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ORIGINAL ARTICLE

Copeptin, miRNA-208, and miRNA-499 as New Biomarkers for Early Detection of Acute Coronary Syndrome

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Abstract
cTn and CK-MB are gold standard biomarkers for acute coronary syndrome (ACS) but are less sensitive in the first 3 h after onset of symptoms. A need thus exists for novel biomarkers for early detection of ACS. We evaluated circulating copeptin, miRNA-208, and miRNA-499 as possible biomarkers for early detection of unstable angina (UA) and non-ST-segment elevation myocardial infarction (NSTEMI). Sixty-five patients with probable ACS that presented within 4 h of the onset of chest pain (23 UA and 42 NSTEMI) and 25 apparently healthy individuals were studied. Two sets of blood samples collected in the first 3 h and at 6 h after onset were analyzed for copeptin levels via ELISA and miRNA-208 and miRNA-499 expression via real-time PCR. Copeptin, miRNA-208, and miRNA-499 expression levels were significantly increased in UA and NSTEMI patients compared with controls ($p < 0.001$) and in NSTEMT compared with UA patients ($p < 0.001$). Levels were also significantly elevated in UA and NSTEMI patients with negative cardiac troponin in the first 3 h ($p < 0.001$). ROC curves displayed AUC for prediction of ACS of 0.96 for copeptin, 0.97 for miRNA-208, and 0.97 for miRNA-499. Their combination improved AUC to 0.98. Copeptin and miRNA-208 and miRNA-499 expression are promising biomarkers for UA and NSTEMI that present in the first 3 h of pain onset. A combination of these markers with cTn may increase the accuracy of diagnosis by avoiding the gray zone of cTn as a biomarker.

Keywords Copeptin · miRNA-499 · miRNA-208 · Unstable angina · Non-ST-segment elevation Myocardial infarction · Acute coronary syndrome

Introduction

Acute coronary syndrome (ACS) is the most common cardiovascular emergency and is a leading cause of morbidity and mortality worldwide, particularly in developed countries. ACS may present as ST-elevation myocardial infarction (STEMI), non-STEMI, and

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unstable angina (UA) pectoris. According to the Egyptian National Hypertension Project and WHO, the prevalence of coronary artery disease in Egypt is 8.3% [1].

Acute myocardial infarction (AMI) is a cardiovascular emergency with acute necrosis of myocardial tissue. Accordingly, early and precise identification of AMI and urgent revascularization of coronary lesions are crucial for preventing or reducing myocardial damage. Routine diagnosis of AMI is based on patient history and clinical symptoms, such as chest pain, dyspnea, nausea and vomiting, syncope, and findings on electrocardiogram (ECG) [2, 3]. Also, biochemical markers of myocardial necrosis, including circulating levels of cardiac troponins (cTns) and creatine kinase-MB (CK-MB), are the most reliable and widely used in clinical diagnosis [4, 5].

cTn and CK-MB are gold standard biomarkers for ACS but display the major drawback of poor detection of myocardial necrosis in the first 4 h after the onset of symptoms. These cardiac biomarkers do not reach peak concentration until 16–18 h from initial myocardial injury [6, 7]. Consequently, establishing new biomarkers with greater sensitivity and specificity can correctly diagnose patients presenting with the onset of symptoms within 1–4 h, especially patients with atypical ECG findings [8].

The C-terminal portion of provasopressin (copeptin) is a pathophysiological biomarker of acute endogenous stress and cardiovascular hemostasis. AMI may induce hemodynamic changes as a result of cardiac underfilling, consequent osmotic alterations, and, particularly, a large stress stimulus. This stimulus is transmitted to the hypothalamus for de novo synthesis of pre-provasopressin and to the hypophysis for secretion of stored arginine vasopressin (AVP) and copeptin [9, 10]. Stress-induced acute response leading to the secretion of copeptin is generated rapidly after AMI onset with maximal concentrations early after the onset of the chest pain [8]. Consequently, copeptin, along with cTnT and CK-MB, may improve diagnostic accuracy in patients presenting with early chest pain, resulting in a more precise diagnosis of high-risk NSTEMI patients [8].

MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded RNAs 19–25 nucleotides in length. These small RNAs regulate gene expression at the post-transcriptional level. Mechanisms of action for these molecules include repressing mRNA translation and inducing mRNA degradation thus terminating protein synthesis [11, 12]. More than 200 miRNAs are found in heart tissues and are crucial for cardiac development and pathological processes, including AMI, arrhythmias, hypertrophy, heart failure, and atherosclerosis [13]. Cardiomyocyte-enriched miRNAs (miRNA-1, miRNA-21, miRNA208a, miRNA208b, miRNA-133a, miRNA-133b, and miR-499) are promising diagnostic biomarkers for ACS reflecting their increased sensitivity and specificity. miRNA-208 is existing in two isoforms, miRNA-208a and miRNA-208b. Both are coded at chromosome 14q11.2. The miRNA-208a gene is located on intron 29 of the *MYH6* gene that encodes α-cardiac muscle myosin heavy chain (MHC), and miR-208b resides on intron 31 of the *MYH7* gene encoding β-cardiac muscle MHC. These miRNAs are concomitantly expressed during cardiogenesis, suggesting that their expression is driven by a mutual regulatory element. miRNA-208 may control thyroid hormone-associated protein 1 and myostatin 2 and negative regulators of muscle growth and hypertrophy and thus induce hypertrophic growth [13]. Moreover, miRNA-208 is required for controlling hypertrophic growth and gene expression in response to stress. Human miRNA-499 is coded on intron 19 of the *MYH7B* gene (chromosome 20q11.2) and is composed of *miRNA-499a* and *miRNA-499b*, which are located on sense and antisense DNA chains of the same intron and are transcribed in antiparallel directions [14]. miRNA-499 regulates late cardiogenesis and is responsible for the terminal differentiation of myoblasts to cardiomyocytes. It downregulates myocyte enhancer factor 2C that favors switching myogenic differentiation preferentially toward

cardiomyocytes [15]. Circulating muscle-derived miRNAs, such as miRNA-208 and miRNA-499, are released from dying cardiomyocytes during AMI, resulting in elevated circulating levels that may aid in early screening [16–18]. Furthermore, serum and plasma miRNAs are remarkably stable, capable of withstanding repetitive freezing and thawing cycles, and are protected from RNase-dependent degradation. The latter property may reflect their packing in microvesicles or the formation of protein–miRNA complexes [16]. Consequently, the present study investigated the predictive value of copeptin and cardiac-specific total miRNA-208 and total miRNA-499 as biomarkers for early testing for AMI, especially for NSTEMI patients with chest pain presenting within the first 4 h of onset.

Materials and Methods

Study Participants

A prospective case-controlled study was conducted with 65 patients with ACS who were admitted to the Department of Cardiology, Faculty of Medicine, Assiut University Hospital. Furthermore, 25 apparently healthy age-matched volunteers were included as a control group. The sample size for detecting significant differences in copeptin, miRNA-208, and miRNA-499 with power 0.95 and hypothetical effect size 0.33 was determined using the G Power program. The study protocol was approved by the Assiut University Faculty of Medicine Institutional Review Board (IRB17101364) and was conducted according to the guidelines of the Helsinki Declaration. Informed consent was obtained from all participants prior to the study.

Our patients were divided according to the ACS type and results from the first set of cardiac markers into two groups. Group I included 42 NSTEMI patients with elevated gold standard biomarkers of AMI. This group was further classified into 34 positive patients in the first set and eight negative patients who were positive in the second set. Group II included 23 UA patients with negative cardiac biomarkers in either the first or second set.

Inclusion criteria of patients were in accordance with the "2019 European Society of Cardiology Universal Definition of MI Guideline" [19]. Only ACS patients who presented within 4 h of the onset of symptoms were included. Exclusion criteria were STEMI patients, ACS patients presenting more than 4 h from pain onset, myocarditis, pulmonary embolism, congestive heart failure, and renal failure.

Baseline demographic and clinic and laboratory characteristics of study participants were gathered. Resting 12 lead surface ECG was recorded for each patient just after hospital admission to diagnose ACS type. Traditional risk factors, such as diabetes mellitus (DM), hypertension (HTN), dyslipidemia, smoking, and obesity, were also recorded.

Methods

Sample Collection

Eight milliliters of peripheral venous blood was obtained from each patient within 4 h of the onset of clinical symptoms (chest pain or dyspnea) and divided into three tubes. Plain tubes were used for serum separation within 30 min of collection via centrifugation at 3000 rpm for 20 min. The supernatant was transferred to Eppendorf tubes and stored at − 20 °C until batch analysis of serum copeptin. Four milliliters of whole blood

in EDTA tubes was freshly processed to collect polymorph nuclear cells (PMCs), which were stored in –80 °C until batch analysis of miRNA-208 and miRNA-499. A diagnostic work-up for myocardial necrosis (CK-MB and cardiac troponin T) was analyzed with the remaining blood upon admission. A second set of enzymes was assayed 6 h after the first whenever the first set was negative for AMI. Healthy volunteers completed physical examinations, body mass index (BMI) assessment, and analysis of circulating LDL, TG, CK-MB, and cTn.

qRT-PCR Analysis of miRNA-208 and miRNA-499

After collection of PMCs, miRNA extraction used a miRNeasy Mini kit (QIAGEN, Catalog No. 217004, USA); miRNA purity and concentration were determined using a nanodrop spectrophotometer (EPOCH, Bio Tek Instruments Inc, USA). To increase poly-A tails of small noncoding miRNA with constant volumes and concentrations of miRNA, a poly-A polymerase enzyme kit (NEB, Catalog No. M0276L, New England) was used. Reverse transcription was conducted using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (USA).

qRT-PCR was conducted under sterile condition using a BiolineMyTaq™ Red Mix (2×) kit (Catalog No. K0251, USA). Two sets of reactions were conducted in the same manner except for forward and reverse primers. One set was used for miRNA-208 and the second for miRNA-499. U6-snRNA was used as an internal control. Primers were designed using the Primer-Blast program from the National Center for Biotechnology Information and reconstructed according to the manufacturer's instructions (Table 1) and obtained from (Invitrogen, UK). The thermal cycler (Applied Biosystems Step One Plus™ Real-Time PCR Systems, CA, USA) was programmed for a hot start step at 95 °C for 1 min, initial denaturation for 15 s at 95 °C, and annealing and extension for 60 s at 60 °C for 40 cycles. Relative expression of miRNA-208 and miRNA-499 was evaluated using Applied Biosystem Step OnePlus™software using the ΔΔCt method (Lava and Schmittgen, 2001) and fold differences calculated as $2^{-\Delta\Delta CT}$.

Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Copeptin Level

Copeptin level was measured in serum using a Human Copeptin ELISA Kit (Sinoxenic Biotech Co., Ltd., Catalog No. SG-10357).

Table 1 Primers used for qRT-PCR reaction

Gene	Primer sequence
miRNA-208 Accession no. (MI0000251)	(forward) 5′ CTTTTGGCCCGGGTTATAC 3′ (reverse) 5′ CTGACATCCTCTAGGCTGG 3′
miRNA-499 Accession no. (MI003183)	(forward) 5′ CGGCTGTTAAGACTTGCAGTG 3′ (reverse) 5′ GGGAAGCAGCACAGACTTG 3′
U-6	(forward) 5′ CGCTTCGGCAGCACATATAC 3′ (reverse) 5′ TTCACGAATTTGCGTGTCAT 3′

Routine Work-up

Routine clinical tests used available commercial kits, CK-MB (Human CKMB ELISA Kit ab193696), cardiac troponin T (Human Cardiac Troponin T ELISA Kit ab223860), LDL-cholesterol (LDL CHOLESTEROL Direct Enzymatic colorimetric, Liquid, REF: 280 001), and TG (Triglyceride Assay Kit, Quantification ab65336).

Statistical Analysis

Data were analyzed using SPSS (Statistical Package for the Social Science, version 20, IBM, and Armonk, New York). Continuous data were expressed as means ± standard deviation (SD), median, and range, whereas nominal data were expressed as frequency (percentage). Chi^2-tests were used to compare nominal data between groups, and Student's *t* test or one-way analysis of variance were used to compare means of continuous variables between study and control subjects. A probability (*p* value) of < 0.05 was considered statistically significant. The thresholds for optimal sensitivity and specificity of copeptin, miRNA-499, and miRNA-208 were determined using a receiver operating characteristic (ROC) curve, plotted by calculating sensitivity and specificity at several cutoff points.

Results

Baseline Characteristics of Patients and Volunteers

Mean BMI was 31 ± 4.3 for patients (with no significant difference between NSTEMI and UA), and 25.5 ± 3.9 for volunteers (Table 2). Approximately, 47% of patients had a family history of MI (42% in NSTEMI and 52% in UA subjects), and 55% showed previous ischemic heart disease (45% of NSTEMI and 73% of UA subjects). DM was diagnosed in 40%, and HTN was found in 72% of patients. The mean onset of chest pain was 3.2 ± 0.8 h prior to presentation and differed significantly between NSTEMI (3.4 ± 0.8) and UA (2.8 ± 0.8) patients.

LDL and triacylglycerol (TG) levels were significantly elevated in patients (150.6 ± 21.7 and 229 ± 54.5, respectively) compared with volunteers (101.6 ± 13.8 and 124.3 ± 18.7, respectively); however, no significant difference between NSTEMI and UA was seen. Circulating cardiac enzymes (cTn and CK-MB) showed significant differences between patient subgroups.

Biochemical Analysis of Circulating Copeptin, miRNA-208, and miRNA-499

Serum level of copeptin was significantly higher in patients than in healthy volunteers. Levels in NSTEMI patients (6.5 ± 2) were higher than in UA subjects (2.7 ± 0.74), *p* value = 0.00 (Table 2). Also, the two patient subgroups displayed significantly higher gene expression of miRNA-208 (188.5 ± 78.6 for NSTEMI and 2.3 ± 0.7 for UA) than healthy volunteers (1.0 ± 0.0), $p = 0.00$. Moreover, miRNA-208 showed a significantly higher fold

Table 2 Baseline characteristic data in NSTEMI patients, UA patients, and healthy controls

Parameter	NSTEMI (1) ***N*** = (42)	UA (2) ***N*** = (23)	Controls (3) ***N*** = (25)	***p*** value 1 Vs 3, 2 Vs 3, 1 Vs 2		
Age (years)	61.4 ± 9.7	53.3 ± 10.3	56.9 ± 15.7	0.15	0.35	***0.003***
Male (***n***, %)	22 (52%)	13 (56%)	13 (56%)	*0.97*	*0.75*	*0.74*
DM (***n***, %)	21 (50%)	5 (22%)	0 (0%)	***0.00***	***0.01***	***0.02***
HTN (***n***, %)	33 (79%)	14 (60%)	0 (0%)	***P1: 0.00***	***P2: 0.00***	P3: 0.13
Smoking (***n***, %)	13 (31%)	10 (43%)	8 (34%)	*0.92*	*0.41*	*0.31*
BMI (mean ± SD)	31.2 ± 8	30.6 ± 3.2	25.5 ± 3.9	***0.00***	***0.00***	0.583
Positive family history (***n***, %)	18 (42%)	12 (52%)	6 (26%)	*0.12*	*0.04*	*0.47*
History of IHD (***n***, %)	19 (45%)	17 (73%)	0 (0%)	***0.00***	***0.00***	*0.06*
HR (mean ± SD)	110. ± 1.5	101 ± 9.0	78.8 ± 7.0	***0.00***	***0.00***	***0.00***
SBP (mean ± SD)	153.9 ± 16.8	147 ± 17.4	113.2 ± 10.2	***0.00***	***0.00***	*0.124*
DBP (mean ± SD)	97.1 ± 8.5	97.3 ± 7.6	76.7 ± 5.1	***0.00***	***0.00***	*0.94*
Onset of pain (h, mean ± SD)	3.4 ± 0.8	2.8 ± 0.8	0.0	***0.00***	***0.00***	***0.007***
LDL-C (mmol/L)	150.6 ± 21.7	151.4 ± 25.2	101.6 ± 13.8	***0.00***	***0.00***	*0.89*
TG (mmol/L)	232.9 ± 53.9	222 ± 56	124.3 ± 18.7	***0.00***	***0.00***	*0.46*
Troponin (ng/ml) (1st set)	0.95 ± .0.98	0.1 ± .0.1	0.1 ± 0.1	***0.00***	*0.6*	***0.00***
Troponin (ng/ml) (2nd set)	1.56 ± 2.8	0.16 ± 0.1	0.1 ± 0.1	***0.01***	***0.5***	***0.01***
CK-MB (IU/L) (1st set)	43.4 ± 20.0	11.4 ± 3.7	4.6 ± 3.8	***0.00***	***0.00***	***0.00***
CK-MB (IU/L) (2nd set)	38.0 ± 6.4	15.2 ± 4.4	0.0	***0.00***	***0.00***	***0.00***
Copeptin (ng/μL)	6.5 ± 2.6	2.7 ± 0.74	2.5 ± 0.8	***0.00***	*0.25*	***0.00***

Continuous data was expressed in the form of mean ± SD (compared with Student's *t* test), while nominal data was expressed in form of frequency (percentage) (compared with Chi^2 test). *p* value was significant if < 0.05

change in NSTEMI patients than in UA patients, $p = 0.00$. miRNA-499 showed the same

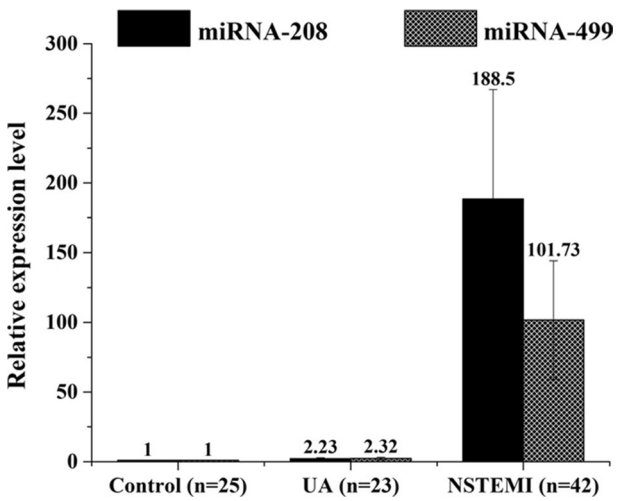

Fig. 1 The relative expression levels of miRNA-208 and miRNA-499 in the studied groups

pattern (Fig. 1).

Correlation Coefficients for Different Markers and Risk Factors

A significant positive correlation between age, SBP, DBP, heart rate, and the onset of pain with both miRNA-208 and miRNA-499 was observed in patients. Copeptin, miRNA-208, and miRNA-499 were positively correlated with the first and second sets of cTn, CK-MB, LDL-C, and TG. Our miRNA markers also presented significant positive correlations with each other (Table 3).

Diagnostic Performance of Copeptin, miRNA-208, and miRNA-499 Levels in ACS

Cutoff values, sensitivity, specificity, and area under the curve (AUC) were established using ROC curves. These curves indicated that a copeptin cutoff point of > 3.4 pmol/L shows 90% sensitivity and 86% specificity for the prediction of AMI. AUC was 0.96. A miRNA-208 ROC cutoff point of > 3.6 displayed 93% sensitivity and 98% specificity for prediction of AMI and an AUC of 0.97. Similar estimates for miRNA-499 ROC have a cutoff point of > 3.1, 95% sensitivity, 94% specificity, and an AUC of 0.978. ROC analysis showed troponin (first set) at a cutoff point of > 0.04 with 81% sensitivity, 79% specificity, and an AUC of 0.93 (Fig. 2).

Table 3 Correlation between copeptin, miRNA-208, miRNA-499, and baseline characteristics

Parameter	Copeptin		miRNA-499		miRNA-208	
Age	$r=0.27$	$p<0.01$	$r=0.31$	$p<\mathbf{0.003}$	$r=0.37$	$p<\mathbf{0.00}$
Gender	$r=-0.02$	$p<0.86$	$r=0.009$	$p<0.93$	$r=-0.06$	$p<0.58$
Family history	$r=-.0.01$	$p<0.89$	$r=-0.03$	$p<0.76$	$r=-0.01$	$p<0.95$
IHD history	$r=0.06$	$p<0.59$	$r=0.002$	$p<0.98$	$r=0.04$	$p<0.68$
Smoking	$r=-0.06$	$p<0.57$	$r=-0.007$	$p<0.95$	$r=-0.06$	$p<0.6$
BMI	$r=0.29$	$p<\mathbf{0.005}$	$r=0.3$	$p<\mathbf{0.004}$	$r=0.23$	$p<0.25$
HR	$r=0.59$	$p<\mathbf{0.00}$	$r=0.66$	$p<\mathbf{0.00}$	$r=0.6$	$p<\mathbf{0.00}$
SBP	$r=0.42$	$p<\mathbf{0.00}$	$r=0.40$	$p<\mathbf{0.00}$	$r=0.41$	$p<\mathbf{0.00}$
DBP	$r=0.42$	$p<\mathbf{0.00}$	$r=0.33$	$p<\mathbf{0.001}$	$r=0.37$	$p<\mathbf{0.00}$
Onset of pain	$r=0.33$	$p<\mathbf{0.01}$	$r=0.45$	$p<\mathbf{0.00}$	$r=0.42$	$p<\mathbf{0.00}$
Troponin 1st set (ng/ml)	$r=0.7$	$p<0.00$	$r=0.64$	$p<0.00$	$r=0.68$	$p<0.00$
Troponin 2nd set (ng/ml)	$r=0.59$	$p<0.00$	$r=0.82$	$p<0.00$	$r=0.82$	$p<0.00$
CK-MB 1st set (IU/L)	$r=0.82$	$p<0.00$	$r=0.84$	$p<0.00$	$r=0.86$	$p<0.00$
CKMB 2nd set (IU/L)	$r=0.64$	$p<0.00$	$r=0.87$	$p<0.00$	$r=0.88$	$p<0.00$
LDL-C (mmol/L)	$r=0.36$	$p<\mathbf{0.00}$	$r=0.32$	$p<\mathbf{0.002}$	$r=0.33$	$p<\mathbf{0.002}$
TG (mmol/L)	$r=0.5$	$p<\mathbf{0.00}$	$r=0.49$	$p<\mathbf{0.00}$	$r=0.47$	$p<\mathbf{0.00}$
Copeptin (ngμ/L)	—-		$r=0.75$	$p<\mathbf{0.00}$	$r=0.76$	$p<\mathbf{0.00}$
miRNA-208 ($2^{-\Delta\Delta ct}$)	$r=0.76$	$p<\mathbf{0.00}$	$r=0.92$	$p<\mathbf{0.00}$	——	
miRNA-499 ($2^{-\Delta\Delta ct}$)	$r=0.75$	$p<\mathbf{0.00}$	—-		$r=0.92$	$p<0.00$

IHD, ischemic heart disease; *BMI*, body mass index; *HR*, heart rate; *SBP*. systolic blood pressure; *DBP*, diastolic blood pressure; *CK-MB*, creatine kinase MB; *LDL-C*, low density lipoprotein cholesterol; *TG*, triacylglycerol. *p* value was significant if < 0.05

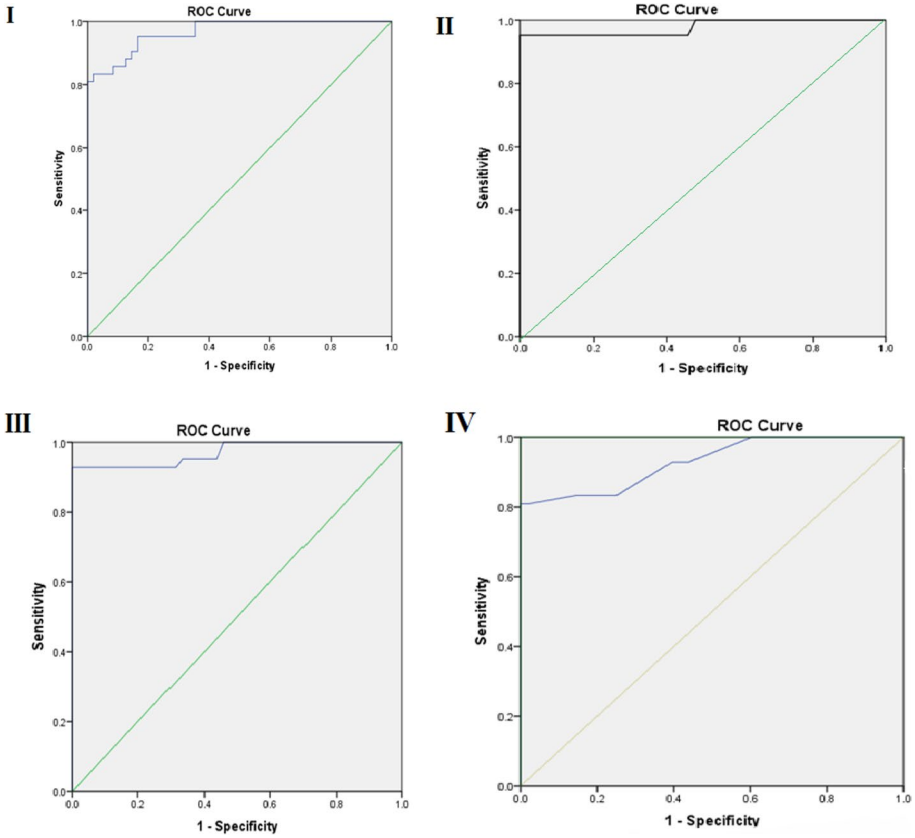

Fig. 2 ROC curves analysis for copeptin, miRNA-499, miRNA-208, and troponin (1st set) in ACS patients

Discussion

ACS is a common illness with severe consequences of mortality, morbidity, and cost to society as myocardial necrosis begins 15–30 min after severe ischemia [20]. Recent improvement in biomarker testing, especially the development of cardiac troponin (cTn) assay is a major advance [21]. As cardiomyocyte damage, detected and quantified by cTn, is a time-dependent phenomenon, and serial measurements of cTn are necessary to definitively rule out NSTEMI [8]. The blind period for troponin and CK-MB during the early onset of AMI remains a major limitation. Consequently, efforts to identify new biomarkers that enable reliable early rule out of an AMI diagnosis are continuing [22]. This study evaluated the utility of copeptin and miRNAs for the early exclusion of NSTEMI.

Prevalence of AMI is higher in males, who are more likely to develop ACS. The weak predominance of males in our patients might be partially explained by female patients being near menopause when incidence increases rapidly to become equal to the incidence in men. This observation is consistent with the loss of cardioprotective properties of estrogen [23, 24]. Unexpectedly, smoking was not a significant risk factor among the patient subgroups and control subjects. About half of our patients were females and were not smokers, which is common in our society. Furthermore, not all males were smokers.

Higher serum copeptin levels were exhibited in AMI patients than in healthy control subjects, and in NSTEMI patients than in UA patients or healthy volunteers. Furthermore, copeptin displayed better sensitivity and specificity than did troponin. Ahmedy reported that adding copeptin to CK-MB and cTn allowed safe exclusion of AMI as a diagnosis with a negative predictive value of > 99% in patients presenting with suspected AMI. This combination might rule out AMI in 58% of patients without frequent blood draws collection [20]. Ay et al., recommended the use of copeptin as a diagnostic marker in patients with suspected AMI in combination with other biomarkers [25]. A hypothesis to explain the rapid release of AVP/copeptin after AMI is the rapid response of the endocrine stress axis, resulting in the release of adrenocorticotropic hormone and cortisol [26]. An alternative trigger for AVP/copeptin secretion from the posterior pituitary is a baroreceptor stimulated by the threat of hypotension as a result of the AMI or direct damage to the cardiac baroreceptors [27].

Copeptin offers early positive results in our patients who had negative cTn and CK-MB in the first set of routine markers but was positive in the second set (eight patients). Thus, copeptin is an effective marker for early diagnosis of NSTEMI, avoiding the gray zone of cTn. These findings are consistent with Khan et al. who reported increased copeptin levels on day 1 of AMI [28]. Moreover, Reichlin and his group evaluated the contribution of copeptin to the management of AMI [27]. They observed elevated copeptin concentrations 4 h after the onset of symptoms. At this time, troponin T was still undetectable in many patients. They also indicated that as copeptin concentrations declined, troponin concentrations increased. These distinct kinetics add value to the use of both markers for the accurate diagnosis of AMI [27]. Recently, Mahmoud et al. showed that copeptin levels were elevated in AMI patients at the time of admission, 0 to 4 h after onset of chest pain. Circulating levels then decreased from 5 to 10 h thereafter [29]. Moreover, we showed a significant positive correlation between copeptin and cTn, CK-MB, LDL, and TG. Similarly, Ay et al. showed a significant positive correlation between levels of copeptin and the presence of AMI and elevated CK-MB and troponin in ACS patients [25].

Plasma miRNA-208 and miRNA-499 showed significantly higher levels in NSTEMI and UA patients than in controls and higher levels in NSTEMI patients than in UA patients. miRNA-208 and miRNA-499 were effective in assessing AMI with a cutoff point that showed 93 to 98% sensitivity and specificity with an AUC of 0.097 or greater. Additionally, miRNA fold changes were measurable soon after the onset of symptoms at times when patients showed negative cTn and CK-MB activity in the first set of routine enzymes. These results were positive in the second set (eight patients examined later). miRNA-208 and miRNA-499 can be used as early markers of AMIthat allow evaluation of the gray zone in cTn analysis. miRNA-499 is involved in cardiomyocyte differentiation, which could explain elevated miRNA-499 expression in NSTEMI patients as it might be released from damaged cardiac cells into the circulation [30]. Our findings are supported by previous studies; however, these studies mostly assessed miRNA plasma levels instead of their expression. Liu et al. studied the pattern of plasma levels of miR-208 and miR-499, which were significantly elevated in AMI patients [18]. These authors reported that miR-208 and miR-499 were possible biomarkers for the prediction of AMI as AUC values were 0.72 and 0.88, respectively. They attributed greater specificity and sensitivity of these miRNA to the cardiac-specific distribution of the genes. Furthermore, Olivieri et al. also published results consistent with our assessment of miRNA-499, where elevated levels were seen in NSTEMI patients with an AUC of 0.86 [31].

Circulating miRNA-208 and miRNA-499 levels were elevated in Greek patients with NSTEMI, and the AUC of miRNA-208 was 0.9996. Similarly, the AUC of miRNA-499

was 0.9992, with 98% sensitivity and 100% specificity, which supports the use of plasma levels of miRNA-208b and miRNA-499 as AMI biomarkers [32]. These findings are consistent with Boštjančič et al. who showed that the miRNA-208 level was significantly elevated after AMI [33]. Still, Wang et al. indicated that miRNA-208 has an advantage over miRNA-499 for AMI diagnosis [34]. Other authors concluded that miRNA-499 may be the more reliable biomarker for AMI diagnosis in patients presenting > 4 h after onset of pain. miRNA-499 was positive in 93% of patients and cTn in only 88% [18, 35].

cTn and CK-MB were positively and significantly correlated with miRNA-208 and miRNA-499. Additionally, miRNA-208 and miRNA-499 showed a similar correlation with LDL-C and TG. Interestingly, miRNA-208 was significantly correlated with miRNA-499 and vice versa. Devaux et al. reported results in agreement with ours, showing significant positive correlations between miRNA-208 and miRNA-499 and gold standards cTn and CK-MB [35].

Accordingly, expression of total miRNA-208 and total miRNA-499 and plasma levels of copeptin is now believed to offer better and early exclusion of AMI, superior to conventional troponin and CK-MB within the first 4 h after the onset of acute chest pain. Adding copeptin and miRNA to the gold standard of cardiac markers may offer better information to clinicians for early decision-making in patients with suspected AMI. Additionally, our study is considered the first to analyze both copeptin and miRNA in the same ACS patients.

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Author Contribution Marwa A Gaber and Omnia HM Omar conceptualized and designed the study protocol development, assessment, and writing of the manuscript. Ayman KM Hassan and Marwan S Mahmoud performed clinical assessments and selection of all cases and revised the manuscript. Marwa A Gaber and Omnia HM Omar performed all lab investigations. Sahar EM El-Deek and Abdel-Raheim MA Meki revised the manuscript. All authors approved the final manuscript as submitted and agreed to be responsible for all aspects of the work.

Availability of Data and Materials Data and materials are available.

Declarations

Ethics Approval and Consent to Participate The study protocol was approved by the institutional ethics committee (IRB local approval number 17101364) and explained to all participants, and only those who gave informed written consent were included in the study.

Guarantor: Marwa A Gaber.

Conflict of Interest The authors declare no competing interests.

References

1. Hassanin, N., Gharib, S., El Ramly, M. Z., Meged, M. A., & Makram, A. (2015). Metabolic syndrome and coronary artery disease in young Egyptians presenting with acute coronary syndrome. *Kasr Al Ainy Medical Journal, 21*(1), 27. https://doi.org/10.4103/1687-4625.155666
2. Anderson, J. L., Adams, C. D., Antman, E. M., Bridges, C. R., Califf, R. M., Casey, D. E., Chavey, W. E., Fesmire, F. M., Hochman, J. S., Levin, T. N., Lincoff, A. M., Peterson, E. D., Theroux, P., Wenger, N. K., & Wright, R. S. (2013). Accf/Aha focused update incorporated into the Accf/Aha 2007 guidelines for the management of patients with unstable angina/non–St-elevation myocardial infarction: A report of the American College of Cardiology Foundation/American Heart Association Task Force on

Practice Guidelines. *Journal of the American College of Cardiology, 61*(23), e179–e347. https://doi.org/10.1016/j.jacc.2013.01.014
3. Oerlemans, M. I., Koudstaal, S., Chamuleau, S. A., de Kleijn, D. P., Doevendans, P. A., & Sluijter, J. P. (2013). Targeting cell death in the reperfused heart: Pharmacological approaches for cardioprotection. *International Journal of Cardiology, 165*(3), 410–422. https://doi.org/10.1016/j.ijcard.2012.03.055
4. Apple, F. S., Jesse, R. L., Newby, L. K., Wu, A. H., Christenson, R. H., & National Academy of Clinical Biochemistry, & IFCC Committee for Standardization of Markers of Cardiac Damage. (2007). National Academy of Clinical Biochemistry and IFCC Committee for Standardization of Markers of Cardiac Damage Laboratory Medicine Practice Guidelines: Analytical issues for biochemical markers of acute coronary syndromes. *Circulation, 115*(13), e352–e355. https://doi.org/10.1161/CIRCULATIONAHA.107.182881
5. Morrow, D. A., Cannon, C. P., Jesse, R. L., Newby, L. K., Ravkilde, J., Storrow, A. B., Wu, A. H., Christenson, R. H., & National Academy of Clinical Biochemistry. (2007). National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: Clinical characteristics and utilization of biochemical markers in acute coronary syndromes. *Circulation, 115*(13), e356–e375. https://doi.org/10.1161/CIRCULATIONAHA.107.182882
6. Bauer, T., Hoffmann, R., Jünger, C., Koeth, O., Zahn, R., Gitt, A., Heer, T., Bestehorn, K., Senges, J., & Zeymer, U. (2009). Efficacy of a 24-H primary percutaneous coronary intervention service on outcome in patients with St elevation myocardial infarction in clinical practice. *Clinical Research in Cardiology, 98*(3), 171–178. https://doi.org/10.1007/s00392-008-0738-6
7. Keller, T., Post, F., Tzikas, S., Schneider, A., Arnolds, S., Scheiba, O., Blankenberg, S., Münzel, T., & Genth-Zotz, S. (2010). Improved outcome in acute coronary syndrome by establishing a chest pain unit. *Clinical Research in Cardiology, 99*(3), 149–155. https://doi.org/10.1007/s00392-009-0099-9
8. Gu, Y. L., Voors, A. A., Zijlstra, F., Hillege, H. L., Struck, J., Masson, S., Vago, T., Anker, S. D., Van Den Heuvel, A. F., Van Veldhuisen, D. J., & de Smet, B. J. (2011). Comparison of the temporal release pattern of copeptin with conventional biomarkers in acute myocardial infarction. *Clinical Research in Cardiology, 100*(12), 1069–1076. https://doi.org/10.1007/s00392-011-0343-y
9. Morgenthaler, N. G. (2010). Copeptin: A biomarker of cardiovascular and renal function. *Congestive Heart Failure, 16*(Suppl. 1), S37–S44. https://doi.org/10.1111/j.1751-7133.2010.00177.x
10. Vargas, K. G., Kassem, M., Mueller, C., Wojta, J., & Huber, K. (2016). Copeptin for the early rule-out of non-St-elevation myocardial infarction. *International Journal of Cardiology, 223*, 797–804. https://doi.org/10.1016/j.ijcard.2016.08.304
11. Pheasant, M., & Mattick, J. S. (2007). Raising the estimate of functional human sequences. *Genome Research, 17*(9), 1245–1253. https://doi.org/10.1101/gr.6406307
12. Cortez, M. A., Bueso-Ramos, C., Ferdin, J., Lopez-Berestein, G., Sood, A. K., & Calin, G. A. (2011). MicroRNAs in body fluids—The mix of hormones and biomarkers. *Nature Reviews. Clinical Oncology, 8*(8), 467–477. https://doi.org/10.1038/nrclinonc.2011.76
13. Shen, E., Diao, X., Wei, C., Wu, Z., Zhang, L., & Hu, B. (2010). MicroRNAs target gene and signaling pathway by bioinformatics analysis in the cardiac hypertrophy. *Biochemical and Biophysical Research Communications, 397*(3), 380–385. https://doi.org/10.1016/j.bbrc.2010.05.116
14. Wilson, K. D., Shen, P., Fung, E., Karakikes, I., Zhang, A., InanlooRahatloo, K., Odegaard, J., Sallam, K., Davis, R. W., Lui, G. K., Ashley, E. A., Scharfe, C., & Wu, J. C. (2015). A rapid, high-quality, cost-effective, comprehensive and expandable targeted next-generation sequencing assay for inherited heart diseases. *Circulation Research, 117*(7), 603–611. https://doi.org/10.1161/CIRCRESAHA.115.306723
15. Pagiatakis, C., Gordon, J. W., Ehyai, S., & McDermott, J. C. (2012). A novel Rhoa/Rock-Cpi-17-Mef2c signaling pathway regulates vascular smooth muscle cell gene expression. *Journal of Biological Chemistry, 287*(11), 8361–8370. https://doi.org/10.1074/jbc.M111.286203
16. Dimmeler, S., & Zeiher, A. M. (2010). Circulating microRNAs: Novel biomarkers for cardiovascular diseases? *European Heart Journal, 31*(22), 2705–2707. https://doi.org/10.1093/eurheartj/ehq221 Oxford University Press.
17. Xu, J., Zhao, J., Evan, G., Xiao, C., Cheng, Y., & Xiao, J. (2012). Circulating microRNAs: Novel biomarkers for cardiovascular diseases. *Journal of Molecular Medicine, 90*(8), 865–875. https://doi.org/10.1007/s00109-011-0840-5
18. Liu, X., Fan, Z., Zhao, T., Cao, W., Zhang, L., Li, H., Xie, Q., Tian, Y., & Wang, B. (2015). Plasma Mir-1, Mir-208, Mir-499 as potential predictive biomarkers for acute myocardial infarction: An independent study of Han population. *Experimental Gerontology, 72*, 230–238. https://doi.org/10.1016/j.exger.2015.10.011
19. Thygesen, K., Alpert, J. S., Jaffe, A. S., Chaitman, B. R., Bax, J. J., Morrow, D. A., White, H. D., Thygesen, K., Alpert, J. S., Jaffe, A. S., Chaitman, B. R., Bax, J. J., Morrow, D. A., White, H. D., Mickley, H., Crea, F., Van de Werf, F., Bucciarelli-Ducci, C., Katus, H. A., & Corbett, S. (2019). Fourth

universal definition of myocardial infarction (2018). *European Heart Journal, 40*(3), 237–269. https://doi.org/10.1093/eurheartj/ehy462
20. El shafey, W. E. D. H., & Ahmedy, I. A. (2016). Diagnostic values of copeptin as a novel cardiac marker in relation to traditional markers in acute myocardial infarction. *Clinical Trials and Regulatory Science in Cardiology, 19*, 13–19. https://doi.org/10.1016/j.ctrsc.2016.05.003
21. Twerenbold, R., Jaffe, A., Reichlin, T., Reiter, M., & Mueller, C. (2012). High-sensitive troponin T measurements: What do we gain and what are the challenges? *European Heart Journal, 33*(5), 579–586. https://doi.org/10.1093/eurheartj/ehr492
22. Reinstadler, S. J., Klug, G., Feistritzer, H. J., Metzler, B., & Mair, J. (2015). Copeptin testing in acute myocardial infarction: Ready for routine use? *Disease Markers, 2015*, 614145. https://doi.org/10.1155/2015/614145
23. Jafar, T. H., Qadri, Z., & Chaturvedi, N. (2008). Coronary artery disease epidemic in Pakistan: More electrocardiographic evidence of ischaemia in women than in men. *Heart, 94*(4), 408–413. https://doi.org/10.1136/hrt.2007.120774
24. Reda, A. A., Mina, M. B., & Hussein, A. N. T. (2018). Pattern of risk factors and management strategies in patients with acute coronary syndrome. *Menoufia Medical Journal, 31*(2), 378.
25. Ay, M. O., Erenler, A. K., Dogan, T., & Yetim, M. (2017). Diagnostic value of copeptin in acute myocardial infarction. *European Review for Medical and Pharmacological Sciences, 21*(7), 1576–1582.
26. Katan, M., Morgenthaler, N., Widmer, I., Puder, J. J., König, C., Müller, B., & Christ-Crain, M. (2008). Copeptin, a stable peptide derived from the vasopressin precursor, correlates with the individual stress level. *Neuro Endocrinology Letters, 29*(3), 341–346.
27. Reichlin, T., Hochholzer, W., Stelzig, C., Laule, K., Freidank, H., Morgenthaler, N. G., Bergmann, A., Potocki, M., Noveanu, M., Breidthardt, T., Christ, A., Boldanova, T., Merki, R., Schaub, N., Bingisser, R., Christ, M., & Mueller, C. (2009). Incremental value of copeptin for rapid rule out of acute myocardial infarction. *Journal of the American College of Cardiology, 54*(1), 60–68. https://doi.org/10.1016/j.jacc.2009.01.076
28. Anderson, J. L., Adams, C. D., Antman, E. M., Bridges, C. R., Califf, R. M., Casey, D. E., Chavey, W. E., Fesmire, F. M., Hochman, J. S., Levin, T. N., Acc, & Aha. (2007). Guidelines for the management of patients with unstable angina/non–St-elevation myocardial infarction. *Journal of the American College of Cardiology, 50*(7), 652–726.
29. El Baky, A., Mahmoud, M., Shaaban, M. A. A., & Ali Ramzy, A. (2018). Clinical role of serum copeptin in acute coronary syndrome. *Egyptian Heart Journal, 70*(3), 155–159. https://doi.org/10.1016/j.ehj.2018.04.008
30. Martinez, M. C., Tual-Chalot, S., Leonetti, D., & Andriantsitohaina, R. (2011). Microparticles: Targets and tools in cardiovascular disease. *Trends in Pharmacological Sciences, 32*(11), 659–665. https://doi.org/10.1016/j.tips.2011.06.005
31. Olivieri, F., Antonicelli, R., Lorenzi, M., D'Alessandra, Y., Lazzarini, R., Santini, G., Spazzafumo, L., Lisa, R., La Sala, L., Galeazzi, R., Recchioni, R., Testa, R., Pompilio, G., Capogrossi, M. C., & Procopio, A. D. (2013). Diagnostic potential of circulating Mir-499-5p in elderly patients with acute non St-elevation myocardial infarction. *International Journal of Cardiology, 167*(2), 531–536. https://doi.org/10.1016/j.ijcard.2012.01.075
32. Agiannitopoulos, K., Pavlopoulou, P., Tsamis, K., Bampali, K., Samara, P., Nasioulas, G., Mertzanos, G., Babalis, D., & Lamnissou, K. (2018). Expression of Mir-208b and Mir-499 in Greek patients with acute myocardial infarction. *In Vivo, 32*(2), 313–318. https://doi.org/10.21873/invivo.11239
33. Boštjančič, E., Zidar, N., Štajer, D., & Glavač, D. (2010). MicroRNAs Mir-1, Mir-133a, Mir-133b and Mir-208 are dysregulated in human myocardial infarction. *Cardiology, 115*(3), 163–169. https://doi.org/10.1159/000268088
34. Wang, G. K., Zhu, J. Q., Zhang, J. T., Li, Q., Li, Y., He, J., Qin, Y. W., & Jing, Q. (2010). Circulating microRNA: A novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *European Heart Journal, 31*(6), 659–666. https://doi.org/10.1093/eurheartj/ehq013
35. Devaux, Y., Vausort, M., Goretti, E., Nazarov, P. V., Azuaje, F., Gilson, G., Corsten, M. F., Schroen, B., Lair, M. L., Heymans, S., & Wagner, D. R. (2012). Use of circulating microRNAs to diagnose acute myocardial infarction. *Clinical Chemistry, 58*(3), 559–567. https://doi.org/10.1373/clinchem.2011.173823

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