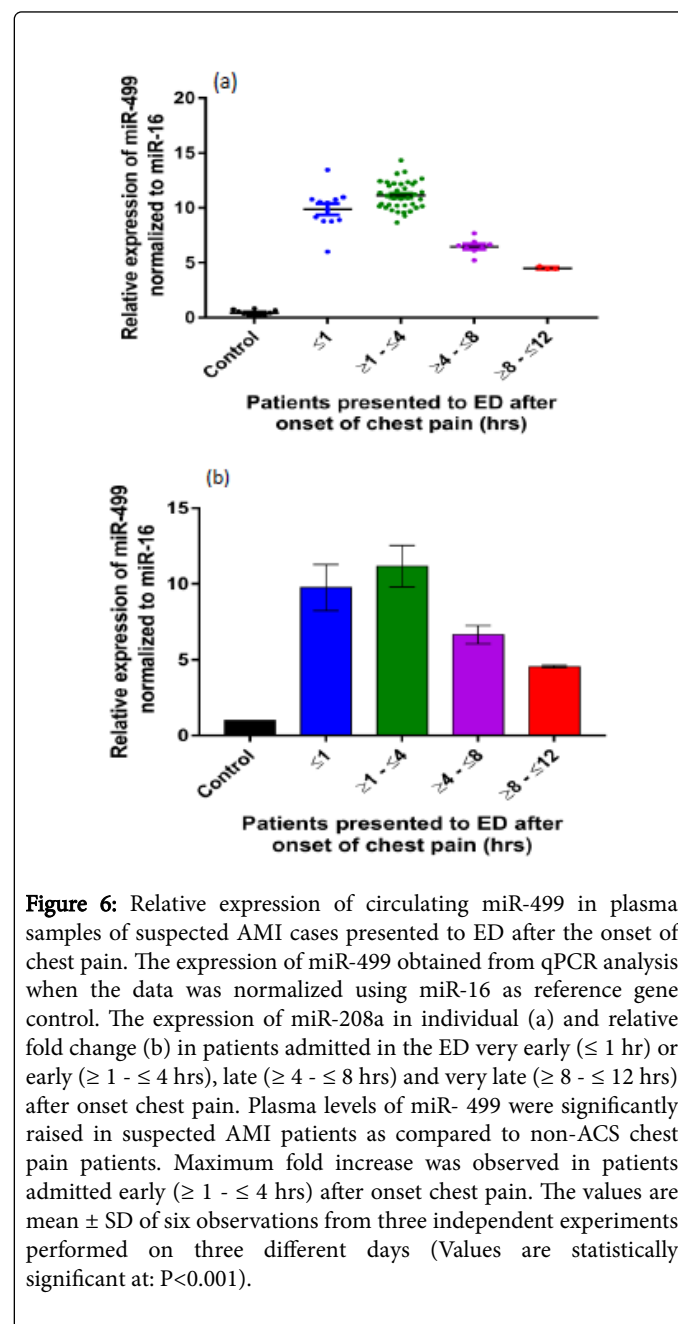

Figure 6: Relative expression of circulating miR-499 in plasma samples of suspected AMI cases presented to ED after the onset of chest pain. The expression of miR-499 obtained from qPCR analysis when the data was normalized using miR-16 as reference gene control. The expression of miR-208a in individual (a) and relative fold change (b) in patients admitted in the ED very early ($\leq$ 1 hr) or early ($\geq$ 1 - $\leq$ 4 hrs), late ($\geq$ 4 - $\leq$ 8 hrs) and very late ($\geq$ 8 - $\leq$ 12 hrs) after onset chest pain. Plasma levels of miR- 499 were significantly raised in suspected AMI patients as compared to non-ACS chest pain patients. Maximum fold increase was observed in patients admitted early ($\geq$ 1 - $\leq$ 4 hrs) after onset chest pain. The values are mean $\pm$ SD of six observations from three independent experiments performed on three different days (Values are statistically significant at: P<0.001).

In our study, the sensitivity and specificity for miR-208 and miR-499 was observed to be 90.0% and 91.21% respectively. Whereas the specificity of both miRNAs decreased from 90% (in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs) after chest pain onset) to 60.00% (in patients presented to ED late ($\geq$ 4 - $\leq$ 8 hrs) after chest pain onset). In contrast, hs-cTnI levels were upregulated from 0% (in patients presented to ED very early ($\leq$ 1 hr), after chest pain onset) to 40% (in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs) after chest pain onset), from 40% to 60% (in patients presented to ED early ($\geq$ 1 - $\leq$ 4 hrs), after chest pain onset and from 60% to 100% (in patients presented to ED very late ($\geq$ 8 - $\leq$ 12 hrs) after chest pain onset), indicating miRNAs showed higher sensitivity than hs-cTnI. Combined determination of miR-208a and

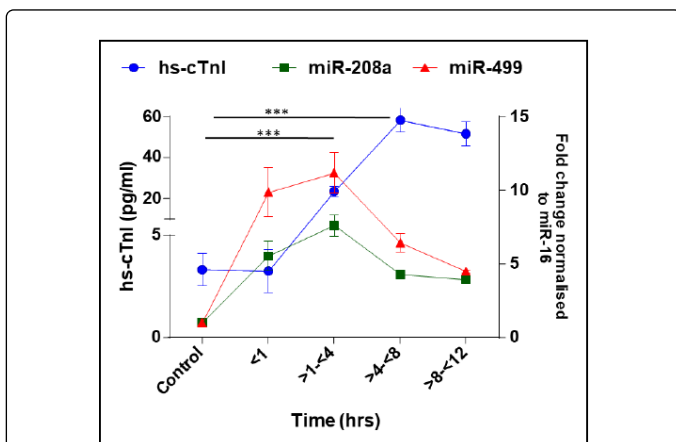miR-499 did not significantly improve the diagnostic value of single markers.

Figure 7: The levels hs-cTn I (pg/ml), and relative fold increase of miR-208a and miR-499 in suspected AMI patients presented to ED very early (≤ 1 hr) or early (≥ 1 - ≤ 4 hrs), late (≥ 4 - ≤ 8 hrs) and very late (≥ 8 - ≤ 12 hrs) after onset of chest pain. Relative fold increase in miR-208a and miR-499 was peaked early (≥ 1 - ≤ 4 hrs) and hs-cTnI peaked late (≥ 4 - ≤ 8 hrs) after the onset of chest pain. The values are mean ± SD of six observations from three independent experiments performed on three different days (***$P<0.001$).
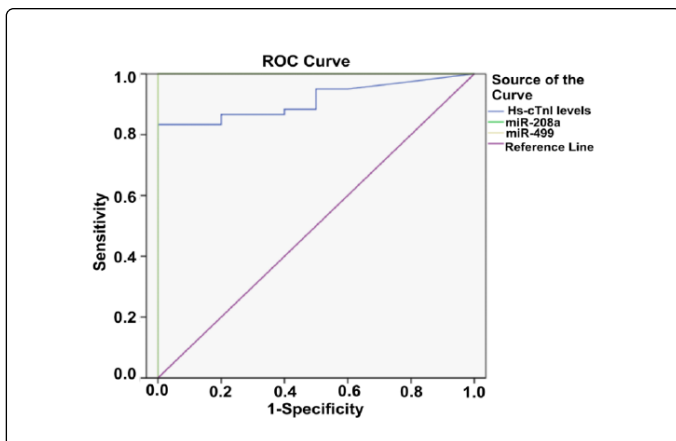

Figure 8: Comparison of sensitivity and specificity of miR-208a, miR-499 and hs-cTnI for the diagnosis of AMI in ACS-NSTEMI patients presented to ED very early (≤ 1 hr) after onset of chest pain. ROC curves were constructed to determine the potential of miRNAs in comparison to hs-cTnI. AUC for hs-cTnI 0.913 (95% CI, 0.846-0.980; $P<0.001$). For both miR-208a and miR-499, AUC was 1.000 (95% confidence interval $P<0.001$) (AUC: Area Under the Curve).

The results demonstrated that both these miRNAs were more sensitive and specific than hs-cTnI, and have great potential to provide sensitive and specific diagnostic value for early rule-out of suspected AMI cases in ACS-NSTEMI patients.
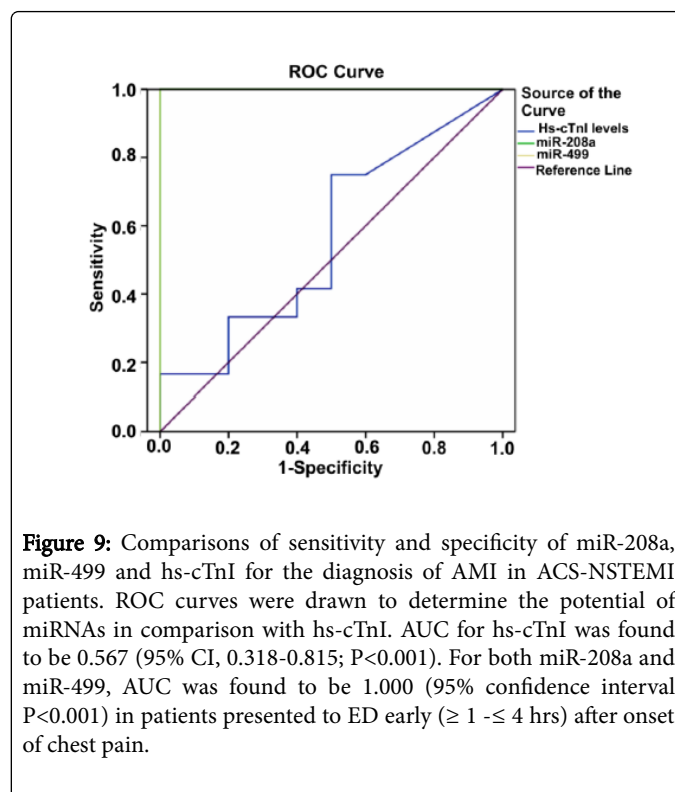

Figure 9: Comparisons of sensitivity and specificity of miR-208a, miR-499 and hs-cTnI for the diagnosis of AMI in ACS-NSTEMI patients. ROC curves were drawn to determine the potential of miRNAs in comparison with hs-cTnI. AUC for hs-cTnI was found to be 0.567 (95% CI, 0.318-0.815; $P<0.001$). For both miR-208a and miR-499, AUC was found to be 1.000 (95% confidence interval $P<0.001$) in patients presented to ED early (≥ 1 -≤ 4 hrs) after onset of chest pain.

Discussion

A rapid and correct diagnosis of AMI has important implications on patients' treatment and prognosis. Although hs-cTns have been used in the diagnosis and risk stratification of AMI, there are still perceived limitations [27], particularly the diagnosis of suspected AMI cases among ACS-NSTEMI patients with acute chest pain symptoms based on clinical presentation and ECG findings are often misleading [4]. Hoeller et al. [28] reported that hs-cTnI levels at presentation should not be used to rule out AMI as 6-23% of confirmed AMI cases had normal levels of hs-cTnI. The kinetic studies of hs-cTnI release from the disrupted cardiomyocytes revealed that acceptable assay sensitivity could detect hs-cTns in patients having a delay of at least 4-6 hrs from symptom onset [29]. Our results are in agreement with Wu et al. [29], wherein these authors demonstrated cTnI levels in blood begins to rise after 4-8 hrs of myocardial injury. In contrast to our study, it has been reported that measurable quantities of cardiac troponin in patients visited to ED after early onset of chest pain [21]. The possible explanation could be due to time difference in patient admission to ED followed by blood collection after onset of chest pain. In our study, we strictly consider time lapse (in hours) between onset of chest pain and patients arrival to ED and blood collection).

Therefore, identifying new molecular biomarkers with better specificity than hs-cTnI can detect molecules released from injured heart muscle at earlier time points. It has been previously reported that the plasma level of miRNAs such as miR-1, miR-133a, miR-133b and miR-499 was significantly elevated in STEMI patients as compared to controls and patients with chest pain [3,11,12,30,31]. Circulating miRNAs (miR-126, miR-223, and miR-197) levels were also shown to be increased in CVD patients [18]. However, there are not many specific studies on miR-208a and miR-499 expression pattern at

different time points after onset of chest pain in suspected AMI cases in ACS-NSTEMI patients.

In the present study, we aimed to investigate the potential prognostic value of circulating miRNAs, miR-208a and miR-499 levels to identify suspected AMI cases in ACS-NSTEMI patients. In our study, AMI risk parameters (triglycerides, total cholesterol, LDL, smoking, hypertension, and creatinine) did not influence the levels of miR-208a, miR-499 and hs-cTnI. We did not observe correlation of hs-cTnI, miR-208a, miR-499 levels with age, gender and other confounding factors associated with hypertension (e.g. Smoking, diabetes, hypertension, LDL etc).

In non-ACS controls cohort, the expression levels of these three biomarkers, hs-cTnI, mir-208a and miR-499 was hardly detectable. However, detectable levels of both miRNA were measured in all the 12 ACS-NSTEMI patent samples collected within 1hr after chest pain onset, supporting the evidence that miRNAs can form early biomarkers to diagnose AMI in ACSNSTEMI patients. However, this observation was limited by the low number of patients presented that early after chest pain onset. At this time point, hs-cTnI levels were undetectable in 75% (9/12) of suspected AMI patients, attributing negative association between miRNAs and hscTnI within 1 hr after the onset of chest pain. This observation also suggests that faster release of miRNAs into the circulation from the injured cardiomyocytes within 1 hr after onset of chest pain as compared to hs-cTnI. For reasons for slow release of hs-cTnI, few studies have revealed that hs-cTnI are bound to myofibrils, whereas miRNAs are bound to cytosolic protein complexes, which affect the patterns of release of these biomarkers during myocardial necrosis [3,6,10]. We believe that these differences might affect the patterns of miRNAs and hs-cTnI release during the progression of myocardial injury, though the release mechanism of miR-208a or miR-499 is unclear at present. Rabinowits et al. [32] has reported that miRNAs are present in microvesicles. It remains to be seen whether cardiac specific miR-208a and miR-499 released from damaged myocardium in the form of microvesicles when they enter into circulation or they may be freed into the bloodstream because of passive release of the cell contents as apoptotic bodies, exosomes and microparticles. Since both miR-208a and miR-499 are cardiac-specific, therefore the levels were minimally affected by non-cardiac tissue injury. Hence, they appear to be superior for the diagnosis of AMI in ACS-NSTEMI patients.

A positive correlation between miRNAs and hs-cTnI release was observed in patients presented to ED late ($\geq 4 - \leq 8$ hrs) and very late ($\geq 8 - \leq 12$ hrs) after onset of chest pain. This could be due to late release of hs-cTn-I, which usually takes 4-6 hrs after onset of chest pain and coincides with peak levels of miRNA [28]. This observation suggests superiority of both miRN-208a and miR-499 over hs-cTn-I for an early prediction of AMI in ACS-NSTEMI patients. To the best of our knowledge, this could be the first systematic study that analyzes the expression of miRNAs at different time points after onset of chest pain and compared with hs-cTnI in suspected AMI cases in ACS-NSTEMI patients.

Next, we performed ROC curve analyses and determined the diagnostic potential of miRN-208a and miR-499 and the results were compared with hs-cTnI levels. miR-208a and miR-499 showed AUC of 1.00 (95% CI 1.00-1.00) with a sensitivity and specificity of 100%, as compared to hs-cTnI, which has an AUC of 0.567 (95% CI 0.318-0.815) in patients presented to ED early ($\geq 1 - \leq 4$ hrs) after onset of chest pain. These results are in consonance with previous study by Wang et al. [2] that miRNA-208a had a high specificity (100%) and sensitivity (90.90%) in the diagnosis of AMI.

Conclusion

The expression of miRNA-208a and miR-499 reached to the peak within first 4 hrs after chest pain onset, gradually decreased and reaches to the baseline level by 12 hrs (i.e., the levels decreased with increased time gap between chest pain onset and patients admission to ED). The miRNA values of suspected AMI patients at 12 hrs were at par with the non-ACS chest pain control patients. In contrast, an opposite trend was observed for hs-cTnI levels. ROC curve results revealed that both miRNAs are superior to hs-cTn-I and can serve as an early warning sign for myocardial ischemia. We believe that further evaluation of the role of these miRNAs in the pathogenesis and progression of AMI will contribute to our understanding of the disease process and lead to new therapeutic and preventative strategies although they need more validation in future studies. If these results holds true for large data sets, the miRNAs explored in this study could be used as diagnostic biomarkers to identify suspected AMI cases in ACSNSTEMI patients.

Study Limitations

Our study has certain limitations which need to be addressed before their application in clinical practice. It is single centre study and sample size may not adequately reflect the population of cardiac patients. Larger studies will be necessary to confirm diagnostic usefulness of these miRNAs in the identification of suspected AMI cases in ACS-NSTEMI patients and unselected populations of patients with AMI. Moreover, miRNAs can only be detected by qRT-PCR, which is a time consuming process. The internal reference for miRNAs quantification is still an issue and needs to be addressed in future studies.

Acknowledgements

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