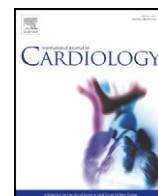




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Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction

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ABSTRACT

Background: Geriatric patients with acute non-ST elevation myocardial infarction (NSTEMI) can frequently present atypical symptoms and non-diagnostic electrocardiogram. The detection of modest cardiac troponin T (cTnT) elevation is challenging for physicians needing to routinely triage these patients. Unfortunately, non-coronary diseases, such as acute heart failure (CHF), may cause cTnT elevation. Circulating microRNAs (miRs) have emerged as biomarkers of MI. However, their diagnostic potential needs to be determined in elderly NSTEMI patients.

Methods: 92 NSTEMI patients (82.6 ± 6.9 years old; complicated by CHF in 74% of cases) and 81 patients with acute CHF without AMI (81.3 ± 6.8 years old) were enrolled at presentation. A third group comprised 99 age-matched healthy control subjects (CTR). Plasma levels of miR-1, -21, -133a, -208a, -423-5p and -499-5p were analyzed.

Results: MiR-1, -21 -133a and -423-5p showed a 3- to 10-fold increase and miR-499-5p exhibited >80-fold increase in acute NSTEMI patient vs. CTR. MiR-499-5p and -21 showed a significantly increased expression in NSTEMI vs. CHF. Interestingly, miR-499-5p was comparable to cTnT in discriminating NSTEMI vs. CTR and CHF patients. Its diagnostic accuracy was higher than conventional and hs-cTnT in differentiating NSTEMI (n = 31) vs. acute CHF (n = 32) patients with modest cTnT elevation at presentation (miR-499-5p AUC = 0.86 vs. cTnT AUC = 0.68 and vs. hs-cTnT AUC = 0.70).

Conclusions: Circulating miR-499-5p is a sensitive biomarker of acute NSTEMI in the elderly, exhibiting a diagnostic accuracy superior to that of cTnT in patients with modest elevation at presentation.

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Abbreviations: AMI, acute myocardial infarction; AUC, area under the ROC curve; CHF, congestive heart failure; cTnT, cardiac troponin T; hs, high sensitivity; LVEF, left ventricular ejection fraction; MI, myocardial infarction; miRNA, microRNA; NSTEMI, non-ST elevation myocardial infarction; qRT-PCR, quantitative real time polymerase chain reaction; ROC, receiver operator characteristic; TTE, trans-thoracic 2D echocardiography; WMSI, wall motion score index.

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1. Introduction

The prevalence of acute coronary syndromes (ACS) increases with age [1]. In elderly patients, the incidence of myocardial infarction (MI) is high, with mortality rates exceeding 30% [2]. However, the recognition of acute myocardial infarction (AMI) in old individuals is often challenging, particularly in the presence of non-ST-elevation MI (NSTEMI) [3]. In geriatric patients the symptoms of NSTEMI may be atypical, the electrocardiogram (ECG) inconclusive because of a preexisting left bundle branch block, the presence of a cardiac pacemaker, and diffused ECG changes due to chronic ischemic cardiomyopathy or previous MIs [3–5]. Further, the diagnostic value of cTnT,

currently regarded as a key marker of cardiac injury, can be flawed because of modest increase at presentation to the hospital that cannot be readily differentiated from non-coronary troponin elevation due to other pathologic conditions, such as CHF [3,6–9]. As a consequence, aged patients have a higher incidence of unrecognized events in an early phase raising the compelling need for new, sensitive and specific early biomarkers of NSTEMI in the geriatric population.

MicroRNAs (miRNAs), a class of evolutionary conserved small non-coding RNAs ~20–23 nt in length, play a role in most cellular processes as modulators of gene-expression. MiRNAs have been recently measured in plasma, serum and urine; where they are protected from degradation and remain remarkably stable [10–13]. Further, in animal models of AMI as well as in STEMI patients, the plasma levels of some cardiac-associated miRNAs, such as miR-1, -133a and -499-5p, are significantly increased [14–21] and the kinetics of some STEMI-associated miRNAs parallel cTnT [22]. These results indicate that some miRNAs represent novel biomarkers of cardiac injury.

Presently, miRNA plasma levels in the geriatric population are still uncharacterized and, specifically, it is unknown which miRNAs are modulated in elderly patients with NSTEMI and how their sensitivity and specificity compare to the commonly used biomarker cTnT. High sensitivity (hs) cTnT assays have been developed [23] and both conventional and hs-cTnT assays are now available for clinical use. The objective of the present work was to examine miRNA plasma levels in geriatric patients with acute NSTEMI, and to compare them to an age-matched control group of healthy subjects. Since in elderly patients NSTEMI is frequently complicated by heart failure, a group of age-matched patients with acute CHF without acute AMI was also included. Moreover, the specificity and sensitivity of miRNA plasma levels were evaluated in a sub-group of NSTEMI and acute CHF elderly patients with comparable conventional and hs-cTnT elevation at presentation.

2. Methods

2.1. Study groups

A total of 272 subjects (113 males and 159 females; 81.1 ± 6.7 years old) were enrolled; 92 patients had acute NSTEMI (82.6 ± 6.9 years old), 81 patients had acute CHF without evidence of AMI (81.3 ± 6.8 years old) and 99 were healthy control subjects (CTR) (79.5 ± 5.4 years old) (Table 1).

NSTEMI and CHF patients were hospitalized in the Coronary Care Unit (CCU) of the Italian National Research Centre on Aging (INRCA) Hospital, Ancona, whereas CTR were selected among subjects participating to a cardiovascular diseases prevention program. All patients and CTR were examined and enrolled from the same medical team. All subjects gave their informed consent before enrolment and the study protocol was approved by the local Ethical Committee.

NSTEMI was diagnosed according to the European Society of Cardiology (ESC) guidelines [24]. Briefly, the diagnosis was made based on the presence of at least 2 of the following criteria: 1) clinical symptoms potentially correlated to AMI, in particular typical chest pain or sudden onset of dyspnea; 2) typical ECG alterations. ECG signs of myocardial ischemia included either transient or persistent ST-segment elevation or depression > 1 mm, persistent and definite T-wave inversion, including the pseudo-normalization of previously negative T waves in ≥ 2 contiguous leads and new onset of complete left ventricular block branch; 3) significant rise and consequently fall of cTnT within the first 48–72 h from the CCU admission. Cardiac troponin is the preferred marker for the diagnosis of MI. As reported by guidelines, CK-MB by mass assay is an acceptable alternative only when cardiac troponin is not available [24].

Moreover, echocardiogram was performed in all enrolled NSTEMI patients to confirm the presence of infarcted area. Regional wall motion (WM) was assessed with a 16-segment model, as recommended by the American Society of Echocardiography, by two expert operators. Each segment was scored as: 1) normal, 2) hypokinetic, 3) akinetic, 4) dyskinetic or 5) aneurysm. The sum of WMSs, averaged over the number of segments with interpretable scores, gave the wall motion score index (WMSI). However, since a prior history of ischemic heart disease (IHD) was present in 71 of 92 (77%) NSTEMI patients, it was difficult to differentiate between acute and previous MI damages. Further, coronary angiography was performed in 35 of 92 (38%) NSTEMI patients; in 21 patients the angiogram showed complete coronary occlusion and in 14 patients it was found evidence of critical coronary artery stenosis. In all patients left ventriculography confirmed the presence of segmental wall akinesis in the distribution of the stenotic/occluded artery.

Interestingly, 68 of 92 NSTEMI patients (74%) presented symptoms and signs of left ventricular failure as complication of NSTEMI. Since a large percentage of enrolled

Table 1

Clinical characteristics and relevant blood biochemistry values of healthy subjects (CTR), patients with NSTEMI (NSTEMI) and patients with CHF (CHF).

	CTR (n 99)	NSTEMI (n 92)	CHF (n 81)
Age (years)	79.5 ± 5.4	82.6 ± 6.9	81.3 ± 6.8
Glucose (mmol/L)	5.46 ± 0.79	$8.97 \pm 2.27^*$	$8.13 \pm 2.79^*$
PCR-hs (mg/L)	2.95 ± 2.26	$7.77 \pm 3.88^*$	$4.60 \pm 1.31^*$
Myoglobin (μ g/L)	48.81 ± 12.02	$366.51 \pm 87.49^*$	95.86 ± 12.97
CK-MB (nmol/L)	0.07 ± 0.02	$0.48 \pm 0.07^*$	0.08 ± 0.05
cTnT-Is (ng/mL)	0.01 ± 0.01	$0.88 \pm 1.76^*$	$0.09 \pm 0.06^*$
NT proBNP (pg/mL)	654.8 ± 359.6	$8435.9 \pm 3208.2^*$	$12878.3 \pm 5404.8^*$
Total cholesterol (mmol/L)	5.64 ± 0.41	$4.45 \pm 0.96^*$	$4.96 \pm 0.91^*$
HDL cholesterol (mmol/L)	1.55 ± 0.35	$1.18 \pm 0.37^*$	1.22 ± 0.39
Triglycerides \pm S.D. (mmol/L)	1.25 ± 0.54	1.49 ± 0.81	$1.37 \pm 0.19^\dagger$
LVEF (%)	58 ± 8	$43 \pm 8^*$	$41 \pm 4^*$
Male, N (%)	40 (40)	39 (41)	34 (42)
cTnT > 0.03 , N (%)	0 (0)	92 (100)*	32 (39)*
$0.03 < \text{cTnT} < 0.10$, N (%)	0 (0)	31 (34)*	32 (39)*
CHF, N (%)	0 (0)	68 (74)*	81 (100)*
RF, N (%)	0 (0)	36 (39)*	29 (36)*
T2DM, N (%)	0 (0)	58 (63)*	39 (48)*
Arterial hypertension, N (%)	52 (52)	75 (81)*	56 (69)*

All values are reported as mean \pm standard deviation. Probability values were calculated by General Linear Model (GLM) age-adjusted. NSTEMI vs CTR, CHF vs CTR, $^\dagger p < 0.05$, * $p < 0.01$.

PCR-hs = high sensitive C reactive protein; CKMB = creatin Kinase MB; cTnT = cardiac troponin T; NT pro-BNP: N-terminal prohormone brain natriuretic peptide; HDL = high-density lipoprotein; LVEF = left ventricular ejection fraction; CHF = congestive heart failure; RF = renal failure; T2DM = type 2 diabetes mellitus.

NSTEMI patients were complicated by CHF, a group of acute CHF patients without evidence of AMI were selected among consecutive patients in whom CHF exacerbation determined the need for urgent hospitalization. Only patients with CHF decompensation due to non coronary etiology (such as exacerbation of pulmonary pathologies due to infectious diseases, acute pulmonary embolism, hypertensive crisis or valvular pathologies, i.e. aortic stenosis) were recruited for this study. Moreover, since NSTEMI may lead predominantly to impairment of systolic function, only patients with systolic CHF of primitive post-ischemic etiology were enrolled, while those with isolated diastolic dysfunction were excluded.

In all acute CHF patients the diagnosis was confirmed by trans-thoracic echocardiography (TTE) and NT-proBNP increase. Left ventricular ejection fraction (LVEF) (%) in NSTEMI and acute CHF patients were 43 ± 8 and 41 ± 4 , respectively.

Conventional cTnT plasma levels (normal range < 0.03 ng/mL) were determined in all NSTEMI and CHF patients. It is noteworthy that 31 of 92 NSTEMI and 32 of 81 acute CHF patients presented with conventional cTnT elevation ranging from 0.03 to 0.10 ng/mL at the time of hospital admission (time 0). However, none of the acute CHF patients presented cTnT rise and fall within the initial 48–72 h after arriving to the hospital and none of them had cTnT values higher than 0.10 ng/mL. On the contrary, all NSTEMI patients exhibited a cTnT rise $\geq 40\%$ of the value at time 0 and a subsequent typical fall in cTnT plasma level within the ensuing 48–72 h.

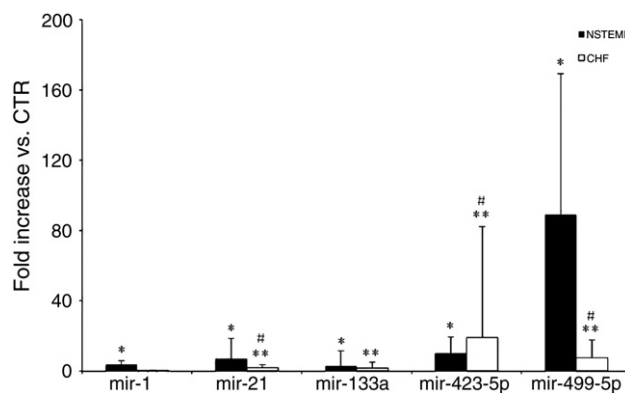


Fig. 1. Mean fold increase expression of circulating miR-1, -21, -133a, -423-5p and -499-5p in NSTEMI (n = 92) and CHF patients (n = 81) vs. CTR subjects (n = 99). * NSTEMI versus CTR $p < 0.05$; ** CHF versus CTR $p < 0.05$; # CHF versus NSTEMI $p < 0.05$. Data are reported as mean fold increase \pm standard deviation. CTR values were set as 1.

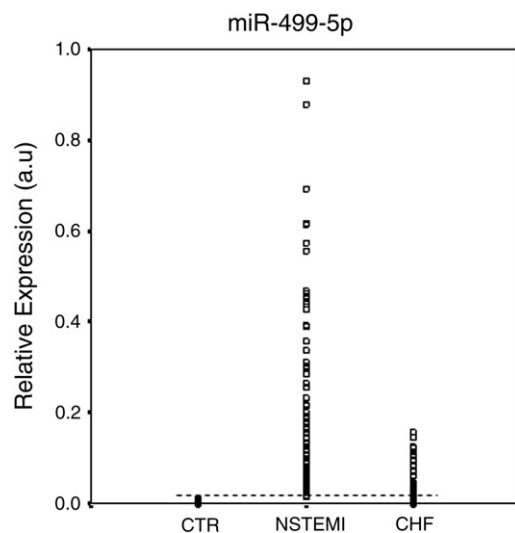


Fig. 2. MiRNA-499-5p relative expression in CTR subjects ($n=99$), NSTEMI ($n=92$) and CHF patients ($n=81$). The horizontal dotted line indicates the cut-off value calculated as 99th percentile of miRNA relative expression values distribution in CTR subjects. Data are reported as $2^{-\Delta\Delta Ct}$. CHF = congestive heart failure; CTR = control healthy subjects; NSTEMI = non-ST elevation myocardial infarction.

In the setting of NSTEMI and CHF patients exhibited modest cTnT elevation (0.03–0.10 ng/mL) at presentation also hs-cTnT plasma levels (normal range <0.015 ng/mL) were determined.

Clinical history and use of medications were collected for all patients.

For both NSTEMI and acute CHF groups, duration of symptoms from onset to hospital admission was 4–9 h. NSTEMI and CHF blood samples were drawn on admission. No patients enrolled in the study received anticoagulant therapy with heparin, which is known to interfere with polymerase chain reaction, at the time of hospital admission and prior to obtaining the first blood sample for miRNA determination. After CCU admission all patients with NSTEMI diagnosis received standard therapy, according to the current guideline of NSTEMI treatment.

Finally, a control group of 99 age-matched healthy subjects was enrolled. All patients with severe hepatic or renal dysfunction, as well as with evidence of malignancy with a short term negative prognosis were excluded from the study.

2.2. RNA extraction and miRNA expression

Peripheral venous blood samples from all subjects were collected in EDTA-coated tubes (Venoject, Terumo Europe NV) 4–9 h after the onset of symptoms. Total RNA was extracted from 100 μ L of plasma. MiRNAs were quantified by qRT-PCR using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Data were analyzed with the iCycler (Bio-Rad Laboratories S.r.l., Italy) with automatic setting for assigning baseline.

There is no current consensus on the use of an internal control for real-time PCR analysis of circulating miRNA. Therefore, we used fixed volumes of starting plasma

(100 μ L), buffer for the elution of RNA (50 μ L) from starting plasma, and input into the RT reaction (5 μ L) in each assay for technical consistency. Moreover, all qRT-PCR data were standardized to miR-17, a miRNA which was previously validated in STEMI patients [20] and fulfilled the following criteria: detectable in all samples, low dispersion of expression levels and null association with MI and acute CHF.

Synthetic cel-miR-39 (Applied Biosystems, Foster City, CA, USA) was spiked into plasma after the addition of denaturing solution and it was used for the normalization of differences in RNA isolation.

MiRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method setting 1 as an arbitrary value for control group. MiRNA relative expression distribution values were calculated as $2^{-\Delta Ct}$, ($\Delta Ct = Ct \text{ miR-X} - Ct \text{ miR-17}$).

The Ct values from qRT-PCR assays greater than 35 were treated as not expressed. The intra- and inter-assay variability of measurements were <3% and <10%, respectively.

2.3. Laboratory assays

Serum concentration of Is- and hs-cTnT was determined by ElectroChemiluminescence ImmunoAssay (ECLIA) using the Modular Analytics E170/Cobas immunoanalyzer (Roche Diagnostics) according to manufacturer's instructions. The intra-assay imprecision was determined by replicate measurements ($n=10$) in a single run. Total CVs were obtained by analyzing two runs per days each in duplication for 10 days. The intra-assay imprecision was 2.9% using a serum pool at 11.5 ng/L. The inter-assay imprecision was 7.3% using a serum pool at 9.5 ng/L. The analytical measurement range was up to 10,000 ng/L and determined to be linear throughout the range. The limit of blank (LoB) was 3 ng/L and the limit of detection (LoD) was 5 ng/L determined in accordance with the CLSI (Clinical and Laboratory Standard Institute) EP17-A requirements.

cTnT <0.03 ng/mL and hs-cTnT <0.015 ng/mL were considered normal. Some patients were identified as having modest cTnT elevation, i.e. cTnT ranging from 0.03 to 0.10 ng/mL and hs-cTnT ranging from 0.015 to 0.10 ng/mL; these ranges were observed in acute CHF patients without evidence of AMI. Interestingly, most non-coronary causes of cTnT elevation induce an increase within the range adopted in the present work [8].

It is noteworthy that the same blood sample was used for miRNAs, cTnT, CK-MB, myoglobin and NT-proBNP determinations, unless otherwise specified.

2.4. Statistical analysis

Data were analyzed with SPSS/Win program (version 17.0; SPSS Inc., Chicago, IL) and reported as mean \pm standard deviation (SD).

Differences among groups were compared using Student's *t*-test and General Linear Model analysis adjusted for age and gender for continuous variables and χ^2 test for categorical variables. Receiver Operating Characteristic (ROC) curve analysis was used to assess the diagnostic accuracy of each miRNA. The area under the ROC curve (AUC) was used as diagnostic index.

The correlations between parameters were calculated with Pearson or Spearman's rho correlation coefficient.

Probability values lower than 0.05 were considered statistically significant. The reported *p*-values were two-tailed in all calculations.

3. Results

Plasma levels of miR-1, -21, -133a, -208a, -423-5p and -499-5p were analyzed in all subjects. MiR-208a was undetectable in the

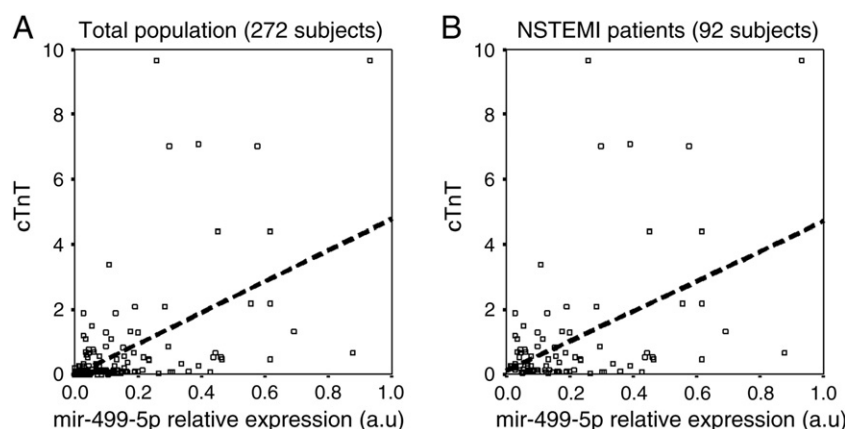


Fig. 3. MiR-499-5p relative expressions correlation with cTnT in total population (panel A) and in the setting of NSTEMI patients (panel B). cTnT values were expressed as pg/mL, and miR-499-5p relative expression values were expressed as arbitrary unit (a.u.). MiR-499-5p and cTnT: Spearman's rho = 0.76 ($p<0.001$) in the total population (A). MiR-499-5p and cTnT: Spearman's rho = 0.57 ($p<0.001$) in NSTEMI patients (B). NSTEMI = non-ST elevation myocardial infarction.

plasma of 30 subjects (i.e. 10 individuals from each of the 3 groups) and, for this reason, no other patients were tested and miR-208a was excluded from additional analyses. All other miRNAs were detectable in all patients. It was found that the mean value of all analyzed miRNAs was significantly elevated in NSTEMI vs. CTR subjects. Moreover, miR-133a, -423-5p, -21 and -499-5p were increased in CHF vs. CTR subjects. Interestingly, miR-21 and -499-5p were increased in NSTEMI vs. acute CHF patients and miR-423-5p was increased in acute CHF vs. NSTEMI patients (Fig. 1).

Importantly, all analyzed miR levels were similar in patients with and without angiographic confirmation of the NSTEMI diagnosis.

MiR-499-5p and miR-21 were the only two miRs that showed significant increased values in NSTEMI patients compared to CTR and, at the same time, that showed significant discrimination between NSTEMI and CHF patients groups. However, miR-499-5p exhibited the highest increase in NSTEMI patients, of about 80-fold, and showed the greatest discrimination between NSTEMI and CHF patients groups (Figs. 1 and 2). Therefore, all subsequent analyses were aimed at comparing miR-499-5p vs. cTnT as biomarkers of acute NSTEMI. In the total population, including NSTEMI, acute CHF and CTR subjects, miR-499-5p relative expression was significantly correlated with cTnT (Spearman's rho 0.76, $p < 0.001$) (Fig. 3A). A significant correlation was also found in the group of patients with NSTEMI (Spearman's rho 0.57, $p < 0.001$) (Fig. 3B).

No significant effect of type 2 diabetes mellitus and systemic arterial hypertension was found on miR-499-5p expression levels (data not shown).

The diagnostic accuracy of miR-499-5p was evaluated with ROC curve analyses and was compared to cTnT. When comparing NSTEMI patients vs. CTR subjects, miR-499-5p and cTnT exhibited identical AUC values, i.e. AUC = 1 (data not shown). For acute CHF patients vs. CTR subjects miR-499-5p exhibited AUC values higher than cTnT (AUC = 0.88 vs. AUC = 0.78, respectively, $p < 0.05$) (Fig. 4A). In the differential diagnosis between NSTEMI and acute CHF patients miR-499-5p and cTnT showed comparable AUC values (AUC = 0.88 vs. AUC = 0.93, respectively, $p > 0.05$) (Fig. 4B). Importantly, 31 of 92 NSTEMI and 32 of 81 acute CHF patients exhibited single point cTnT plasma level ranging from 0.03 to 0.10 ng/mL. This range was chosen because it was observed in acute CHF patients without evidence of AMI and most non-coronary causes of troponin elevation induce a cTnT increase frequently within this range. In order to improve the diagnostic accuracy of cTnT in these patients hs-cTnT levels were evaluated, showing values ranging from 0.015 to 0.10 ng/mL. In this setting, miR-499-5p showed a diagnostic accuracy superior to both cTnT and hs-cTnT (AUC of 0.86 vs. an AUC of 0.70 and of 0.68 for hs-cTnT and cTnT, respectively, $p < 0.05$) (Fig. 4C).

4. Discussion

The present work examined the plasma level of selected miRNAs to establish their potential role as acute NSTEMI biomarkers in elderly patients. Prior studies had demonstrated that circulating levels of some miRNAs increase in patients with AMI [15–20] and acute CHF [25], however circulating miRNA levels in geriatric patients with AMI and/or CHF have not been previously evaluated. Our study focused on miR-1, -133a, -208a and -499-5p because the circulating level of these miRNAs has been reported to increase both in patients and animal models with AMI [16,19,20]; miR-423-5p was evaluated because it was found elevated in the circulation of patients with CHF [25]; miR-21 was included because it is highly expressed in the heart [26–28] and it exhibits a significant age-dependent increase in some tissues (E.G. Lakatta, L. Jiang, M. De Simone, Y. Zhang, K. G. Becker, W. H. Wood III, M. Wang, P. Fasanaro and M. C. Capogrossi, unpublished results).

We found that miR-1, -21, -133a, -423-5p and -499-5p mean plasma levels were significantly higher in NSTEMI patients than in CTR subjects; miR-133a, -423-5p, -21 and -499-5p were higher in acute CHF patients than in CTR subjects; miR-21 and -499-5p plasma levels were higher and miR-423-5p was lower in NSTEMI than in acute CHF patients. Under our experimental conditions, miR-208a resulted undetectable. This is in agreement with a recent study in which we reported that circulating miR-208a was barely detectable in some STEMI patients but never in healthy controls [20–29].

We and others had previously examined miRNAs plasma level in patients with STEMI, showing an increase of miR-1, -133a and -499-5p plasma levels [15,18–22]. The present work extends these results to a setting of geriatric NSTEMI patients, with an average age higher

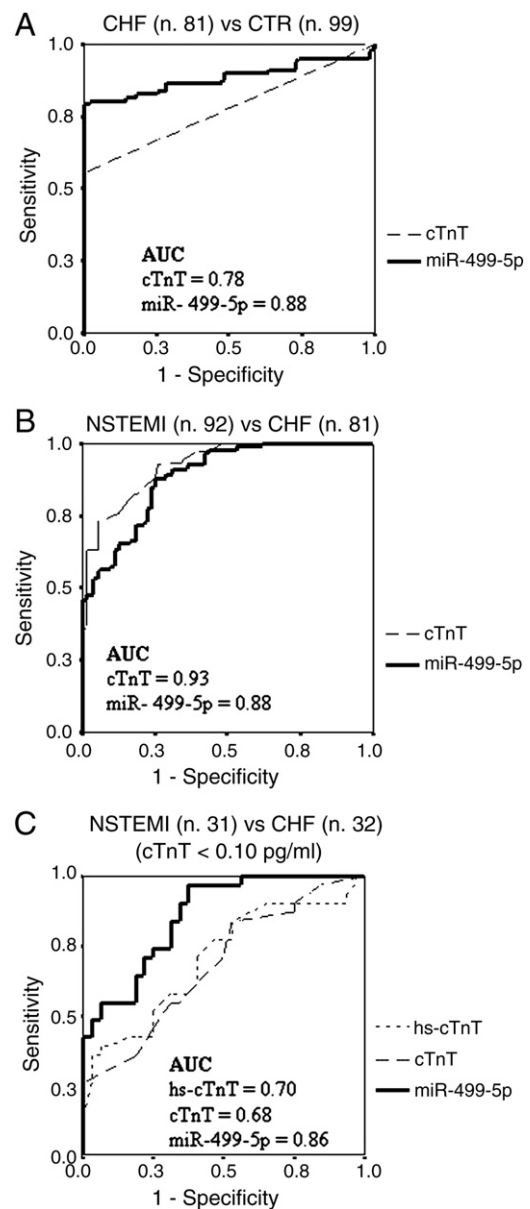


Fig. 4. Diagnostic accuracy of miR-499-5p and standard and hs-cTnT as assessed by ROC curve analysis. A. Sensitivity and specificity of miR-499-5p and cTnT in CHF vs. CTR. B. Sensitivity and specificity of miR-499-5p and cTnT in NSTEMI vs. CHF. C. Sensitivity and specificity of miR-499-5p and cTnT and hs-cTnT in the setting of NSTEMI and CHF patients with cTnT elevation < 0.10 ng/mL. AUC (area under the curve) values were reported for each analyzed parameters. CHF = congestive heart failure; CTR = control healthy subjects; NSTEMI = non-ST elevation myocardial infarction. hs = high sensitivity.

than 80 years. Importantly, the major finding of the present work relates to the expression of miR-499-5p in NSTEMI and acute CHF patients with single point modest increase in cTnT at the time of hospital admission. In these patients miR-499-5p exhibited sensitivity and specificity superior to cTnT both conventional and high sensitivity, in differentiating NSTEMI patients from those with acute CHF. It is noteworthy that elderly NSTEMI patients frequently present with atypical symptoms, such as dyspnea and/or epigastric discomfort without chest pain, uninterpretable ECG and a modest increase in cTnT, ranging from 0.03 to 0.10 ng/mL. These levels can also be found in patients without AMI because of a variety of non coronary causes including acute CHF, pulmonary embolism, shock, pericarditis, sepsis, cerebrovascular accident, cardiac trauma and renal failure [8]. For these reasons, NSTEMI patients with modest single increase in cTnT at presentation are frequently misdiagnosed in the earliest phases of the acute event [30]. The excellent performance of hs-cTnT assays in the early diagnosis of AMI was confirmed also in elderly patients [31]. However, elevated hs-cTnT levels are common in elderly patients with diagnoses other than AMI and its specificity is not superior to that of cTnT in the elderly population (Olivieri et al., manuscript in preparation). Such a diagnostic uncertainty after the cTnT measurement at presentation may have clinical implication, determining an inappropriate treatment in those initial hours following coronary occlusion in which revascularization would provide the greatest benefit [32]. An increase in serial cTnT concentrations is necessary to confirm the AMI diagnosis, requiring almost 12 h from the first examination. In the present work the time course of circulating miRNA plasma levels was not determined because, after obtaining the first blood sample upon arrival to the hospital, all patients received heparin which interferes with the PCR amplification utilized to quantify miRNAs in the plasma.

Importantly, recent data published by an independent research group showed that circulating levels of miR-499-5p was significantly elevated in cTnT-positive patients with acute coronary syndrome compared with patients with coronary artery disease, reinforcing our results [33].

An important and novel finding of the present work is the increase in miR-21 in NSTEMI vs. CTR and vs. acute CHF patients; this is the first report to show that circulating miR-21 increases in patients with acute MI. Two studies in humans with STEMI [18,20] and one in patients with CHF [25] identified miR-21 in the circulation during the screening procedure; however, since this miRNA was not modulated in the presence of acute MI and CHF, respectively, its expression level was not validated by qRT-PCR. In contrast to the paucity of information on circulating miR-21 and cardiovascular diseases, there is a wealth of data on this miRNA expression and function in the heart. It is known that miR-21 is highly expressed in cardiac fibroblasts and modulates fibrosis [34–36]. Further, miR-21 expression markedly increased in a mouse model of cardiac ischemia/reperfusion injury and it was predominantly expressed by cardiac fibroblasts in the infarcted region [37]; miR-21 is also increased in failing human hearts [38,39] and in the presence of cardiac hypertrophy [28]. Finally, miR-21 expression exhibits a marked age-dependent increase in some non-human primates tissues (E.G. Lakatta, L. Jiang, M. De Simone, Y. Zhang, K. G. Becker, W. H. Wood III, M. Wang, P. Fasanaro and M. C. Capogrossi, unpublished results) and its expression is enhanced by interleukin-6, a cytokine which increases with age [40]. Thus, it is possible that elderly individuals with a depressed LV function may express miR-21 in the heart at significantly higher levels than younger subjects and that, in response to ischemic cardiac injury, only elderly patients release high concentrations of this miRNA into the bloodstream.

In conclusion, circulating miR-499-5p emerged in a geriatric population as a promising complementary biomarker of acute NSTEMI. MiR-499-5p single point evaluation may have relevant clinical implication, contributing to reduce the time to diagnosis and consequently

determine an appropriate treatment in the initial hours following coronary occlusion.

5. Limitations

Some limitations of the present work need to be acknowledged. The results on miR-499-5p as biomarker of AMI in patients with modest increase in cTnT at presentation are limited to the differential diagnosis between NSTEMI and acute CHF; additional studies will be required to establish whether this miRNA diagnostic accuracy applies to other causes of cTnT elevation in the absence of MI. The present work represents a single-center study involving a limited number of patients. Multicenter large-scale studies will be required to determine the potential use of circulating miRNAs as diagnostic biomarkers of NSTEMI and CHF in geriatric patients.

Moreover, since we aimed at comparing NSTEMI patients complicated by CHF to patients with acute CHF and without evidence of AMI, we presented the retrospective comparison of these two distinct cohorts.

Further, the mechanisms underlying the release of cardiac-associated miRNAs into the bloodstream after myocardial injury remain to be elucidated. Specifically, it would be of interest to ascertain whether the mechanisms of release are purely passive, as for conventionally used necrosis-associated biomarkers, or if an active release process is involved [41,42]. Finally, it is unclear why miR-423-5p was higher in acute CHF than in NSTEMI patients; both groups exhibited a decrease in LVEF%, although cardiac function was marginally more depressed in systolic CHF than in NSTEMI patients. The presented data do not allow us to establish whether miR-423-5p was higher in patients with systolic CHF than in NSTEMI patients because of a worse ventricular function or because of relation to different pathologic mechanisms.

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Author contributions

All authors contributed significantly to the submitted work: (1) conception and design of the study: RA and FO; (2) data collection, analysis and interpretation: FO, RA, ML, YDA, RL, GS, LS, RL, LLS, RG, RR, RT, GP, MCC and ADP; (3) drafting of the manuscript and revising it for intellectual content: FO, RA, YDA, GP, MCC and ADP.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [43].

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