School of Sciences and Engineering

Role Of MicroRNA-208a As A Diagnostic And Prognostic Marker In Patients With ST-Elevation Myocardial Infarction

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DEDICATION

To my beloved wife, daughter and family; my dear godfathers; Mr. Ashraf Ali & Prof. Dr. Atef Redwan; and my faithful friends, you are all what I need, beside my faith, in my holey journey to success

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List of Abbreviations

ACC	American college of cardiology
ACEI	angiotensin converting-enzyme inhibitors
ACS	acute coronary syndromes
AHA	American heart association
AMI	acute myocardial infarction
ARBs	angiotensin receptor blockers
BMS	bare metal stents
Bpm	beat per minute
BUN	blood urea nitrogen
CABG	coronary artery bypass grafting
CBC	complete blood count
cDNA	complementary DNA
CIN	contrast induced nephropathy
CK-MB	creatine kinase-MB
cTns	cardiac troponins
DBP	diastolic blood pressure
DES	drug eluting stents
DM	diabetes mellitus
ECG	electrocardiographic
ED	emergency department
EMS	emergency medical system
ESC	European society of cardiology
FMC	first medical contact
FTT	fibrinolytic therapy trialists
Hb	hemoglobin
HIT	heparin induced thrombocytopenia
HR	heart rate
hs-CRP	high sensitivity C-reactive protein
hs-TnT	high sensitivity- troponine T
INR	international normalization ratio
IRB	institutional review board
ISFC	international society and federation of cardiology
KFT	kidney function tests
LBBB	left bundle branch block
LDL-C	low density lipoprotein cholesterol
LFT	liver function tests
LMWH	low molecular weight heparin
lncRNAs	long non coding RNAs
LV	left ventricular
MACE	major adverse cardiac events
MI	myocardial infarction
miR-Base	miRNA database
miRISC	miRNA-induced silencing complex
miRNAs	microRNAs

miRNAs	Micro-RNAs
MONICA	MONItoring of trends and determinants in CArdiovascular disease
MRA	mineralocoticoid receptor antagonists
mRNAs	messenger RNAs
ncRNAs	non-coding RNAs
NHLBI	national heart, lung, and blood institute
NRI	net reclassification indexes
NSTEMI	non–ST-segment elevation myocardial infarction
NT pro-BNP	N- terminal pro B-type natriuretic peptide
PCI	percutaneuos coronary angiography
piRNAs	Piwi-interacting RNAs
pre-miRNAs	precursor miRNAs
pri-miRNAs	primary miRNAs
RBBB	right bundle branch block
RISC	RNA-induced silencing complex
ROC	receiver operating curve
rRNAs	ribosomal RNAs
RT-PCR	real-time quantitative polymerase chain reaction
SBP	systolic blood pressure
SK	streptokinase
snoRNAs	small nucleolar RNAs
SNP	single nucleotide polymorphism
snRNAs	small nuclear RNAs
SPSS	statistical package for social sciences
STEMI	ST-segment elevation myocardial infarction
TIMI	thrombolysis in myocardial infarction
t-PA	tissue plasminogen activator
tRNAs	transfer RNAs
UDMI	universal definition of myocardial infarction
UFH	unfractionated heparin
URL	upper reference limit
UTR	3’untranslated regions
WHF	world heart federation
WHO	world health organization

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Abstract

Acute Myocardial infarction (AMI) is the leading cause of death worldwide. Diagnosis of AMI depends on presenting symptoms, electrocardiogram (ECG), and cardiac troponins (cTns). Troponins are not good markers for patients presenting at the first hour of chest pain, so there is still a need for a marker that can be detected within the first hour of presentation, with high specificity and sensitivity, guide the medical decisions and give good insight on the expected prognosis.

Previous studies reported miR-208a as a cardiac specific microRNA that can be detected in the plasma as early as 15 min of cardiac insult and is stable in plasma for hours. But no study has investigated its role as a predictor of primary percutaneuos coronary angiography (PCI) outcome.

The study objective was to investigate the diagnostic role of miR-208a in AMI and its ability to predict the outcome of primary PCI and in-hospital major adverse events (MACE).

In the present study, 75 patients presented by chest pain to Zagazig University hospitals were enrolled into 2 groups. Group 1 included 40 patients diagnosed with ST-elevation myocardial infarction and underwent primary PCI, of them 21 had sufficient reperfusion and 19 had no-reflow. Group 2 had negative cTns. Institutional review board approval and consents according to the Helsinki declaration were obtained. Plasma expression of miR-208a was assessed in both groups and patients were followed along their hospital stay (range =6-96 hours).

MicroRNA-208a was found to be a good marker for diagnosis of MI (AUC= 0.926) which was not inferior to cTns. There was no value of adding miR208a to the classic clinical model. miR-208a can predict in-hospital MACE (AUC=0.871) and no reflow (AUC=0.875) after primary PCI and is significantly superior to cTns in these outcomes.

We conclude that miR-208a is a good diagnostic and prognostic marker of myocardial infarction and can predict no reflow after primary PCI.

Chapter One: Introduction and Literature Review

1.1. Introduction

Acute Myocardial infarction (AMI) is the leading cause of death worldwide (Wang et al., 2010). Diagnosis of MI mainly depends on the skillful interpretation of the symptoms and signs and depiction of the electrocardiographic (ECG) findings in the context of the cardiac biomarkers; and serum level of cardiac troponins (cTns) or Creatine kinase-MB (CK-MB) (D'Alessandra et al., 2010).

1.2. ST-Elevation Myocardial Infarction

1.2.1. Definition

Myocardial infarction is irreversible necrosis of cardiomyocytes due to prolonged cessation of oxygen supply which is known as heart attack. The imbalance in oxygen supply and demand occurs usually after thrombus formation on top of plaque rupture within the coronary vasculature that leads to an abrupt reduction of blood supply to a portion of the myocardium (Costa et al., 2008).

Acute coronary syndromes (ACS) are wide spectrum of clinical presentations due to ongoing myocardial ischemia characterized by chest pain with or without changes of ECG and cardiac injury biomarkers. Myocardial infarction is on top of the spectrum which is characterized by elevated cardiac biomarkers in a special temporal profile. When this elevation is associated with elevation of ST segment in the ECG it is named ST-elevation myocardial infarction (STEMI), otherwise it will be considered non–ST-elevation myocardial infarction (NSTEMI). The ST elevations noticed in ECG denote active and ongoing transmural myocardial ischemia and injury (Wang et al., 2004).

The development in biosensors and the possibility to establish a clear temporal profile of biomarkers in relation to the ongoing pathology with good correlation to clinical presentation, outcome and with high sensitivity and specificity encouraged the task force of the European Society of Cardiology (ESC) and the American college of Cardiology (ACC) to report the first definition of Myocardial infarction based on the biomarkers (Antman et al., 2000). Consensus about biomarker based definition with

refining and adoption of five subtypes lead to the development of universal definition of myocardial infarction (figure 1). The fourth universal definition of myocardial infarction (UDMI) came into action mid 2018 (Thygesen et al., 2018).

1.2.2. Epidemiology of ST Elevation Myocardial Infarction

The incidence rate of myocardial infarction varies considerably between countries, yet remains high. In Sweden; where the Swedish registry is considered the most comprehensive European one; the incidence rate of STEMI was 58 per100 000 per year in 2015 (Jernberg, 2016). The rate may increase up to 144 per 100 000 in other European countries (Widimsky et al., 2010). There is a global trend of decrease in the incidence of STEMI and increase in NSTEMI over the past years (McManus et al., 2011; Sugiyama et al., 2015).

Ischemic heart disease in general is the leading cause of death worldwide. The incidence rate is more common in males than females, developing than developed countries, and low income than high income populations. Few of the developed countries; Sweden as example; show overall decrease in deaths related to STEMI but the incidence is increasing elsewhere and global incidence is increasing (WHO, 2019). In Egypt, Ischemic heart disease is the leading cause of death and cardiovascular diseases account for 46% of total deaths while non communicable diseases represent 25% of premature deaths in 2010 (Abdul Rahim et al., 2014; WHO, Egypt, 2019; STEPS, 2019).

Considerable variance in outcomes may depend importantly upon patients' age, race and gender, as well as the treatment facility itself. Being black, female or of advanced age confers relatively higher mortality in the US, while STEMI mortality is consistently lower in hospitals that manage higher patient volumes and have busier procedural centers (Kang et al., 2012; Kyto et al., 2015).

1.2.3. Etiology

Acute Coronary Syndrome (ACS) is usually a result of imbalanced cardiomyocytes supply and demand which is caused primarily from disruption of what is called

vulnerable plaque; an atherosclerotic lesion that was previously non severe and may be hemodynamically insignificant yet prone to rupture (Bangalore et al., 2010).

The process of coronary atherosclerosis is the main drive for development of more than 90% of acute coronary syndrome cases. The plaques developed in a very long and sophisticated inflammatory and proliferative process and become vulnerable due to different mechanical and biological causes resulting in either plaque rupture or plaque erosion. As a result, activation of platelets and coagulation pathways leads to thrombus formation and coronary occlusion (Bonaca et al., 2012).

Usually the vulnerable lesions are stenoses of less than 70% located in the proximal portions of the coronary vasculature. The dynamics of flow and the shear stress affecting the endothelium at these points accelerate the pathogenesis of vulnerable plaque (Chatzizisis et al., 2007). The turbulence of blood flow at the coronary branching points leads to faster development of atherosclerosis at bifurcations (Wang et al., 2004). Atheromas with high inflammatory content and abundant macrophage around large lipid-rich core with a thin fibrous cap are more rupture prone particularly (Bonaca et al., 2012).

Risk factors for development of coronary atherosclerosis are either modifiable or non modifiable. They include Age, sex, family history of premature coronary artery disease, smoking, diabetes mellitus, hypertension, dyslipidemia, sedentary life style, psychological stress, elevated homocysteine levels and the presence of peripheral vascular disease (Bonaca et al., 2012).

Although atherosclerosis represent the main cause of acute coronary syndromes, other etiologies are as important because the plan of management may differ accordingly. Coronary vasculitis, ventricular hypertrophy, coronary embolism, coronary trauma, vasospasm, increased oxygen requirements due to fever or hyperthyroidism, decreased oxygen supply as in severe anemia and aortic dissection are the most common non atherosclerotic drives (Haaf et al., 2011):

1.2.4. Pathophysiology

It is widely accepted that the majority of ACS events are caused by an episode of acute athero-thrombosis. Episodes of angina or myocardial infarction can occur in other cases than coronary atherosclerosis, but, in these particular cases, both treatment and prognosis are different from those present when ACS is associated with underlying atherosclerotic changes (Antman et al., 2008).

The characteristic pathological hallmark of the disease is the atherosclerotic plaque, a focal deposit of extracellular matrix (collagen, elastic fibers and proteoglycans), cells (inflammatory and non-inflammatory) and lipids within the intima of the arterial wall (Falk, 2006). Because of composition and structural instability, certain plaques are known as "vulnerable"; they are mainly composed of a large, necrotic, lipid core, containing infiltrated macrophages and other inflammatory cells, and covered by a thin fibrous cap (Naghavi et al., 2003).

When a plaque's fibrous cap ruptures, the lipid rich core which is highly thrombogenic is exposed to the circulation and triggers a large and complex cascade of inflammatory and prothrombotic responses (figure 2) (Davì and Patrono, 2007). The subsequent platelet activation and aggregation superimposes a clot upon the plaque area and may lead to an abrupt obstruction of the arterial lumen; distal ischemia results and is the mechanism responsible for the majority of ACS episodes. Particularly in STEMI, the ischemia is a consequence of an acute reduction in blood flow caused by a plaque-associated thrombus in a proximal segment of an epicardial artery (Wang et al., 2004).

The resulting myocardial necrosis triggers histological and structural changes in both infarct and non-infarct related coronary arterial segments, and the extent and severity of the ischemia are affected by several factors, such as the presence of collateral blood supply to the infarct area, the location of infarct and its extent, whether or not the ischemia extends from epicardial to endocardial surfaces, and the pre-existing ventricular functional status (Davì and Patrono, 2007).

Ultrastructural evidence of cellular damage and death is evident within 20 minutes of the cessation of blood flow. However, the gross morphological features are not

apparent until 6 hours post infarction. The cell damage progresses and becomes increasingly irreversible over the following 12 hours which provides a window of chance for salvage of some myocardium through thrombolysis and reperfusion (Greene and Harris, 2008).

The hemodynamic stressing of both the healthy myocardium and the scar tissue leads to progressive stretching and thinning of the ventricular wall which may start from the first day after infarction and progress over months being more evident with large transmural infarctions especially those of the anterior wall, a process called ventricular remodeling. The remodeling increases the chance of complications including congestive heart failure, arrhythmias and free wall rupture (Greene and Harris, 2008).

1.2.5. Management

1.2.5.1. Diagnosis

Prompt timely diagnosis of MI is critical for early initiation of the management plan. Diagnosis is based mainly on the presenting symptoms, ECG signs and laboratory findings. The most common presentation is persistent chest pain with radiation to the left arm, neck, jaw or epigastria. The symptoms are not always typical. Sometimes, shortness of breath, vomiting, palpitations or syncope is the main complaint. 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 (Henrikson et al., 2003; De Torbal et al., 2006; Ibanez et al., 2017).

In the context of the 4th universal definition of myocardial infarction biomarkers are the corner stone for diagnosis. Acute myocardial injury is evidenced by at least one value of cardiac troponins rise above the 99th percentile upper reference limit (URL). If associated of any of: symptoms of myocardial ischemia, new ischemic ECG changes, development of pathological Q waves, imagining evidence of new ischemic wall motion abnormality or loss of viable myocardium or identification of coronary thrombus by angiography or autopsy; this will be consistent with MI (Thygesen et al., 2018). It has to be noted that primary PCI in the context of clinical suspension and

ECG findings has never to be delayed waiting for the laboratory results (Ibanez et al., 2017).

1.2.5.2. Logistics of management:

The main goal of management of patients with STEMI is early and complete restoration of blood flow in the infarct-related artery and improvement of myocardial perfusion in the infarct zone. Two different but efficient reperfusion strategies are widely used to open the occluded culprit vessel: pharmacological (fibrinolytic drugs) or mechanical (primary PCI).

Primary PCI is the strategy of choice within the first 12 hours of symptom onset under the standard conditions; 120 minutes maximum delay and experienced team (West et al., 2011). Otherwise, the choice and implementation of each approach depends on several factors including patient characteristics, ready availability of an interventional laboratory, or availability of prompt transportation for a patient to an interventional site (Antman et al., 2005).

The most important element is to implement treatment rapidly, regardless of which method to achieve reperfusion is selected. Time between FMC and STEMI diagnosis must be minimized to less than 10 minutes (Ibanez et al., 2017).The management of a patient presenting with STEMI can start in either pre-hospital or hospital settings, depending on where the first medical contact occurs through emergency medical system (EMS) providers or at the hospital emergency room (Antman et al., 2008).

1.2.5.3. Primary percutaneuos coronary intervention (Primary PCI):

Primary PCI is the mechanical reperfusion method using balloons, stents and/ or thrombectomy aiming at achieving the maximal ideal opening of the thrombosed artery and achievement of sufficient coronary flow. As long as there is access to well equipped facility with trained stuff in the recommended time frame for primary PCI, this will be the treatment modality of choice for patients presented with STEMI (Boersma, 2006; Ibanez et al., 2017).

Primary PCI is not complication free. In 2 - 3 % of patients local vascular complications include: bleeding, hematomas, pseudoaneurysms, and arteriovenous

fistulae. Bleeding is major in about two thirds of patients and may require transfusion (Grines et al., 1993; Aversano et al., 2002; Piper et al., 2003). The incidence of bleeding increases to almost 7% when considering all types of bleeding, among which intracranial hemorrhage is the most dangerous but considerably of lower incidence compared to fibrinolysis (0.05% vs. 1%, P<0.001) (Keeley, 2003). Recently, lower doses of heparin, more fine catheters, increased operators experience and use of radial access has led to declining incidence of bleeding.

Up to 2% of patients suffer from severe contrast induced nephropathy (CIN) after PCI (Bartholomew et al., 2004). Patients with cardiogenic shock, underlying renal insufficiency and the elderly are more CIN prone (Hochman et al., 1999; DeGeare et al., 2000; Sadeghi et al., 2003). Radiographic contrast material may induce anaphylaxis as well in very rare cases (Goss et al., 1995). Arrhythmia may occur as a complication of primary PCI with ventricular tachycardia or fibrillation incidence of 4.3%. It is associated with longer hospital stays but with similar long term prognosis to those without arrhythmias (Mehta et al., 2004).

No-reflow is failure of restoring coronary perfusion despite successful mechanical opening of the infarct artery. The real incidence is not known because it is usually under-reported but, it is suggested to range between 12-20 %. Immunologic and mechanical theories of the occlusion have been proposed. Up to the best of knowledge, no evidence based marker can predict the occurrence of no-reflow. Patients with no-reflow has worse prognosis (Kumar et al., 2019).

1.2.6. Complications

Complications of myocardial infarction are both mechanical and electrophysiological. Mechanical complications include heart failure, mitral regurgitation, ventricular wall aneurysm, rupture free wall and cardiogenic shock. Thromboembolism is not uncommon with high possibility of reinfarction, pulmonary embolism and stroke. Electrophysiological complications includes both tachy and bradyarrhythmias. Dressler's syndrome, pericarditis and sudden cardiac death may occur (Chang et al., 2010).

1.2.7. Prognosis

Around 30 % of STEMI patients die in the first 24 hours of the index event, half of them before hospital arrival; however, there is a steady decline in these rates in the past years. Within the first year almost 10 % of survivors die and half of them will be rehospitalized. Many variables control the prognosis including the size of infarct, the success of reperfusion and the residual functions. Better prognosis is linked to successful reperfusion within the prespecified timeframes, preserved ventricular functions and long term treatment with ACE inhibitors, Beta blockers and Statins. In contrary, elderly patients with diabetes, previous vascular incident, failed or delayed reperfusion and/ or reduced ventricular functions are expected to have poor prognosis (Terkelsen et al., 2010).

There is wide range of tools for prognostication including clinical, laboratory and imaging tools. TIMI risk score for STEMI, GRACE score and Killip class of heart failure are widely used clinical models. Laboratory markers including cTns, N-terminal pro B-type natriuretic peptide (NT pro-BNP) and high sensitive C-reactive protein (hs-CRP) have been extensively studied with good predictive power but yet not sufficient to guide clinical decisions. Many studies are linking inflammatory markers to occurrence of no-reflow phenomenon during primary PCI but no single marker has been proved to be clinically evidenced marker to predict the occurrence of such disabling complication (Jaber et al., 1999; Reffelmann and Kloner, 2002; James et al., 2003; Antman et al., 2008; Haaf et al., 2011).

1.3. MicroRNA

1.3.1. Definition

MicroRNAs are tiny, regulatory, non-coding RNAs, around 22 nucleotides in length which regulate gene expression post-transcriptionally (Yates et al., 2013). The first miRNA to be described in literature was the 21-nucleotide long “lin-4”.It was reported in December 1993 after discovery in the nematode *C. elegans* by two separate teams of investigators (Lee et al., 1993; Wightman et al., 1993).

1.3.2. Existence of miRNAs

MicroRNAs are present in the different body fluids as a consequence of active and passive processes. Apoptotic or necrotic cells passively releases miRNAs as part of cellular debris. On the other hand, viable cells actively secrete miRNAs in the form of shed microvesicles and exosomes or conjugated to proteins (Shah and Calin, 2013).

1.3.3. Regulation of miRNAs

Fine tuning mechanisms precisely silence miRNAs in a highly developed pathways affecting miRNA stability, miRNA processing or target specificity (Yates et al., 2013). In short, regulation of miRNA and its expression takes place at several levels and is affected by several factors, including genomic sequence itself, transcriptional regulation, regulation at Drosha level, regulation at Dicer level, post-dicing regulation, and epigenetic regulation (Ha and Kim, 2014).

Most miRNA genes promoters have not been localized yet. However, it can be depicted by mapping analysis of CpG islands. Given the fact that some miRNAs are encoded within introns, its logic to infer that they share the same promoters of the host gene. But, some miRNAs tend to have distinct promoters from those of the host genes and sometimes multiple transcription start sites (Ozsolak et al., 2008).

The regulation of miRNA production usually targets the two endoribonucleolytic cleavage steps taking place during miRNA synthesis (Yates et al., 2013). The pri-miRNAs processing by Drosha-DGCR8 in the nucleus can be inhibited in a complex manner (Guil and Cáceres, 2007; Viswanathan et al., 2008). The cytoplasmic Dicer machinery is also mandatory for miRNA maturation with its knockout in experimental models leading to accumulation of pre-miRNA (Hutvágner et al., 2001). Several mechanisms have been reported that either influence Dicer processing of miRNA or alter its activity (Heo et al., 2009; Heo et al., 2012). Dicer can alternatively process miRNA producing different isoforms (isomiRs) that have different target sequences (Fukunaga et al., 2012; Lee and Doudna, 2012).

Different downstream regulatory processes such as miRNA 3’ tailing, miRNA editing, and miRNA uridinylation can alter the mature miRNA length and affect its

specificity and activity (Heo et al., 2012; Yates et al., 2013; Ha and Kim, 2014). Other different mechanisms for the variable expression of miRNA were determined as well, including single nucleotide polymorphism (SNP), epigenetic control, transcription factors, and miRNA biogenesis pathway dysregulation (Krol et al., 2010; Ha and Kim, 2014; Reddy, 2015).

1.3.4. Function of miRNA

Complementarity is the key for miRNAs suppression of translation of the target mRNA with either degradation of the target mRNA in cases of perfect complementarity is strict or block of translation with less perfect cases (Hutvagner and Zamore, 2002). The seed sequence (nucleotides 2 to 8) of a miRNA recognizes the target binding sites of mRNA which is in the 30 untranslated regions and pairs to it (Radojicic et al., 2011; Yates et al., 2013). However, the exact order of the regulatory events; translation inhibition, mRNA deadenylation, and degradation is still debatable (Yates et al., 2013).

Each miRNA can bind many target sequences. Hence, through using bioinformatics approaches, the potential binding targets of miRNAs can be determined and confirmatory laboratory experimental models can establish the target sites. Binding of the target transcript with miRNA associated with RISC complex promotes removal of both 5'-caps and poly-adenylated tails from both ends of the transcript, thereby exposing the coding sequences to exoribo-nucleolytic activities, mediating both "5'→3'" and "3'→5'" degradation (Yamashita et al., 2005).

1.3.5. MicroRNA in Diseases

As miRNAs are key regulators of wide range of cellular processes, it is very expected that their dysregulation will be the trigger for many disease processes. In patients with diabetes, cardiovascular disease, kidney disease, and wide range of cancers, miRNAs are dysregulated. The expression profiles of certain miRNAs can be used as a fingerprint guiding the diagnosis of certain diseases and predicting drug responses and prognosis (Table 1) (Paul et al., 2017).

On the other hand, the miRNAs as regulators of cellular processes can be targeted for either restoring their functions or silencing them in different pathologies (Tables 1 and 2) (van Rooij and Kauppinen, 2014). miRNAs related to cancers (onco-miRs) are targets of intervention in many experimental studies with new treatment possibilities expected (Figure 3) (Paul et al., 2017).

Table 1: Clinical value of measurement of miRNAs in some diseases

miRNA	Diseases	Clinical Value	Study
miR-155	Cancer	Cancer, sensitizes tumor cells to irradiation, has prognostic value	**Gasparini et al, 2014**
miR-21	Cancer breast	Cancer breast, contribute to proliferation and metastasis, has prognostic value	**Mandal et al, 2012**
has-mir-126∗, miR-let 7, hsa-let-7a-2, let-7f-1	Cancer lung	Down-regulated	**Naeini and Ardekani, 2009**
miR-211-5p, 374a-5p, 340-3p, 376c-3p, 5095, and 1225-3p	Aging	Expression profiles of six miRNAs may be useful biomarkers of aging	**Smith-Vikos et al, 2016**
miR-192, miR-216a and miR-217	Diabetes	Involved in pathogenesis of diabetic nephropathy	**Simpson et al, 2016**
miR-1 and miR-133	Heart hypertrophy	Inversely related to cardiac hypertrophy and also help in regulating cardiomyocyte size and function	**Care et al, 2007**
miR-1, miR-133a, miR- 499, and miR-208a	Myocardial infarction	Upregulated	**Condorelli et al, 2014; Paul et al, 2017**
miR-499	Heart Failure	Upregulated	**Corsten et al., 2010**
miR-145, miR-34a, miR-155, miR-326	Multiple sclerosis	Identify patients with relapsing-remitting disease	**Keller et al, 2009**
miR-29b-1, miR-29a, miR-9	Alzheimer's disease	Involved in disease pathogenesis	**Nunez-Iglesias et al, 2010**

Table 2: miRNAs-based therapeutics

Target miRNA	Type of drug	Clinical value	Study
miR-15	antimiR*	reduced infarct size and cardiac remodeling in mouse	**Hullinger et al, 2012**
miR-26a	adeno-associated virus -mediated delivery of vector-based miRNA expression constructs	blunted tumorigenesis in a mouse model of hepatocellular carcinoma	**Kota et al, 2009**
miR-122	IV antagomir-122	Lowering of serum cholesterol by 40%	**Krützfeldt et al, 2005**
miR-34	Mimic	Unresectable primary cancer liver	**Wiggins et al, 2010**
miR-33	Antimir	Treatment of atherosclerosis	**Rayner et al, 2011**
miR-208a	Antimir	Anti-diabetic (normalized glucose response)	**Grueter et al, 2012**
miR-122	Antimir	Genotype-independent lowering of HCV titer	**Janssen et al, 2013**

1.3.6. Analytical aspects of miRNA

Different types of samples were used in the studies on miRNA, including serum, whole blood, cells, cell cultures and tissues. The miRNA studied included extracellular free fraction, intracellular fraction (Essandoh and Fan, 2014), and serum-derived exosomes (Reithmair et al., 2017). In blood samples, miRNAs are bound to proteins and lipoprotein complexes or enclosed in microvesicles. Thus, they are relatively resistant to action of RNAses and more stable in comparison with other biomarkers (Shah and Calin, 2013).

MicroRNAs detection is still not an easy consistent process that yields accurate reproducible results because they are very short and highly homologous (Leshkowitz et al., 2013). The challenge of detection has led to the development of various technologies of detection with different pros and cons of each (Tian et al., 2015). MicroRNAs were initially detected by northern blot techniques using miRNA-specific DNA probe (Lee et al., 1993). But, northern blotting is a complex method with low

sensitivity, and harbors the risk of contamination and all the disadvantages of radio-labeling (Koscianska et al., 2011).

Later on, many methods were used for studying miRNA, such as quantitative PCR, microarrays, and sequencing (Table 3). Though surpassing northern blot performance, yet each has its areas of weakness. Thus, the need for efficient, reproducible, less time consuming and more economic technologies for detection of miRNA is a matter of extensive investigation (Tian et al., 2015).

Table 3: Comparison of currently available miRNA detection methods (Vester and Wengel, 2004; Baker, 2010; Lingam et al., 2014; Mestdagh et al., 2015; Tian et al., 2015)

Method	Advantages	Disadvantages
Quantitative PCR	Good for small scale experiments Highest sensitivity Established protocols	Unsuitable for large scale work Specificity problems with similar sequences
Microarrays	Suits large scale experiments Established protocols Cost per test is low	Specificity problems with similar sequences Small sample volume Least sensitive
Sequencing (NGS)	Suits discovery phase research Whole genome analysis High resolution	Sophisticated technique Less sensitive than PCR
Multiplex miRNA profiling	Suits multiplex studies High sensitivity and specificity No specialized lab equipment	Doesn't suit small scale experiments
Isothermal exponential amplification-based methods	No PCR needed Good signal amplification Good sensitivity	Complicated probe design Multiple enzymes used
Rolling cycle amplification- based methods	No PCR needed Good signal amplification In situ detection possible	Complicated probe design Nonspecific amplification Background noise
Nano-Particles and nano-fluidics based methods	Simple probe design Easy modification Biocompatibility Easy cellular-uptake Suits POCT	Specialized nanotechnology platform needed Time
Capillary-electrophoresis-based methods	Simple probe design Multiple target detection	Specialized equipment need

Chapter Two: Materials and Methods

This prospective case control study was carried out in the Cardiology Department of Zagazig University Hospitals in collaboration with Cardiovascular Research Department of Verona University, Italy. The institutional review board (IRB), Faculty of Medicine-Zagazig University as well as the Ethical committee of University of Verona approved the study. Authors declare no conflict of interests. The study started in January 2018 and ended by August 2019.

2.1. Patients

The study included 75 adult patients. Informed consents were obtained from all patients to use their clinical data and samples for research and analysis using a dedicated form according to the Declaration of Helsinki.They were classified into 2 groups: Group 1 (patient group): 40 patients admitted with chest pain diagnosed to have STEMI and underwent Primary PCI. This group is subdivided into: Subgroup (a): 21 cases with sufficient reperfusion (TIMI III, TIMI II), and Subgroup (b): 19 cases with insufficient reperfusion (TIMI I, TIMI 0). Group 2 (control group): 35 cases admitted with chest pain and found to have negative troponin T.

2.2. Inclusion criteria

Patients who were admitted within 12 hours of the onset of chest pain were included.

2.3. Exclusion criteria

1- Previous myocardial infarction within 1 week.
2- CABG or PCI within 1 week.
3- Congestive heart failure at time of presentation.
4- Cardiogenic shock at time of presentation.
5- Regular haemodialysis, as circulating miRNAs may be washed out or fragmented.
6- Ongoing malignancy, because some of the circulating miRNAs in malignancy have complementary sequences to those released in MI).

2.4. Clinical work up

Both study groups were subjected to full history and clinical data acquisition, either by interview of patients or from the clinical files of inpatients using a dedicated data collection sheet. Group 1 patients have undergone primary percutaneous coronary angiography. Routine laboratory investigations for both groups included complete blood count (CBC), liver function tests (LFT), serum creatinine and blood urea nitrogen (BUN), international normalization ratio (INR), CK-MB, and High sensitivity Troponin T. For both groups, serum expression of miR-208a was assessed using real-time quantitative polymerase chain reaction (RT-PCR).

Samples for routine investigations and myocardial injury biomarkers were collected and handled according to instructions of the Hospital Laboratories service manual. Results of routine investigations were acquired from laboratory information system for both groups.

For miRNA determinations, 3 mL fresh blood were aspirated from antecubital veins of study patient and control groups and delivered into sterile plain vacutainer tubes. Samples were processed within half an hour from collection and micro RNA was extracted immediately, reverse transcripted and stored frozen in -80^{o} C. Haemolysed blood samples were discarded and new samples were collected, if available.

2.5. Methods

2.5.1. Definitions

- **Smoking:** study subjects were classified according to their smoking habits into non-smokers (never smoked or quit smoking more than 6 months ago "exsmokers") and smokers.
- **Hypertension:**

- History of previous diagnosis and control with medication.
- Or, systolic Blood pressure ≥140 mmHg and/or diastolic pressure ≥ 90 mmHg on at least 2 occasions (Williams *et al.*, 2018).

- **Diabetes Mellitus:** Either type I or type II.

- History of previous diagnosis and control with anti-diabetic drug therapy.

- Or, fasting plasma glucose ≥ 126 mg/dL, and/or two hour post glucose load ≥ 200 mg/dL (Sacks et al., 2011).

- **Obesity:** body mass index (BMI) of 30 kg/m^2 and higher (BMI = weight/ height in metres squared).
- **Positive family history:** any parent, or sibling who had a heart attack before and until the time of patient presentation.
- **MACE:** occurrence of: re-infarction, heart failure, arrhythmias, stroke and/ or death.

2.5.2. Primary percutaneuos coronary angiography

A detailed discussion with the patient and his relatives was done to outline the indication for the procedure, steps of the procedure as well as the serious risks and complications then a written consent was signed by the relatives.

Coronary angiography was done using Seldinger's technique through the right radial artery, where the culprit lesion was determined. Reperfusion of culprit lesion only by either balloon dilatation & stent application or direct stenting was performed. Thrombolysis in myocardial infarction (TIMI) score was estimated (Chesebro et al., 1987) as follows:

- TIMI Grade 0 - Complete occlusion of the infarct-related artery.
- TIMI Grade 1 - Some penetration of contrast material beyond the point of obstruction but without perfusion of the distal coronary bed.
- TIMI Grade 2 - Perfusion of the entire infarct vessel into the distal bed but with delayed flow compared with a normal artery.
- TIMI Grade 3 - Full perfusion of the infarct vessel with normal flow.

2.5.3. Analytical Methods

Complete blood count was done using automated cell counter “Sysmex XS”(Sysmex Corporation, Japan). Liver and kidney functions tests were measured spectrophotometrically using automated analyzer “Roche Cobas 8000-c702” (Roche Diagnostics, Germany). While, INR was estimated using Sysmex CS 2100 coagulometer, (Sysmex Corporation, Japan). High sensitivity Troponin T was

measured by electrochemiluminescence method using automated analyser "Roche Cobas –e411" (Roche Diagnostics, Germany).

Serum expression of miRNA-208a was assessed in three sequential steps: using the following steps: miRNA extraction, reverse transcription of miRNA to complementary DNA, and amplification and detection of cDNA by real time PCR.

For miRNA extraction from serum samples "miRNeasy Mini kit" (Qiagen, Germany) was used. The kit depends on phenol/guanidine for samples lysis and silica membrane for purification. Fresh serum samples were acquired and 500 µL Qiazol lysis reagent were added to 100 µL sample. The homogenate was left for 5 minutes at room temperature. Then, 100 µL chloroform were added and the tube was closed securely and vortexed forcibly for 15 seconds. The homogenate was left for 2 minutes at room temperature then centrifuged for 15 min at 12,000 x g at 4^{o}C in cold centrifuge. The upper aqueous phase was transferred to a new collection tube, and 300 µL of 100% ethanol was added and mixed thoroughly by repeated pipetting.

Afterwards, 700 µL of the mix was transferred into an RNeasy Mini spin column in a 2 mL collection tube. Followed by centrifugation at ≥8000 x g (≥10,000 rpm) for 15 seconds at room temperature and flow-through was discarded. Of the buffer RWT 700 µL were added to the mix and centrifuged at ≥8000 x g (≥10,000 rpm) for 15 seconds at room temperature and the flow-through was also discarded. Then, 500 µL Buffer RPE was added to the mix and centrifuged at ≥8000 x g (≥10,000 rpm) for 15 seconds at room temperature with discarding the flow-through (This step was done twice). RNeasy Mini spin column was then centrifuged in a new 2 mL collection tube for 2 minutes at full speed. 40µL RNase-free water were added to the RNeasy Mini spin column in a new 1.5 mL collection tube then centrifuged for 1 minute at room temperature at ≥8000 x g (≥10,000 rpm) so RNA is eluted. DNA contamination was checked spectrophotometrically by measuring ratio of 260/280 absorbance.

To avoid contamination of samples, the whole work was conducted in a laminar flow hood, with a preliminary disinfection with chlorine 5% and sterilizing UV rays. Work area for extraction was separate from work area for amplification reactions. Vinyl gloves were always worn to avoid RNase contamination. Only clean RNase-free

disposable filtered plastic pipette tips were used when dealing with the supplied reagent.

For reverse transcription "miScript II RT Kit" (Qiagen, Germany) was used. All the following steps were done on ice to avoid RNA degradation. Reagents were thawed in room temperature. The master mix was prepared by adding: 4 μL of 5x miScript HiSpec Buffer, 2 μL of 10x miScript Nucleics Mix, 2 μL of miScript Reverse Transcriptase Mix, 2 μL of Template RNA, and 10 μL of RNase-free water. Mixture was incubated at 37ºC for 60 min. Reaction mixture was incubated at 95ºC for 5 min to inactivate miScript RT, then placed immediately on ice and stored in -80^{o}C.

Quantitative RT-PCR was done using specific miScript Primer Assays and the miScript SYBR Green PCR Kit (Qiagen, Germany). Two separate tubes were prepared for each sample: one for miRNA target and one for the normalization gene; human RNU6B (RNU6-2) for later assessment of miRNA expression. Reagents and cDNA samples were thawed at room temperature, and primers reconstituted using Tris EDTA 8.0 buffer according to manufacturer recommendations.

Amplification mixture was prepared as follows in accordance with the kit manufacturer recommendations by adding: 10 μL of 2x QuantiTect SYBR Green PCR Master Mix, 2 μL of 10x miScript Universal Primer, 2 μL of 10x miScript Primer Assay (according to miRNA tested), and 4 μL of RNase-free water. cDNA concentrations were measured using Qubit ® 3Flourometer (ThermoFisher Scientific Inc., USA). cDNA was diluted to concentration of 50 pg ~ 3 ng/μL cDNA using RNase-free water. Two μL of the template cDNA after dilution was added to the amplification mixture. Sample wells were sealed carefully and wells were centrifuged for 1 min at 1000 X*g* at room temperature to remove bubbles and assure reaction mixture positioned at bottom of wells.

Real time PCR was done on "Stratagene Mx3005P" platform (Agilent Technologies, USA). Activation step was done for 15 min at 95^{o}C "to activate the Taq DNA Polymerase" followed by 40 cycles of: denaturation at 94^{o}C for 15 seconds, annealing at 55^{o}C for 30 seconds and extension at 70^{o}C for 30 seconds. Collection of fluorescence data was done at extension phase.

At end of cycles, reaction wells were examined visually to check for evaporation of samples or well damage. Using the software of the thermal cycler, the following steps were done: visual check of amplification curves for homogeneity, determination of baseline fluorescence values, setting the threshold fluorescence and visual check of dissociation curves for non-specific fluorescence signals. Next, threshold cycle (CT) values for each sample were obtained and the miRNA expression fold changes were calculated using the Livak formula (Livak and Schmittgen, 2001), where:

$$\text{Fold Change} = 2^{\wedge\ -\Delta\Delta CT}$$

$$\Delta\Delta C_T = \Delta CT_{patients} - \Delta\ CT_{control}$$

$$\Delta C_T\,(\text{relative expression}) = CT_{\text{miRNA of interest}} - CT_{\text{house keeping RNA}}$$

2.6. Statistical Analysis

The acquired data were analyzed using Microsoft Office Excel 2007 and Statistical Package for Social Sciences version 24 (SPSS: An IBM Company). For descriptive statistics, the arithmetic mean, standard deviation (SD), median and range were calculated for numerical variables; while, the frequency, distribution and percentage were used for categorized variables.

For Comparative Statistics, Pearson Chi-square ($\chi 2$) was used for comparison between qualitative variables to determine whether the variables are independent or not. The student's (t) test was used for comparisons between quantitative variables to determine the significance of the difference between samples' means. Mann Whitney test was used for non-parametric data to determine whether or not the difference between two independent populations' medians is zero, where the null hypothesis is that it is.

Single test (t) test was used to test whether or not the values given in a single population can be attributed to chance compared to a given set value, where the null hypothesis is that it is. Diagnostic and prognostic performance of tests was done using receiver operating curve (ROC). Threshold of significance: was fixed at 5% level (p-value), where P values <0.05 indicate significant results

Chapter Three: Results

3.1. Demographic data and risk factors

The two study groups are homogenous in terms of demographic data, risk factors and history. No significant difference between the two groups is observed regarding sex, age categories and risk factors including; smoking, hypertension, diabetes mellitus, obesity and family history. It has to be noted that none of the candidates of both groups was known to have history of 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 (Table 4).

3.2. Clinical findings

Despite the noticeable difference in clinical findings in both groups, with a tendency for higher blood pressures in the patient group, there was no statistical significance of these observations. Also, the difference between the two groups in time from onset of symptoms to the admission of patient to the ED is not significant (table 4).

3.3. Routine Laboratory Results

Both groups had more or less normal blood picture, liver function tests, kidney function tests and INR. Although most measurements are not significantly different between the two groups, the WBCs count and bilirubin level are significantly higher in the patient group (table 4).

Table 4: Demographic data, clinical findings, laboratory results of patients` groups

	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/µL	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/µL	123 – 410 (264)	41 – 396 (248)	0.05238
Total bilirubin (mg/dL)	0.6 –1.8 (1)	0.6 – 1.2 (0.8)	0.00054*
CK-MB, IU/L	2 – 96 (58.5)	1 - 5.5 (2.8)	< 0.00001*
hs-cTnT IU/L	0.01 – 10.5 (4.85)**	0.01-0.02 (0.01)	< 0.00001*

CK-MB indicates creatine kinase-MB; hs-TnI, high sensitivity Troponin I and WBCs white blood cells.

** 1 patient of the group one was found to have negative hs-cTnT at admission and ST elevation in ECG and had undergone primary PCI.

3.4. Special Investigations

MicroRNA-208a was significantly up-regulated in STEMI patients compared to control group (fold change median =32.54, range: 11.51 – 88.91, P= 0.0001).

3.5. Diagnostic performance of microRNA-208a

Statistically miRNA-208a has been proved to be a good a marker for diagnosis of Myocardial infarction with area under the curve of 0.926, sensitivity of 92.5% and specificity of 80% (95% CI 0.841 to 0.974; P value 0.001) (Figure 4). The diagnostic performance of miR-208a is not significantly different from the routine cardiac biomarkers (table 5, figure 5) but the reclassification power of miR-208a was not proved to be of statistical significance (table 6).

Table 5: Comparison of diagnostic performance between miR-208a and routine cardiac biomarkers:

Marker	miRNA 208a			
	Areas difference	95% CI	Z score	P
hs-Troponin I	0.0614	-0.0038 to 0.127	1.847	0.06
CK-MB	0.0439	-0.0287 to 0.117	1.186	0.235

Table 6: Reclassification performance of miRNA 208a over a multiparameter clinical model[a]:

Class	Clinical model			Clinical model + miRNA 208a			NRI %
	Test – ve	**Test +ve**	**Correctly classified %**	**Test – ve**	**Test +ve**	**Correctly classified %**	
Control (n=35)	34[b]	1[c]	97.14	34[b]	1[c]	97.14	0.025
STEMI (n=40)	1[c]	39[b]	97.5	0[c]	40[b]	100	P= 0.3

a Parameters included in the clinical model were: age, sex, hypertension, hypercholesterolemia, smoking habit, and hs-cTnT. Net reclassification indexes (NRI). P value is indicated.
b No. of correct classifications.
c No. of false classifications.

3.6. Analysis of patient subgroups

3.6.1. Demographic data and risk factors

The two subgroups are homogenous in terms of demographic data, risk factors and history. No significant difference between the two subgroups is observed regarding patient sex, age categories and risk factors including; smoking, hypertension, diabetes mellitus, obesity and family history. It has to be noted that 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 (table 7).

3.6.2. Clinical findings in both groups

Despite the noticeable difference in clinical findings in the subgroups, with a tendency for no-reflow group (group b) to have higher heart rates, there was no statistical significance of these observations. Also, the difference between the two groups in time from onset of symptoms to the admission of patient to the ED is not significant (table 7).

3.6.3. Routine Laboratory Results

Both groups had more or less normal blood picture, liver function tests, kidney function tests and INR with no statistically significant difference between them regarding routine laboratory results.

3.6.4. Special Investigations

It is obvious that miRNA-208a was significantly up-regulated in subgroup b compared to subgroup a (table 7, figure 6).

Table 7: Demographic data, clinical findings, laboratory results of patients` subgroups

	Subgroup a (n = 21)	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	97.95 ± 14.57	108.95 ± 20.50	0.0562
	Range (median)		P
Time to admission, hours	1-5 (3)	1-7 (3)	0.152914
miR-208a Fold change*	11.51 – 56.7 (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.

3.6.5. Prognostic performance of the test

The incidence of MACE was significantly higher in the no reflow group of patients (subgroup b) X^2 = 17.377 and P = 0.000031 which is strong significant (figure 7). miR-208a has been proved to be a good a marker for prediction of no reflow as well as in hospital MACE in patients with STEMI (table 8, figures 8,9). miR-208a is superior to routine cardiac biomarkers as a predictor of no-reflow (table 9, figure 10) and MACE (table 10, figure 11).

Table 8: miRNA208a diagnostic performance:

Prediction	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*

Table 9: Comparison of no-reflow prediction performance between miR-208a and routine cardiac biomarkers:

Marker	miRNA 208a			
	Areas difference	95% CI	Z score	P
hs-Troponin T	0.231	0.0314 to 0.430	2.268	0.0233*
CK-MB	0.257	0.0625 to 0.451	2.591	0.0096*

Table 11: Comparison of MACE prediction performance between miR-208a and routine cardiac biomarkers:

Marker	miRNA 208a			
	Areas difference	95% CI	Z score	P
hs-Troponin T	0.367	0.109 to 0.626	2.786	0.0053*
CK-MB	0.323	0.135 to 0.511	3.366	0.0008*

Chapter Four: Discussion

Myocardial infarction is on the top of mortality causes in the world which is mainly treated with primary PCI or fibrinolytic drugs (Keeley et al., 2006). Up-to-date cardiology guidelines recommend early prompt diagnosis and immediate mechanical restoration of coronary blood flow and establishment of effective myocardial reperfusion in STEMI patients (Ibanez et al., 2017).

Despite the great improvement in the detection methods for Troponin T; the gold standard test in myocardial infarction; there is a decrease in test specificity which is associated with longer stay at ED, more healthcare costs and undue interventions (Summers et al., 2018). The hs-TnT essays are less accurate in the first hour of pathology development (Thygesen et al., 2018). Also, the prognostic role of hs-TnT is less well established and controversial to a great extent (Giannitsis et al., 2001; Bjorklund, 2004; Wang et al., 2014; Cediel et al., 2017).

A great improvement has been encountered in the equipments and methodology used for primary PCI, yet it is not always successful and still in some patients the coronary flow cannot be restored and the myocardium is deprived of blood. When mechanical opening of the coronaries is not associated with restoration of normal blood flow, this is a no-reflow situation which is linked to high mortality and morbidity. Thoroughly studying of the no-reflow phenomenon proved it to be a result of a complex process involving endothelial disruption due to ischemic insult, microvascular occlusion, extensive inflammatory reaction with leukocytes plugging of microvasculature, uncontrolled coagulation with failure of thrombolysis, more profound injury due to reactive oxygen radicals and mechanical occlusion due to interstitial edema**.** However, no-reflow phenomenon is still unpredictable with no marker being suitable for anticipating no-reflow (Reffelmann and Kloner, 2002).

Micro-RNAs are tiny, regulatory, non-coding RNAs, around 22 nucleotides in length which regulate gene expression post-transcriptionally (Yates et al, 2013). As the pathogenesis of myocardial infarction involves tissue ischemia, edema and necrosis with release of necrotic yield into circulation, miRNAs are supposed to have a promising diagnostic role. As long as no-reflow is linked to more severe pathology, miRNAs are supposed to reflect this.

So, this prospective case-control study aimed to study diagnostic and prognostic values of serum expression of miRNA-208a in MI patients. The study included 75 adult subjects. Informed consents were obtained from all of them to use their clinical data and samples for research and analysis using a dedicated form according to the Declaration of Helsinki.

Subjects were divided into 2 groups, of matched sex and age. The first one was the Patients group that included 40 adult patients admitted with STEMI and underwent primary PCI of which 19 had no-reflow. The second group was the Control group that included 35 subjects presented with chest pain but with negative markers of cardiac injury.

In this study we report six main findings. First, the circulating level of mir208a was significantly higher in STEMI group than the control group. Secondly, mir208a has a good diagnostic accuracy (AUC 0.926). Thirdly, the diagnostic accuracy of the studied miRNA is comparable to that of the routine cardiac biomarkers including CK-MB and hs- TnT. Fourthly, at our study, there is no added value for combining miRNA to the routine clinical model. Fifthly, miR208a showed good performance in prediction of in hospital MACE which was significantly superior to CKMB and hs-TnI. Finally, miR208a showed good performance in prediction of no-reflow.

In the present study there was no statistically significant difference among the study groups as regard demographic and clinical characteristics. This was concordant with previous studies (Widera et al., 2011; Devaux et al., 2012; Li et al., 2013). This does not reflect the real relation between some risk factors and development of myocardial infarction in the whole population. However, this can be explained in our study by small sample size, which makes our study not powered enough to detect the difference.

The time from onset of chest pain till admission and obtaining samples in our study was 1-5 hours for the STEMI group and 1-7 hours 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 hours for the STEMI group and 3-12 hours for the control group. 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 (Sayed et al., 2013; Paiva and Agbulut 2017). 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 hour of presentation. From health economics and clinical points of view this is more convenient (Mills, 2019). Secondly, the accuracy of hs-TnT 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 (Thygesen et al., 2018).

The STEMI group had a significantly higher TLC in comparison with the control group. This Finding from our point of view reflects active inflammatory process linked to the acute MI and is concordant with the reports by Korhan et al. While, Meder et al. showed no difference in WBCs count which may be attributed to the small sample size they used. 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 miRNA208a in regulating cell proliferation and migration and we think it has to be deeply investigated (Feng et al., 2016; Liu et al., 2019; NCBI, 2019). It has to be mentioned that miR208a is known to be cardiomyocyte specific and not expressed by leukocytes (Corsten et al., 2010).

Leukocytosis is considered a regular response to ischemia and reperfusion. Necrotic tissue is infiltrated with WBCs so, it is a common finding to have high TLC in acute STEMI. The pathogenesis of myocardial necrosis involves neutrophils as early cells to be recruited to the damaged area. Leukocytes secrete procoagulants locally which contribute to oxidative and proteolytic injury. Moreover, leukocytes and platelet-leukocyte aggregates reduce downstream micro vascular perfusion and contribute to thrombosis and widespread myocardial injury by distal embolization (Menon et al., 2003). Thrombolysis trials identified leukocyte count as a prognostic tool which is

linked to short- and long-term adverse clinical outcomes. The higher the neutrophil count, the more extensive the myocardial infarction and the more probable congestive heart failure (Kawaguchi et al., 1996; Sweetnam et al., 1997; Huang et al., 2009).

Although, in subgroups analysis, there was no significant difference in the TLC count which is not aligned with the aforementioned pathophysiological hypothesis. This can be attributed to the small size of the subgroups which is not powered enough to demonstrate the difference and the early time of presentation of the studied subjects that allows less time for this difference to be evident. It has to be clarified that our study did not focus on TLC relation to MI and accordingly neutrophils/ lymphocyte ratio was not considered.

Fu et al. and Shen et al. reported higher levels of bilirubin in patients with acute coronary syndromes in comparison to normal subjects and our study shows a concordant finding. They explain this elevation as a response to the inflammatory and oxidative stress. However, no direct relation between miRNA-208a and bilirubin levels could be proved by reviewing literature or studying the signaling pathways affected by the study target.

Our study shows significant higher concentrations of miRNA-208a in the STEMI group in comparison to the control group. Similar findings were reported by Devaux et al., Liu et al. (2015) and Liu et al. (2017). The diagnostic performance of miRNA-208a is still controversial. While in our study it showed good performance with AUC of 0.926 which is comparable to that reported by Li et al. and Wang et al.; Devaux et al. and Liu et al. reported lower diagnostic power of the marker with AUC of 0.76 and 0.72 respectively. In the study by Devaux et al., this can be attributed to the later time of presentation; while, we suppose the methodology used by Liu et al. to be implicated. Liu et al. had their samples frozen before extracting the RNA and this usually gives inaccurate yield of miRNAs.

Despite the good diagnostic performance of our marker, it is of no value when combined to the routine clinical model. A similar finding was reported by Li et al. and Devaux et al. This is an expected finding for many reasons. First, the sample size used despite powered to detect the difference between groups, is still not enough to show the real added value of the test. Secondly, the clinical model used is extremely

powerful especially after introduction of the last generations for hs-Troponins testing. Finally, studies recruited patients already diagnosed to have STEMI, this is not considering patients who were discharged or misdiagnosed or received treatment late because of the delayed diagnosis using the current clinical model.

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 that compare STEMI patients to a control group of unstable angina. This makes our findings in the diagnostic context more valuable.

In the present study, miRNA208a 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.. While these studies show troponin to be superior as MACE predictor, our study shows miRNA-208a to be superior to the routine cardiac biomarkers. This may be attributed to two main factors; the very early time of presentation of our sample population in comparison with other studies and the short term period of follow up of patients for MACE in our study; only during hospital stay. CORTEZ-DIAS et al. studied the short and long term prognostic value of some miRNAs including miR-208a but they did not give clear conclusion about miR-208a value in predicting short term prognosis.

To the best of our knowledge, this is the first study to investigate the value of miRNA208a in no-reflow phenomenon and almost the second to investigate the role of any miRNA in no-reflow. Databases search showed only one article addressing the value of miR-30e in no-reflow prediction, where Su et al. found their marker to be an independent predictor of no-reflow. In our study, miRNA-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. Our marker has good predictive value with AUC= 0.871 which is significantly higher than the predictive value of routine cardiac biomarkers.

The current study had some 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. Our study did not include patients with NSTEMI, recent MI, cardiogenic

shock or heart failure at time of admission. Although these categories were excluded for the fear of having false elevation of miRNA levels, these categories has to be studied independently. The study included patients presented within short time of onset of symptoms. This may be considered as a double edged weapon. From one point of view, our results cannot be generalized to patients presented at later time. On the other side, it strengthens the usefulness of our marker in early hours of infarction.

We recommend extending the study to include large cohort of population, including all categories of ACS, follow up 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 worthy studying. Network analysis of miR-208a interactions may help to better explain its role in no-reflow and suggest better modalities for its management.

Future perspective:

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 prediction of no-reflow. Although still far from clinical application due to long time needed for the assay (at least 2 hours), however, the recent emergence of fast thermal cyclers may enable the clinical use of miR-208a.

In the context of STEMI, time is very tight for the intervention decision and even high sensitivity cTnT assays are not helping in the first hour of chest pain. In some cases, also ECG is not conclusive. Recent studies address the possibility of using micro-fluidic nano-based detectors of microRNAs which offer short assay time, bedside availability and low prices. Such technologies give a great hope for miRNA based diagnostics in the future.

Figures

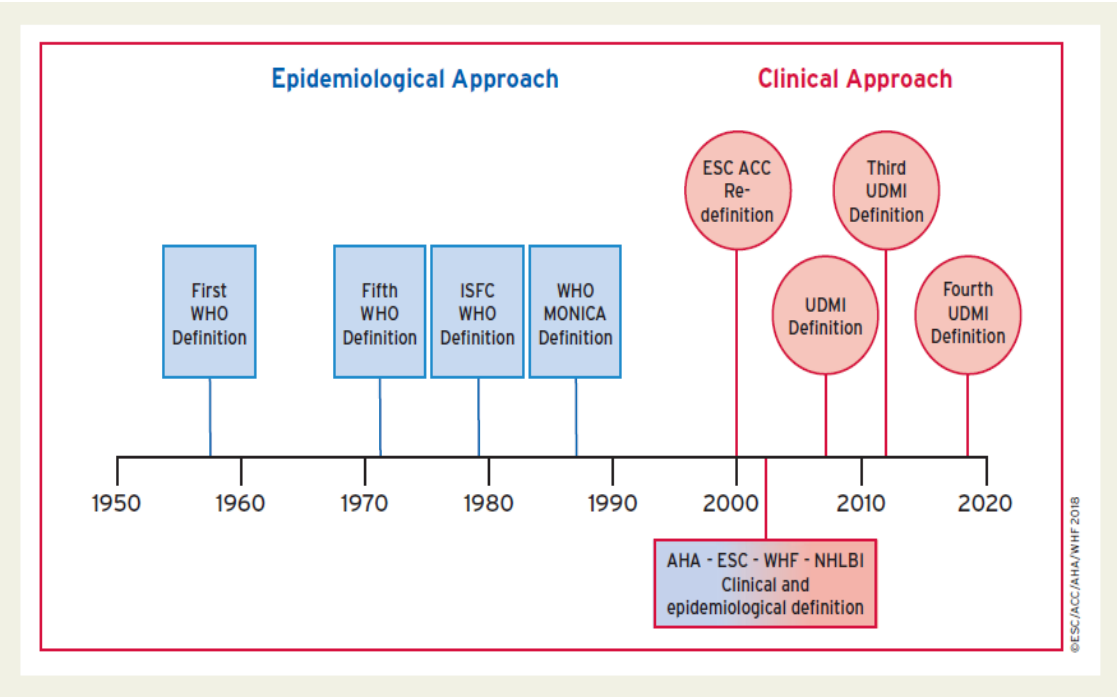

Figure 1: History of documents on the definition of myocardial infarction. ACC = American College of Cardiology; AHA = American Heart Association; ESC = European Society of Cardiology; ISFC = International Society and Federation of Cardiology; MONICA = MONItoring of trends and determinants in CArdiovascular disease; NHLBI = National Heart, Lung, and Blood Institute; UDMI = Universal Definition of Myocardial Infarction; WHF =World Heart Federation; WHO=World Health Organization. (Reproduced with permission from Thygesen et al., 2018).

The definition of STEMI changed over the past 70 years from being pathology based to be biomarker based. Troponins especially high sensitivity assays are corner stone for diagnosis currently.

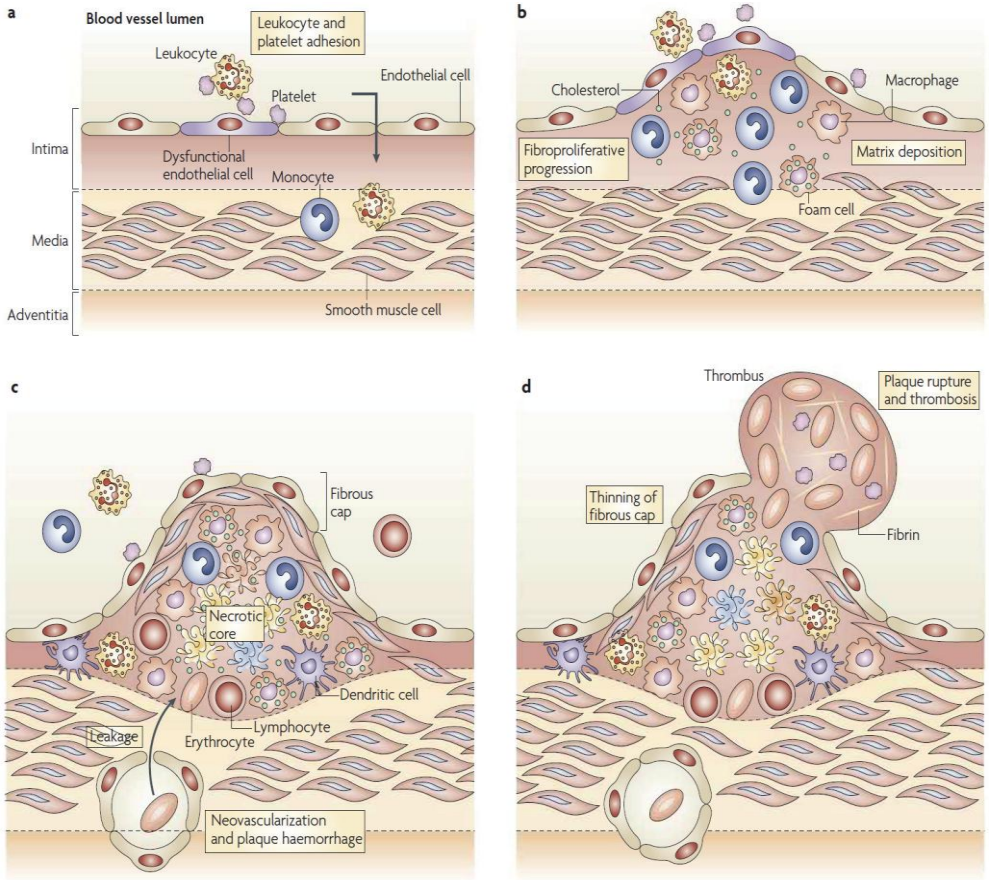

Figure 2: Inflammation mediated arterial plaque formation (reproduced with permission from Libby, 1995). a: Leukocytes and platelets adhesion to the dysfunctional endothelium. b: activated foam cells induce fibro-proliferative reaction. c: formation of necrotic core of the plaque and neovascularization. d: plaque rupture and thrombosis.

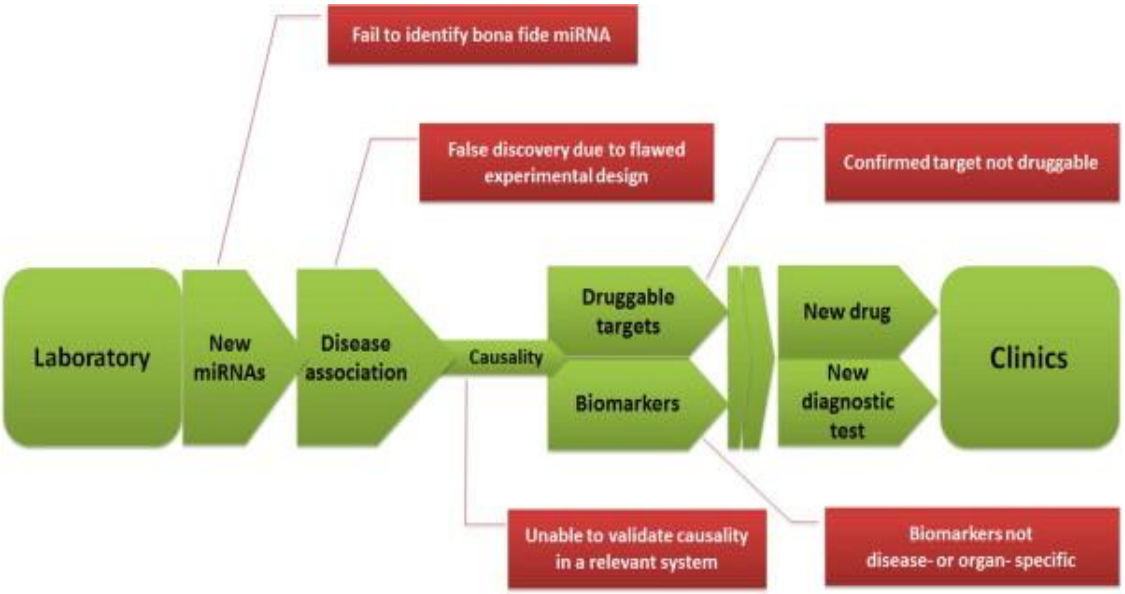

Figure 3: Pathway of developing new miRNA-based drugs and lab tests (reproduced with permission from Li and Kowdley, 2012). The figure describes the steps required for microRNA to reach clinical practice either as a marker for diagnosis or as a therapeutic target. Each step (green) requires long experimentation and many reasons of failure (red) are present.

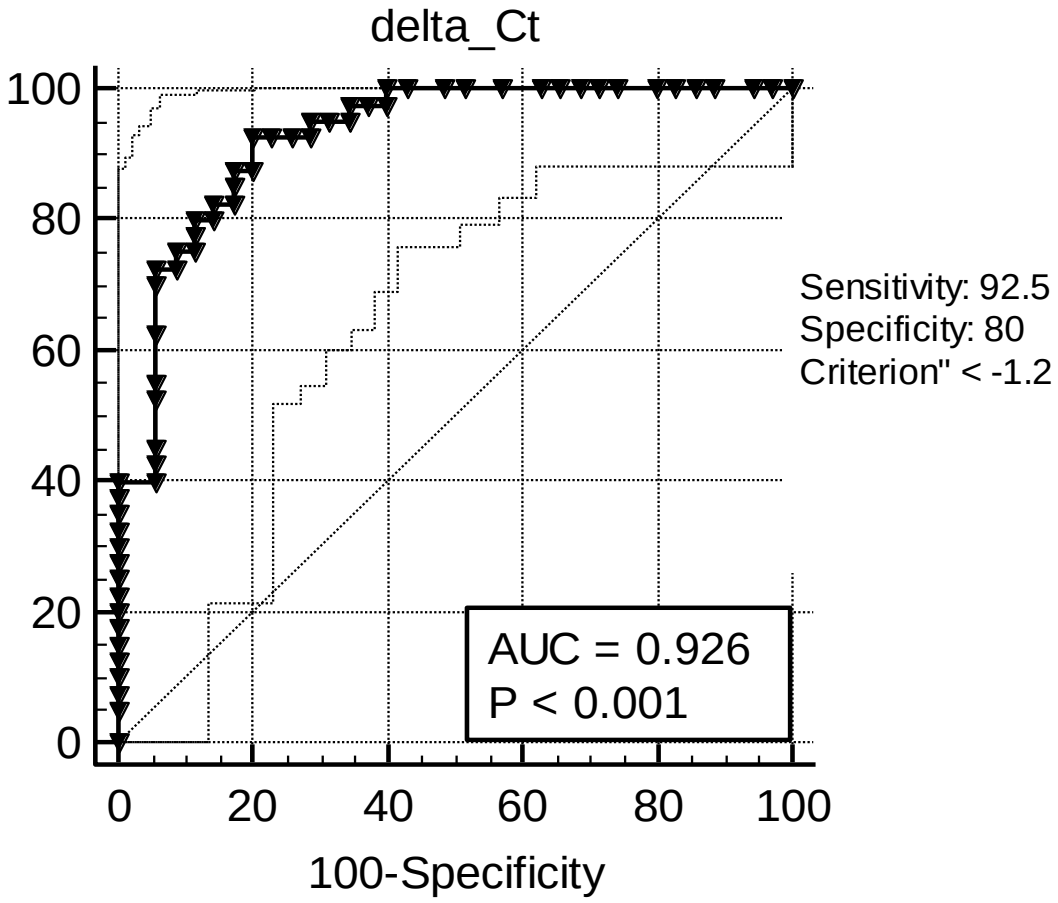

Figure 4: ROC analysis of miR-208a diagnostic performance. The target marker showed good diagnostic performance (AUC=0.926) with high sensitivity and specificity (92.5 % and 80 % respectively).

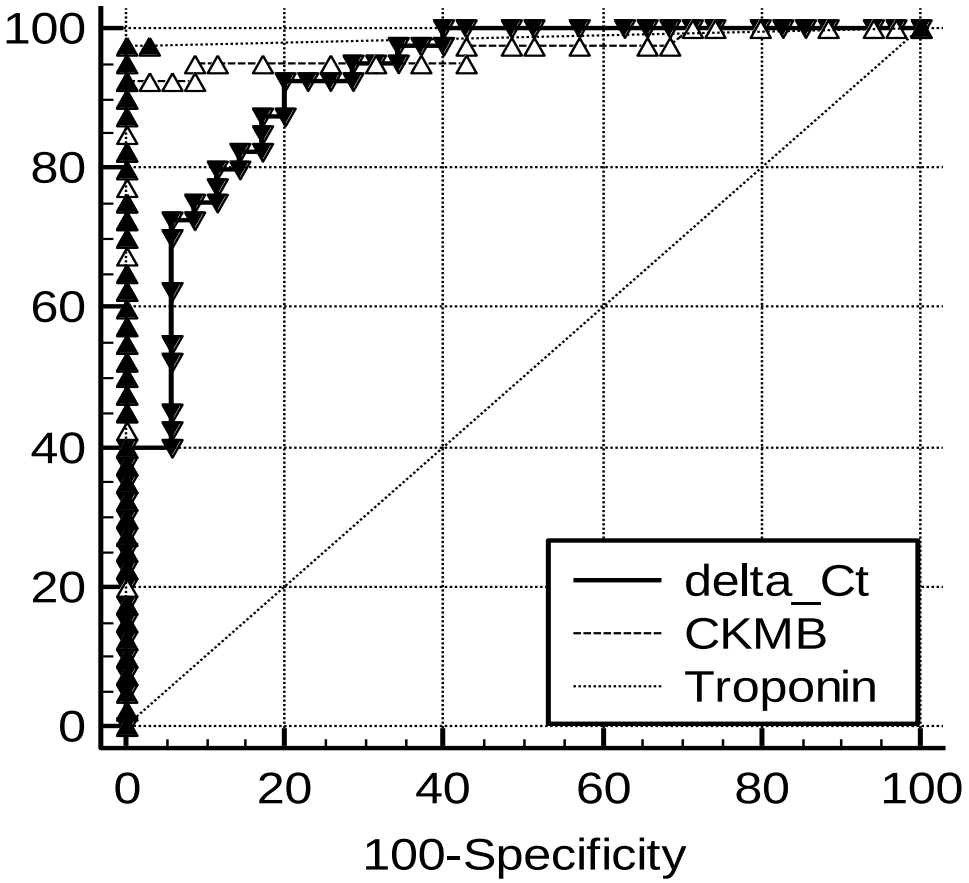

Figure 5: ROC comparison of miR-208a diagnostic performance against routine cardiac biomarkers. MicroRNA-208a is not inferior to hs-cTnT as a diagnostic marker.

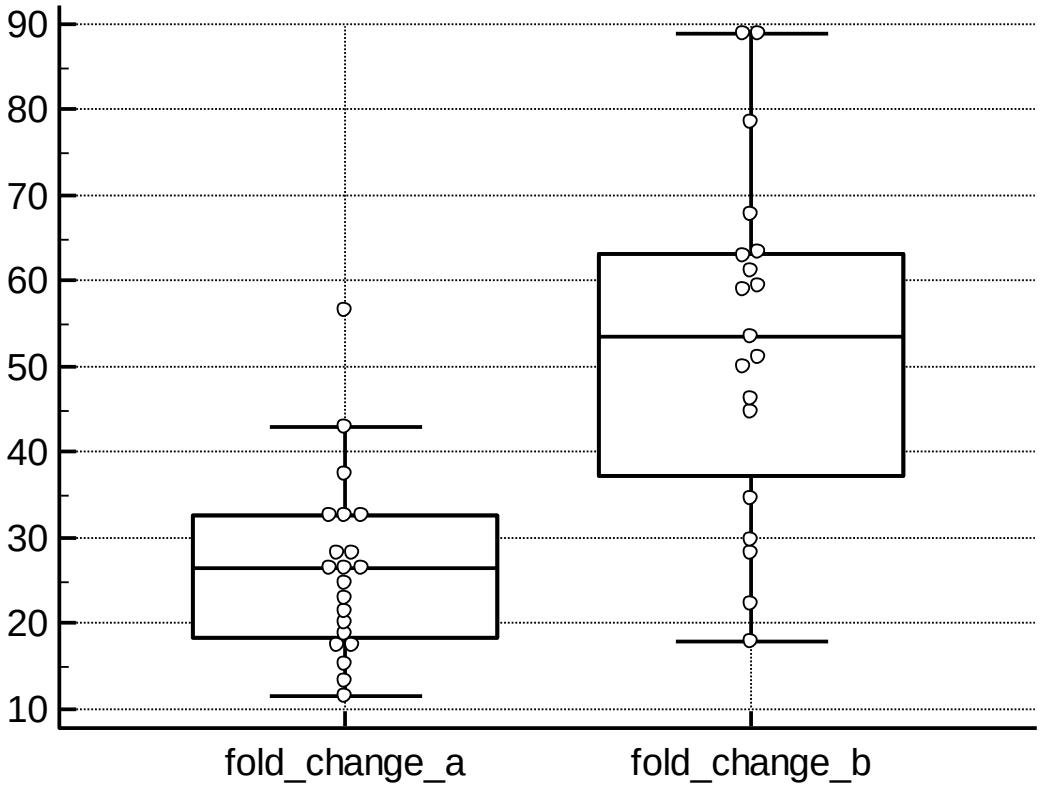

Figure 6: box plot of fold change of miR-208a expression in both of the study subgroups. The expression of miR-208a is much lower in the subgroup a (successful reperfusion) than subgroup b (no-reflow).

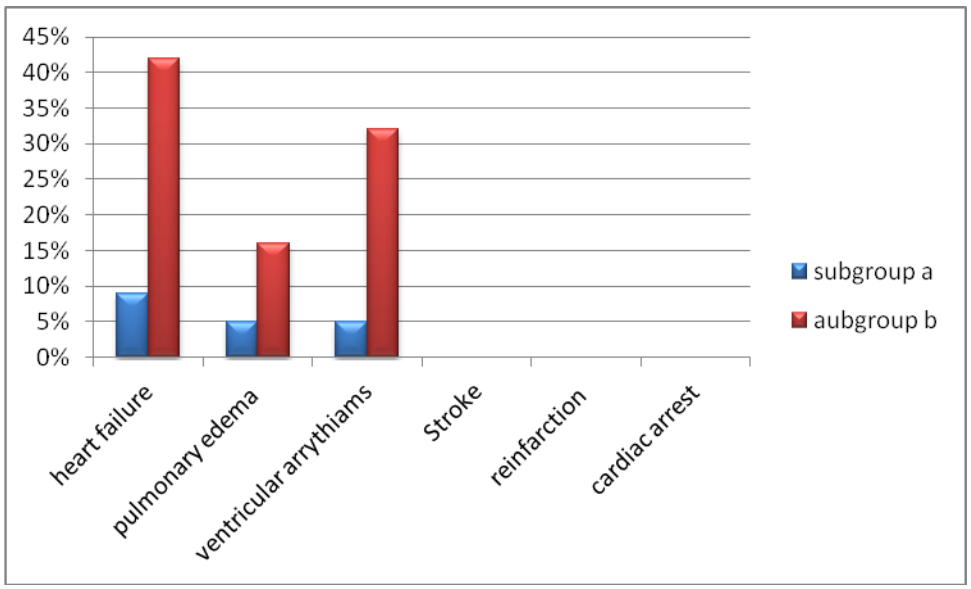

Figure 7: Incidence of MACE in the study subgroups and their distribution. Heart failure, pulmonary edema and ventricular arrhythmias are significantly more frequent in the no-reflow group (subgroup b). Both groups did not have stroke, reinfarction or cardiac arrest.

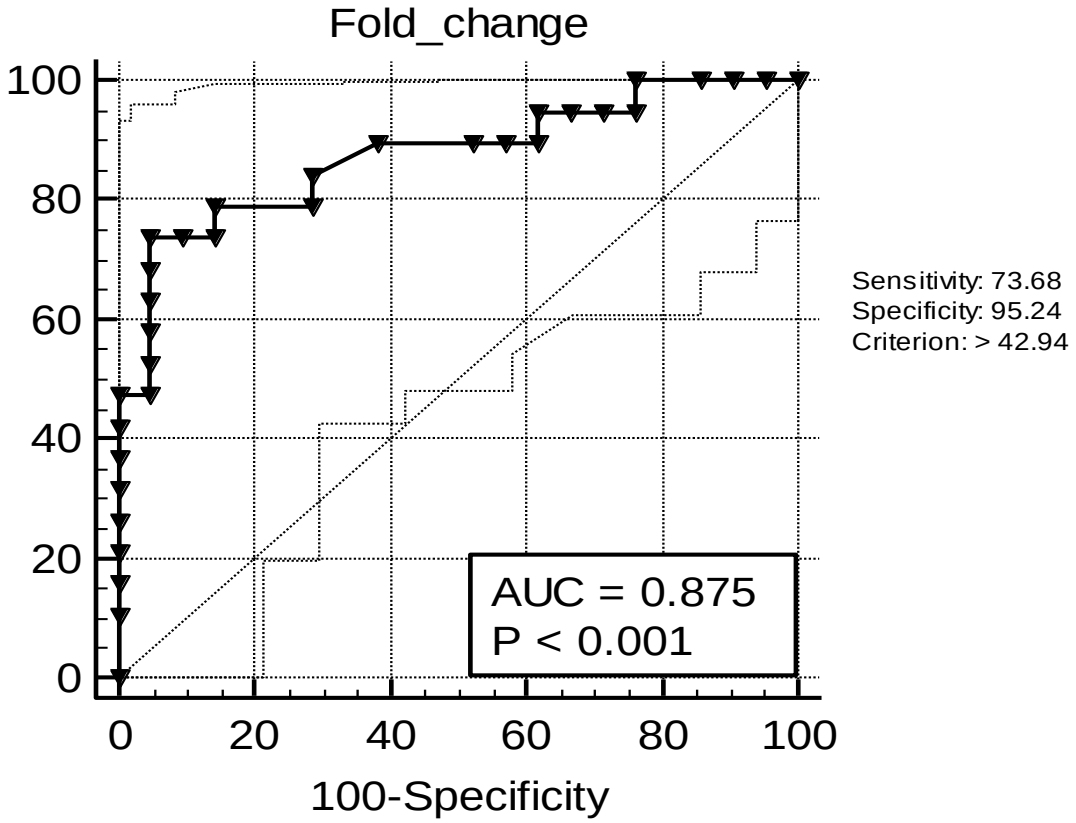

Figure 8: Receiver operating curve for no-reflow prediction performance of miRNA208a. The target marker showed good prediction performance (AUC=0.875) with high sensitivity and specificity (73.7 % and 95.2 % respectively).

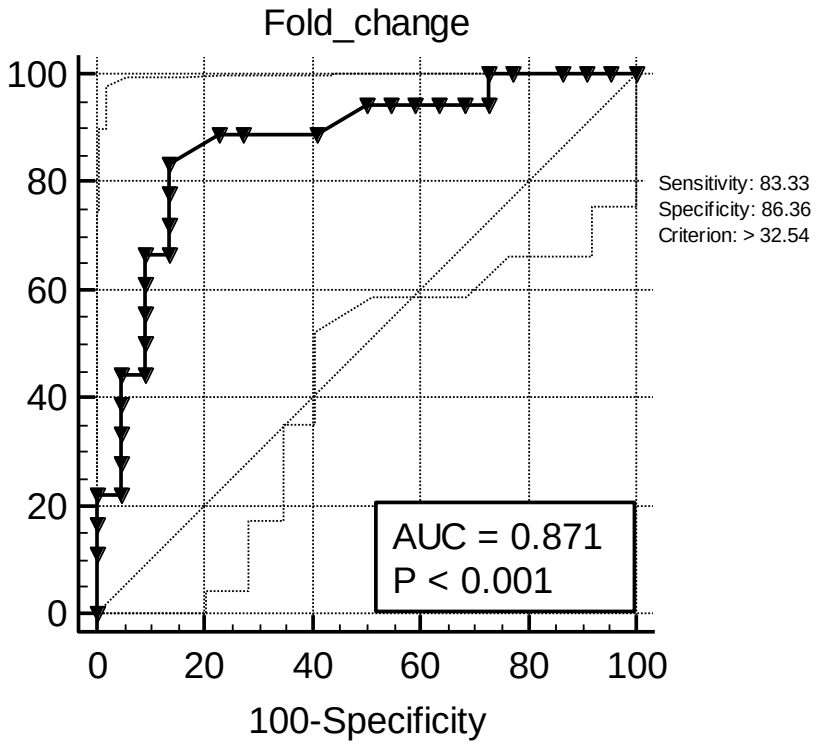

Figure 9: Receiver operating curve MACE prediction performance of miRNA208a. The target marker showed good prediction performance (AUC=0.871) with high sensitivity and specificity (83.3 % and 86.4 % respectively).

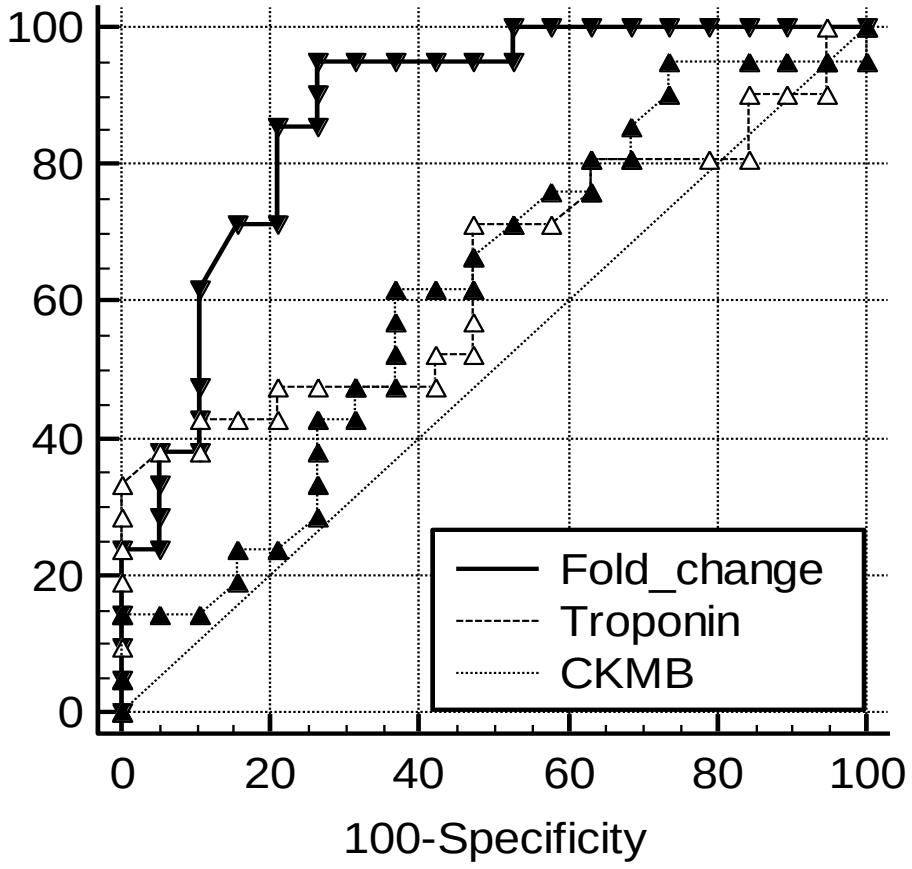

Figure 10: Receiver operating curve analysis of no-reflow prediction performance of miRNA208a, CKMB and hs-Troponin T. microRNA-208a is significantly higher to routine cardiac biomarkers in no-reflow prediction.

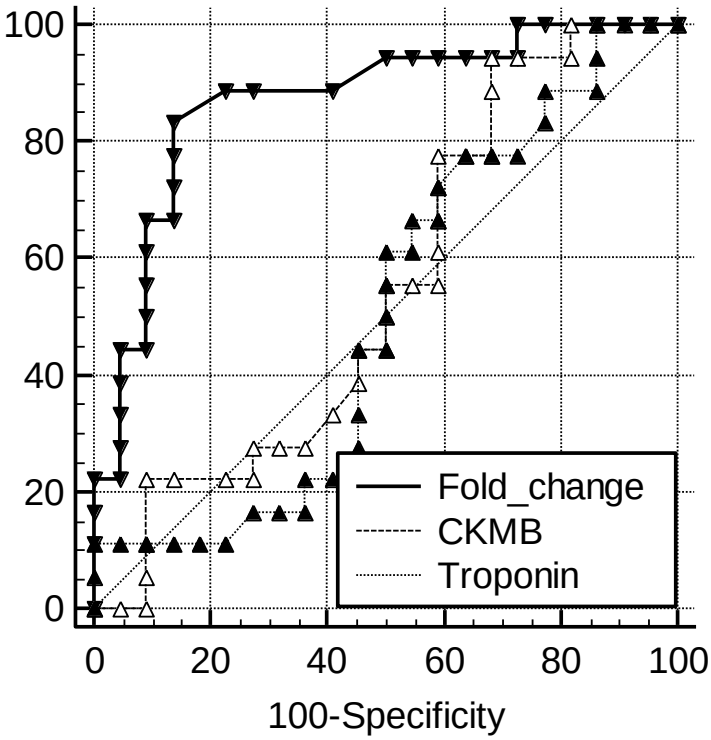

Figure 11: Receiver operating curve analysis of MACE prediction performance of miRNA208a, CKMB and hs-Troponin T. microRNA-208a is significantly higher to routine cardiac biomarkers in MACE prediction.

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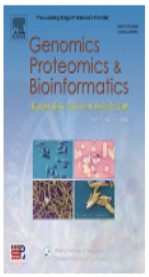

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Title of Protocol:

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Dear Dr: Abobakr Mohamed Mohamed Hammad Salama

The IRB has reviewed and assessed the above named study regarding the potential risks and benefits based on the Declaration of Helsinki. The "ratio" of risk to benefit is reasonable, given the goals of the study. The variables assessed, including the proposed subject populations, proposed procedures and scientific background are supporting the study. The IRB approved that it is within the ethical guidelines as outlined in the Declaration of Helsinki.
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your research project is now approved, effective nov 9, 2017. This project will require annual review and will expire on nov 9, 2018Research that has not received approval for continuation by this date may not continue past midnight of the expiration date.
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Sincerely
CHair IRB

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استمارة موافقة المشارك (المريض) علي المشاركة في البحث

مطلوب منك المشاركة كعينة فى مشروع بحثى بعنوان: – تركيز الحمض النووي الريبوزي متناهي الصغر 208 أ كوسيلة تشخيصية و تنبؤية في مرضى الاحتشاء القلبي المصاحب بارتفاع قطعةST .

تحت اشراف الباحث الرئيسى د/ أبوبكر محمد محمد حماد بالاشتراك مع أ.د – مصباح طه حسانين و أ.د- منار الزكي و أ.د.- سمية حسن عبد الله ود.- وائل علي خليل و أ.د. حسن عزازي

الغرض من الدراسة: اثبات امكانية استخدام الحمض النووي الريبوزي متناهي الصغر 208 أ كوسيلة تنبؤية و تشخيصية لاحتشاء القلب.

الإجراءات / الأعمال المطلوبة من المشارك خاصة بالبحث:

بعد موافقتك, سيتم عمل التالى لك: - سحب عينة دم وريدية مقدارها 5 مل.

المخاطر: من المشاركة فى هذه الدراسة ضئيلة ولا تتعدى بعض الالم أثناء سحب عينة الدم أو حدوث عدوى او تسرب دموي في موضع سحب العينة و نادرا التعرض لعدوى نتيجة استخدام الحقن. و للحد من هذه المخاطر سيقوم أفضل التمريض المدربين لدينا بسحب عينة الدم باستخدام محقن(سرنجة)معقم يستخدم لمرة واحدة فقط.

عدد الحالات المشاركة/الفترة الزمنية المطلوبة للمشاركة: 63 مريض خلال فترة سنة

الفوائد للمشارك: ليس لك اى فوائد من المشاركة

الفوائد للمجتمع: مشاركتك فى البحث سيخدم المجتمع بتقديم معلومات اضافية بالنسبة للحالات المماثلة مما قد يؤدى الى تحسين الخدمة المقدمة لمثل تلك الحالات

بدائل المشاركة: عدم المشاركة بالبحث

الحق فى الانسحاب الآمن من الدراسة: يمكن لك الانسحاب من البحث فى اى وقت بدون عواقب او تاثير على المتابعة الصحية لك.

تكاليف المشاركة فى البحث: ستقدم لك كل الخدمات بدون تكاليف

التعويضات للاصابة الجسمانية نتيجة البحث: اذا اصبت جسديا بسبب المشاركة سيتم توفير العلاج المناسب بالمستشفى و لن يكون هناك تعويض مادى

حماية خصوصية المشارك و سرية البيانات:

- سجل الدراسة التى يمكن التعرف عليك من خلالها سيتم الاحتفاظ بسريته.
- بتوقيع استمارة الموافقة فانك تجيزى جمع واستخدام والكشف عن المعلومات الصحية الخاصة بك لغرض استكمال الدراسة البحثية.

- إلا عندما يقتضي القانون، فلن يمكن الكشف عن اسمك أو الرقم القومى الخاص بك او عنوانك او رقم هاتفك ، لاى شخص او جهة غير المنوطين بالبحث بداخل جامعة الزقازيق.
- اذا تم استخدام المعلومات الصحية الخاصة بك خارج جامعة الزقازيق سيتم وضع كود رمزى لك و مفتاح هذا الكود سيتم حفظه.
- إذا غيرت رأيك في وقت لاحق ولا تريدى جمع أو تبادل المعلومات الصحية الخاصة بك، يجب عليك مخاطبة الباحث الرئيسى كتابتا والمدرج اسمه في استمارة الموافقة. ولكن سنحتفظ بحقنا فى استخدام المعلومات التي جمعناها بالفعل و يمكننا الاتصال بك لمتابعة الحالة حيث اننا بحاجة لمعرفة ما يحدث لجميع الحالات التى بدأت الدراسة بحثية، وليس فقط الحالات التي اتمت المشاركة الى النهاية. ويمكن نشر نتائج هذه الدراسة في المجلات العلمية دون تحديد اسمك او اى بيانات خاصة بك.

معلومات اضافية:

1-لقد تم اخبارك ان المشاركة تطوعية و ان لك الحق فى رفض المشاركة او الانسحاب منها فى اى وقت بدون التاثير على الخدمة الصحية المقدمة لك من قبل مستشفيات جامعة الزقازيق.

2- لمعرفة المزيد عن هذه الدراسة يمكنك الاتصال بالدكتور/ أبوبكر محمد تليفون رقم01287779366

3- فى حالة حدوث متاعب صحية نتيجة المشاركة فى الدراسة يمكنك الاتصال بالدكتور/ أبوبكر محمد تليفون رقم 01287779366

4- فى حالة وجود شكوى، يرجى الاتصال بمكتب لجنة مراجعة اخلاقيات البحوث الدكتور/ _____ تليفون رقم

لقد تم شرح الغرض من هذه الدراسة البحثية، والإجراءات الواجب اتباعها، والمخاطر والفوائد لك. و لقد سمح لك بطرح الأسئلة و تمت الاجابة على كل الاسئلة.لقد تم اخبارك بمن عليك الاتصال به إذا كان لديك أسئلة إضافية. لقد وافقت على المشاركة التطوعية كعينة فى هذا البحث. سوف تحصلين على نسخة من استمارة الموافقة بعد توقيعها.

إذا كنت توافق على الاشتراك فى هذه الدراسة، قم بالإشارة فى المكان المناسب فى الجزء التالى:

_____: لقد تم شرح طريقة البحث ومخاطرة وكيفية تجميع العينات وعمل الفحوصات وكل المعلومات الواردة فى هذه الدراسة.

_____: لقد قرأت وفهمت المعلومات الواردة فى هذه الدراسة.

اسم المشارك: _____الرقم القومى: التليفون.....العنوان__

توقيعه: _____

تسلم نسخة للمشارك وتحفظ النسخة الأصلية مع الباحث فى ملف المشارك