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ORIGINAL ARTICLE

MicroRNA-208a: a Good Diagnostic Marker and a Predictor of no-Reflow in STEMI Patients Undergoing Primary Percutaneuos Coronary Intervention

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Abstract

MicroRNA-208a is a cardiac specific oligo-nucleotide. We aimed at investigating the ability of microRNA-208a to diagnose myocardial infarction and predict the outcome of primary percutaneuos coronary angiography (PCI). Patients ($n = 75$) presented by chest pain were recruited into two groups. Group 1 ($n = 40$) had ST elevation myocardial infarction (STEMI) and underwent primary PCI: 21 patients had sufficient reperfusion and 19 had no-reflow. Group 2 ($n = 35$) had negative cardiac troponins (cTns). Plasma microRNA-208a expression was assessed using quantitative polymerase chain reaction and patients were followed for occurrence of in-hospital major adverse cardiac events (MACE). MicroRNA-208a could diagnose of MI (AUC of 0.926). After primary PCI, it was superior to cTnT in prediction of no-reflow (AUC difference of 0.231, $P = 0.0233$) and MACE (AUC difference of 0.367, $P = 0.0053$). Accordingly, circulating levels of miR-208a can be used as a diagnostic marker of MI and a predictor of no-reflow and in-hospital MACE.

Keywords MicroRNA · Myocardial infarction · Primary PCI · No-reflow

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Abbreviations

CABG	coronary arteries bypass grafting
cDNA	complementary DNA
CK-MB	creatine kinase MB
cTnI	cardiac Troponin I
cTns	cardiac Troponins
ECG	electrocardiogram
ED	emergency department
hs-Tns	high sensitivity troponins
MACE	major cardiac adverse events
MI	myocardial infarction
miR	microRNA
PCI	percutaneuos coronary angiography
qRT-PCR	quantitative real time polymerase chain reaction
SPSS	statistical package for social sciences
STEM	ST elevation myocardial infarction
TIMI	thrombolysis in myocardial infarction
URL	upper reference limit
WBCs	white blood cells

Introduction

Ischemic heart disease is the leading cause of death worldwide [1]. Timely diagnosis of MI is critical for early initiation of

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management which is a pivotal variable of outcome. Presenting symptoms include chest pain (with radiation to the left arm, neck, jaw or epigastria) and/or shortness of breath, vomiting, palpitations or syncope. The relief of chest pain by administration of nitrates has been used for a very long time as a diagnostic clue but it is less emphasized recently because it may be misleading [2, 3].

In the context of the 4th universal definition of myocardial infarction biomarkers namely cardiac troponins (cTns) represent the corner stone for diagnosis. Acute myocardial injury is evidenced by at least one value of cTns rise above the 99th percentile upper reference limit (URL). If associated with any symptomatic, ECG and/ or imaging evidence of ischemia, this will be consistent with MI type 1 [4]. Primary Percutaneuos coronary intervention (PCI) based strategy is the recommended initial approach for MI patients. Failure to restore blood flow to the myocardium despite physical relief of the occlusion is known as no-reflow which is an independent predictor of death and morbidities [3].

MicroRNAs (miRNAs) are regulatory, non-coding RNAs, around 22 nucleotides in length which are involved in post transcriptional gene regulation [5]. miRNAs are predicted to target 60% of all genes regulating the expression of one-third of all human genes [6]. They are released by cells into circulation either passively or actively [7]. The expression level of miRNAs can be used to guide the diagnosis of certain diseases and predict drug responses and prognosis. miR-1, miR-133a, miR-499, and miR-208a have been shown to be over expressed in myocardial infarction [8, 9]. While miR-1, miR-133a, miR-499 may be less specific to myocardial infarction, miR-208a is shown in many studies to be more specific and may be promising as marker [10].

So, this study was designed to explore the role of miR-208a as a marker for diagnosis of AMI as well as a predictor for outcome of primary percutaneuos coronary angiography especially no-reflow phenomenon.

Patients and Methods

The data supporting the study findings are available from the corresponding author upon request.

Study Population

This study was carried out in Cardiology Department, Zagazig University Hospitals. Approval for the study was obtained from institutional review board. Authors declare no conflict of interests. The study started in January 2018 and ended in August 2019. The study included 75 adult patients. Informed consents were obtained from all patients to use their clinical data and samples for research and analysis according to the guidelines of the Declaration of Helsinki.

Patients admitted within 12 h of the onset of chest pain were enrolled. Exclusion criteria included recent myocardial infarction, previous coronary arteries bypass grafting surgery (CABG), or PCI within 1 week, congestive heart failure or cardiogenic shock at time of presentation, regular hemodialysis and/or ongoing malignancy. Patients were classified into 2 groups: Group 1 included 40 patients diagnosed with STEMI and underwent primary PCI and Group 2 included 35 cases with chest pain but negative troponin T (cTnT). Group 1 is subdivided into: subgroup (a) including 21 cases with sufficient reperfusion (thrombolysis in myocardial infarction TIMI III, TIMI II) and subgroup (b) including 19 cases with insufficient reperfusion (TIMI I, TIMI 0). Both study groups were subjected to detailed history taking and clinical examination. The patients were followed for occurrence of major adverse cardiac events (MACE) during hospital stay (3 days average).

Blood Samples

Samples for routine investigations and myocardial injury biomarkers were collected and handled according to instructions of hospital laboratories service manual. For miRNA determination, 3 mL fresh blood was aspirated from antecubital veins of study population at first presentation prior to PCI and delivered into sterile plain vacutainer tubes. Tubes were centrifuged for 10 min at 12000 g and miRNA was extracted from the serum immediately afterwards as described below.

Measurement of cTnT Levels

Cardiac Troponin T levels were measured in patients' serum by electrochemiluminescence using Roche Cobas-e411automated analyzer using Roche Elecsys® Troponin-T-HS kit.

Quantification of Circulating miR208a

Serum expression of miR-208a was assessed using quantitative real time polymerase chain reaction (qRT-PCR). miRNA extraction was done using "miRNeasy Mini kit"(Qiagen, Germany) according to the supplier protocol and DNA contamination was checked spectrophotometrically by measuring ratio of 260/280 absorbance. For reverse transcription of miRNA "miScript II RT Kit" (Qiagen, Germany) was used according to the supplier protocol. Complementary DNA (cDNA) was then incubated at -80 °C. Amplification and detection by real time PCR was performed using target specific "miScript "Primer Assays (forward primers) and the "miScript SYBR Green PCR Kit "(Qiagen, Germany) according to the supplier protocol. Fold changes of miRNA expression were calculated using the formula: Fold Change = $2^{\wedge -\Delta\Delta CT}$ [11].

Statistical Analysis

The acquired data were analyzed using Microsoft Office Excel 2007 and Statistical Package for Social Sciences version 24 (SPSS: An IBM Company). Data are presented as mean ± Standard deviation (SD) for normally distributed data otherwise range and median are used. Normal distribution of data was confirmed by Shapiro-Wilk test. For comparison of groups, the Mann-Whitney test, Pearson $\chi 2$ test or the Student t test was performed. Diagnostic/Prognostic Performance of tests was done using receiver-operating curves (ROC), AUC comparison was done using the Delong et al. method [12], while the cut-off values were calculated using the Youden J statistics [13]. McNemar's test was used to compare the diagnostic performance of miR-208a to hs-cTnT. The threshold of significance was fixed at 5% level (*p value*), where *P* values <0.05 indicate significant results.

Results

The patients' characteristics are shown in Table 1. The two study groups are homogenous in terms of demographic data, risk factors and history with no significant difference. None of the candidates of both groups was known to have hyperlipidaemia. Those who had previous infarction and/ or revascularization; whether PCI or CABG; were clinically free of events for more than 2 months before admission. The white blood cells (WBCs) count was found to be significantly higher in the patient group as well as the routine cardiac biomarkers. There was no statistically significant difference in the rest of clinical and routine laboratory findings.

miR-208a was significantly up-regulated in STEMI patients compared to control group (miR-208a fold change median = 32.54, range: 11.51–88.91). ROC analysis of relative expression values shows that miR-208a has reasonable sensitivity and specificity for MI diagnosis (AUC = 0.92, sensitivity = 92.5, Specificity = 80). The diagnostic performance of miR-208a is comparable to the routine cardiac biomarkers; creatine kinase-MB (CK-MB) (area difference 0.0439, $P = 0.235$) and cTnT (area difference 0.0614, $P = 0.06$) (Fig. 1-a). McNemars' test shows that miR-208a diagnostic power is similar to hs-cTnT (p = 1.000).

The STEMI subgroups were found to be homogenous in terms of demographic data, risk factors and history. Subgroups' characteristics are shown in Table 2. No significant difference in clinical findings at time of presentation was noticed. miR-208a was significantly over expressed in subgroup (b) compared to subgroup (a) (Table 2, Fig. 2). ROC analysis of miR-208a expression in patients' subgroups shows

Table 1 Characteristics of patients

	Group 1 ($n = 40$)	Group 2 ($n = 35$)	*P* value
	Mean ± SD	Mean ± SD	
Age, y	57.37 ± 12.47	60.2 ± 11.1	0.25
Male sex, n (%)	30 (75%)	28 (75%)	0.605
Current Smoker, n (%)	27 (67.5%)	19 (54.3%)	0.2411
Hypertension, n (%)	16 (40%)	14 (40%)	1.0000
Diabetes, n (%)	19 (47.5%)	18 (51.4%)	0.8230
Family history, n (%)	4 (10%)	3 (8.57%)	0.8325
Obesity, n (%)	7 (17.5%)	4 (11.4%)	0.4584
Previous MI, n (%)	4 (10%)	2 (5.7%)	0.4949
Previous revascularization, n (%)	6 (15%)	2 (5.7%)	0.1937
Blood pressure, mmHg			
Systolic	135.75 ± 18.7	129.3 ± 16.6	0.08186
Diastolic	76.25 ± 8.37	74 ± 9.37	0.23404
Heart rate, bpm	103.2 ± 18.3	105.7 ± 23.8	0.9124
	Range (median)		P Value
Time to admission, hours	1–5 (3)	1–7 (3)	0.5157
WBCs × $10^3/\mu L$	3.5–9.9 (7.4)	2.7–9.3 (6.4)	0.0438
Hemoglobin, g/dL	16.2–10.6 (13.05)	15–10.2 (12.7)	0.5892
Platelet count, $10^3/\mu L$	123–410 (264)	41–396 (248)	0.05238
CK-MB, IU/L	2–96 (58.5)	1–5.5 (2.8)	< 0.00001
hs-TnT IU/L	0.01–10.5 (4.85)	0.01–0.02 (0.01)	< 0.00001

CK-MB indicates creatine kinase-MB; hs-TnT, high sensitivity Troponin T and WBCs white blood cells

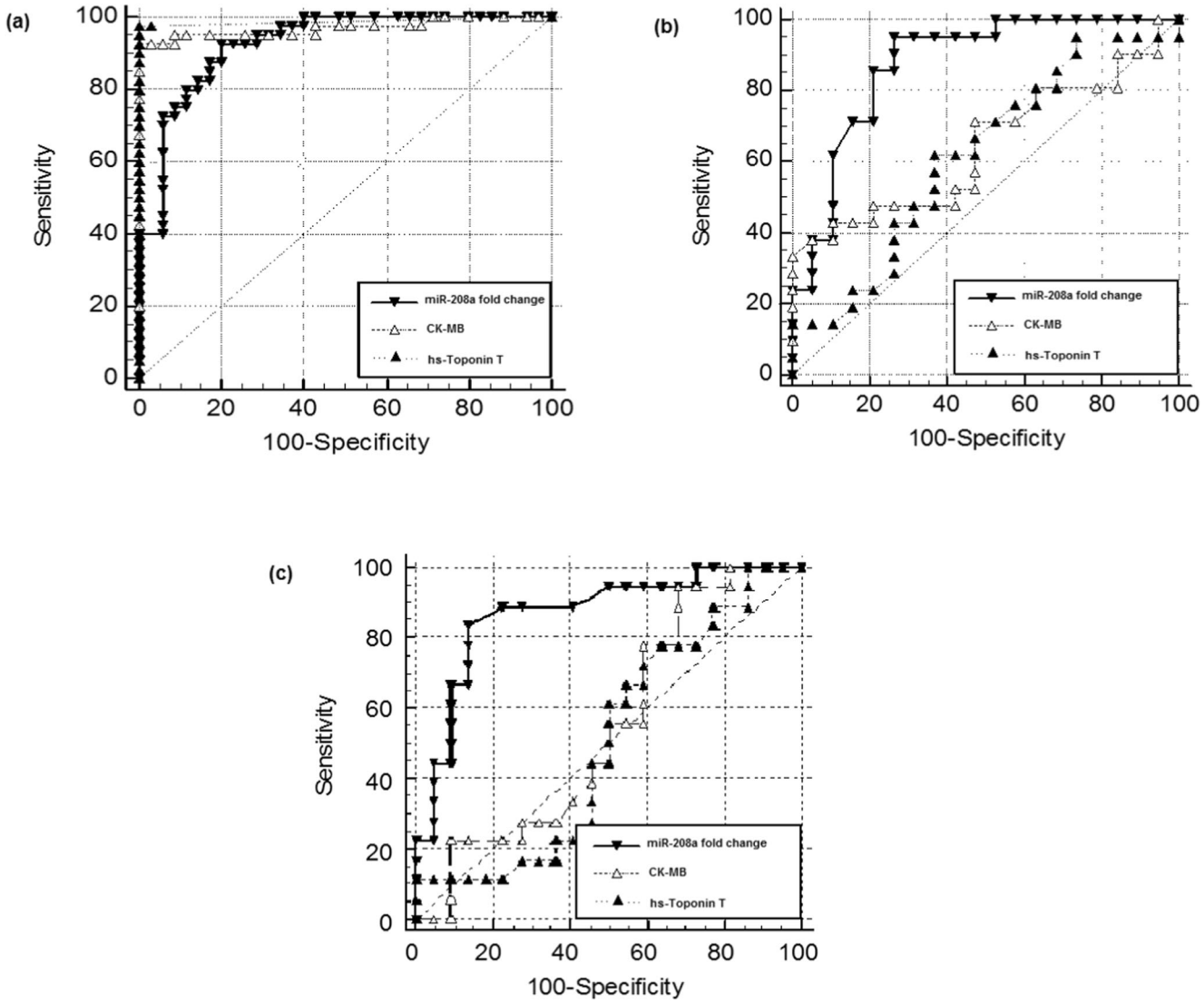

Fig. 1 (a) ROC comparison of miR-208a diagnostic performance against routine cardiac biomarkers. MicroRNA-208a is not inferior to hs-cTnT as a diagnostic marker. (b) Receiver operating curve analysis of no-reflow prediction performance of miRNA208a, CKMB and hs-Troponin T. MicroRNA-208a shows significantly higher prediction of no-reflow as compared to routine cardiac biomarkers. (c) Receiver operating curve analysis of MACE prediction performance of miRNA208a, CKMB and hs-Troponin T. microRNA-208a is significantly higher to routine cardiac biomarkers in MACE prediction

it to be a good predictor of no-reflow as well as in-hospital MACE after primary PCI and significantly superior to cTnT and CK-MB (Tables 3, 4 – Fig. 1-b&c).

Discussion

Myocardial infarction is one of the main causes of mortality in the world which is mainly treated with primary PCI or fibrinolytic drugs. Up-to-date cardiology guidelines recommend early diagnosis and immediate mechanical restoration of coronary flow and establishment of myocardial reperfusion in patients presenting with STEMI [3]. Despite the great improvement in the detection of cardiac troponins; the gold standard test in myocardial infarction; there is a decrease in test specificity which is associated with longer stay at emergency department (ED), more healthcare costs and undue interventions [14]. The high sensitivity cTns (hs-Tns) essays are less accurate in the first hour of pathology development [4]. Also, the prognostic role of hs-Tns is less well established [15–18].

A great improvement has been encountered in the techniques and materials used for primary PCI, yet it fails to normalize the coronary flow and myocardial perfusion in some patients. This phenomenon described as no-reflow is associated with an increased mortality as well as morbidity. Studies have suggested possible mechanisms for no-reflow phenomenon such as endothelial ischemic damage, microvascular

Table 2 characteristics of patients' subgroups

	Subgroup a ($n = 20$)	subgroup b ($n = 19$)	P value
	Mean ± SD	Mean ± SD	
Age, y	61.04 ± 12.11	53.32 ± 11.86	0.06
Male sex, n (%)	16 (76.19%)	14 (73.68%)	0.855
Current Smoker, n (%)	13 (61.90%)	14 (73.68%)	0.427
Hypertension, n (%)	8 (38.09%)	8 (42.11%)	0.796
Diabetes, n (%)	9 (42.85%)	10 (52.63%)	0.53645
Family history, n (%)	2 (9.52%)	2 (10.53%)	0.9159
Obesity, n (%)	6 (28.57%)	1 (5.26%)	0.05269
Previous MI, n (%)	3 (14.28%)	1 (5.26%)	0.316443
Previous revascularization, n (%)	5 (23.80%)	1 (5.26%)	0.100914
Blood pressure, mmHg			
Systolic	139.29 ± 16.9	131.84 ± 20.22	0.212724
Diastolic	77.62 ± 7.68	74.74 ± 9.05	0.282966
Heart rate, bpm	1–5 (3)	1–5 (2)	0.152914
	Range (median)		P
Time to admission, hours	1–5 (3)	1–7 (3)	0.5157
miR-208a Fold change*	11.51–56.66 (26.43)	17.92–88.90 (53.60)	< 0.0001

*Cut-off value for change in expression is 1. Values >1 indicate up-regulation, while values <1 indicate down-regulation

miR-208a indicates microRNA-208a

obstruction, leukocyte occlusions, mechanical compression due to interstitial edema, reactive oxygen radicals and coagulation. However, no-reflow phenomenon is still unpredictable with no marker being suitable for anticipating no-reflow [19].

As the pathogenesis of myocardial infarction involves tissue ischemia, edema and necrosis with release of necrotic yield into circulation, miRNAs are suggested to have a promising diagnostic role [5].

In this study we report five main findings. First, the circulating level of miR-208a was significantly higher in STEMI group than the control group. Secondly, miR-208a has a good diagnostic accuracy (AUC = 0.926). Thirdly, the diagnostic

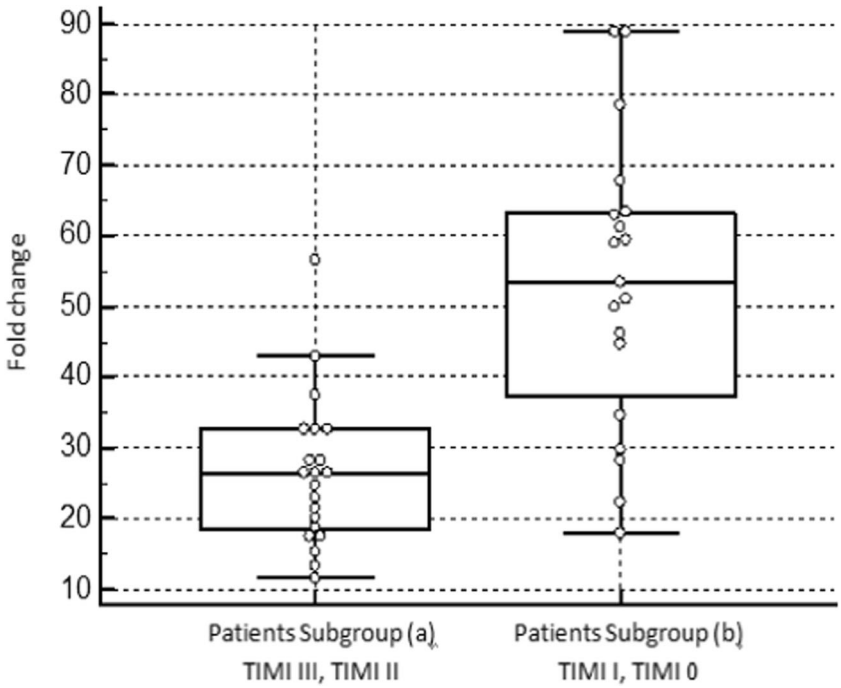

Fig. 2 Box plot of the fold change values of miR-208a in subgroup a (21 patients with TIMI III, TIMI II flow after primary PCI) and subgroup b (19 patients with TIMI I, TIMI 0 flow after primary PCI)

Table 3 miRNA-208a prognostic performance

	Cut-off (>)	AUC	Sensitivity	Specificity	95% CI	p value
No-reflow	42.94	0.875	73.68	95.24	0.732 to 0.958	< 0.0001
MACE	32.54	0.871	83.33	86.36	0.727 to 0.956	< 0.0001

MACE indicates major adverse cardiac events

accuracy of the studied miRNA is comparable to that of the routine cardiac biomarkers including CK-MB and hs- TnT. Fourthly, miR-208a showed good performance in prediction of in-hospital MACE which was significantly superior to CK-MB and TnT. Finally, miR-208a showed good performance in prediction of no-reflow.

The time from onset of chest pain till admission and obtaining samples in our study was 1–5 h for the STEMI group and 1–7 h for the control group. While very few studies on miRNAs in myocardial infarction reported the time to admission we find that Devaux et al. reported significantly longer times to admission which were 3–14 h for the STEMI group and 3–12 h for the control group [20]. Almost all studies that followed the temporal profiles of miRNAs in myocardial infarction reported them to be detected within the first hour of infarction, peaks early, drop after primary PCI to be nearly undetectable in circulation by discharge time [21–25]. The later presentation of the patients in the study by Devaux et al. may explain the less promising results they reported.

While we did not intend to choose shorter periods of onset of symptoms while recruiting our patients to the study, we find this parameter to be more in line with our vision for the possibility of future use of miR-208a for two reasons. First, most of the recent guidelines and studies for best clinical practice recommends early rule in/out of infarction within 1 h of presentation. From health economics and clinical points of view this is more convenient [26]. Secondly, the accuracy of hs-Tns assays increases with longer periods from onset of symptoms which makes it extremely difficult for any other biomarker to compete with and may be not worth investigating, but its role in the first hour of onset of symptoms is still controversial [4].

Table 4 Comparison prognostic performance of miR-208a and routine biomarkers

		miRNA 208a		
		Areas difference	95% CI	P
No-reflow	hs-Troponin I	0.231	0.0314 to 0.430	0.0233
	CK-MB	0.257	0.0625 to 0.451	0.0096
MACE	hs-Troponin I	0.367	0.109 to 0.626	0.0053
	CK-MB	0.323	0.135 to 0.511	0.0008

CK-MB indicates creatine kinase-MB; hs-TnI, high sensitivity Troponin I; MACE, major adverse cardiac events and miR-208a, microRNA-208a

The STEMI group had a significantly higher WBCs counts in comparison with the control group. This finding could reflect active inflammatory process linked to the acute MI and is concordant with other reports [27]. Meder et al. showed no difference in WBCs count which may be attributed to the small sample size they used [28]. Although this finding may be considered as just an ancillary finding, from the molecular pathology point of view it may be linked to the role of miR-208a in regulating cell proliferation and migration and we think it warrants further investigation [29, 30]. It is of note that miR-208a is known to be cardiomyocyte specific and not expressed by leukocytes [31].

Our study shows significant higher concentrations of miR-208a in the STEMI group in comparison to the control group. Similar findings were reported by Devaux et al. 2015, Liu et al. 2015 and Liu et al. 2017 [23, 32, 33]. The diagnostic performance of miR-208a is still controversial. In our study it showed good performance with AUC of 0.926 which is comparable to that reported by Li et al. 2013 and Wang et al. 2010. However, Devaux et al. 2015 and Liu et al. 2015 reported lower diagnostic power of the marker with AUC of 0.76 and 0.72; respectively. In the study by Devaux et al. 2015, this can be attributed to the later time of presentation while we suspect that the methodology used by Liu et al. 2015 is the cause as they had their samples frozen before extracting the RNA and this usually gives less accurate results [10, 20, 22, 33].

It has finally to be noted that for most of the aforementioned studies, miRNA performance is compared between STEMI patients and healthy control group. Our study is one of very few studies which compared STEMI patients to a control group of unstable angina. This makes our findings in the diagnostic context more valuable. Several studies have suggested the use of miR-1, miR-16, miR-499 and/or miR-133a as a standalone or combined diagnostic tests of MI [10, 20–22]. While some of the aforementioned markers showed good sensitivity, most of them lack specificity and are of low diagnostic power compared to the cardiac troponins.

In this study, miR-208a showed very good performance as a prognostic marker. It can predict in hospital MACE with good accuracy (AUC = 0.871). Supporting results were reported by Gidlöf et al. and Widera et al. [21, 34]. While these studies showed troponin to be superior as MACE predictor, our study shows miR-208a to be superior to both CK-MB and cTnT. This may be attributed to two main factors; the very early time of presentation of patients enrolled in this study in

comparison with other studies and the short term period of follow up of patients for MACE; only during hospital stay (average 3 days). CORTEZ-DIAS N et al. studied the short and long term prognostic value of some miRNAs including miR-208a but they did not clearly discuss the value of miR-208a in predicting short term prognosis [35].

To the best of our knowledge, this is the first study to investigate the value of miR-208a in no-reflow phenomenon and the second to investigate the role of any known miRNA in no-reflow. Only one article addressed the value of miR-30e in no-reflow prediction, where Su et al. found this marker to be an independent predictor of no-reflow [36]. In our study, miR-208a is proved to be a good predictor of no-reflow with significantly higher plasma levels in patients with TIMI I or TIMI 0 flow after primary PCI. miR-208a has good prognostic performance with AUC = 0.871 which is significantly better than that of routine cardiac biomarkers. According to literature, we doubt the specificity of miR-30e and believe that miR-208a is more specific and reliable, but still real experimental evidence is needed.

The current study had few limitations. All the studied subjects were presented to the ED with chest pain and had significant ECG changes; this explains the overestimated AUC for troponins and makes the studied cohort less representative of the whole population. This study did not include patients with Non -STEMI, recent MI, cardiogenic shock or heart failure at time of admission. Although these categories were excluded to eliminate the possibility of them having false elevation of miRNA levels, they may be considered for future studies. The study included patients presented within short time from onset of symptoms which may be viewed in two different ways. On one hand, the reported results cannot be generalized to patients presented at later times. On the other hand, they strengthen the usefulness of miR-208a in early hours of infarction. Finally, we conducted our analysis on miR-208a not miR-208b which has a similar (but not identical) nucleotide sequence to miR-208a. Many studies have investigated either miR-208a, miR-208b or both. Accordingly, we would suggest extending future studies to include both sequences for comparison of their diagnostic and prognostic performance.

Conclusion

The present study highlights the value of miR-208a as a diagnostic and prognostic marker in patients presented with STEMI. It is a good marker for diagnosis of STEMI and prediction of no-reflow and in-hospital MACE after primary PCI. Although still far from clinical application due to long time needed for the assay (at least 2 h) yet the introduction of new fast technologies for nucleic acids assays could support its application in clinical sittings. We recommend extending the study to include large cohort of population, including all categories of acute coronary syndromes, follow up of patients for longer time and temporal profiling of the marker. A more in depth study to consider the extent of cardiac damage and the infarction territory may be pursued.

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Compliance with Ethical Standards

Conflict of Interest All authors declare no conflict of interest.

Human Subjects/Informed Consent Statement All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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