# Correlation of Circulating miR-765, miR-93-5p, and miR-433-3p to Obstructive Coronary Heart Disease Evaluated by Cardiac Computed Tomography

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**Epigenetic-sensitive mechanisms may be correlated both to pathogenesis and prognosis of coronary heart disease (CHD). We prospectively investigated some plasma circulating microRNA levels in patients undergoing cardiac computed tomography for suspected CHD (n = 95).**

**We show that let-7c-5p, miR-765, miR-483-5p, miR-31-5p, and miR-206 were upregulated in CHD patients (n = 66) versus healthy subjects HS (n = 29); moreover, let-7c-5p, miR-765, miR- 483-5p showed higher expression in obstructive CHD (n = 36) compared to no obstructive CHD patients (n = 66). Remarkably, miR-765, miR-93-5p, and miR-433-3p showed an upregulation in patients with critical coronary stenosis. Multivariate regression analysis demonstrated that miR-765, miR-31-5p, and miR-206 were independently associated with CHD while circulating levels of miR-765 (p = 0.035), miR-433-3p (p = 0.043), and miR-93-5p (p = 0.041) were significantly higher in critical stenosis patients. Receiver operating characteristic curve analysis revealed a good performance for miR-765, miR-93-5p, and miR-433-3p on predicting CHD severity. In conclusion, our study represents a combined epigenetic/imaging approach useful to support the diagnosis and prediction of CHD.**  **(Am J Cardiol 2019;124:176−182)**

Several studies revealed a wide number of epigenetic modifications involved in the pathogenesis and progression of coronary heart disease (CHD).[1−3] In the epigenetic hallmarks, microRNAs (miRNAs), acting as flexible modulators of gene expression, could represent attractive biomarkers useful in clinical practice.[1] MiRNAs are tissue specific and are released into the bloodstream following organ injury or can act as intercellular communicators.[1] MiRNAs are involved in pathophysiological processes such as atherosclerosis and CHD.[1] Cardiac computed tomography (CCT) is a useful diagnostic tool for the detection of suspected obstructive CHD. Third-generation CCT shows a great potential both in the visualization and quantification of atherosclerotic plaques thanks to its high spatial resolution and excellent negative predictive value providing a reliable tool to exclude CHD when the clinical diagnosis is doubtful.[4,5] To date, we need to improve the conventional risk scores by assessing new noninvasive biomarkers that will guide clinical decision making and to stratify patients for early and/or modified treatment strategies. However, only few studies have associated circulating miRNA expression levels with CCT imaging parameters in the setting of CHD.[6]

Here, we evaluated the expression pattern of circulating miRNAs in patients with suspected CHD as compared with control subjects (HS) with the aim to integrate epigenetic-sensitive molecular findings with morphological and clinical parameters derived by CCT.

## Methods

The study was approved by institutional ethics committee (IRCCS Fondazione SDN, protocol no. 7-13). All recruited individuals provided informed consent, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki. During a period of 36 months, 250 consecutive patients were enrolled. Patients with known history of cancer, active infections, chronic, or immune-mediated diseases, were excluded from the study. Furthermore, subjects with cardiomyopathy, known CHD, previous percutaneous transluminal coronary angioplasty and coronary artery bypass grafting, systemic atherosclerosis such as lower extremity peripheral arterial disease or supra-aortic arterial disease were not included in the study population. The remaining 95 subjects without a history of cardiovascular events and referred to our institution for suspected CHD were included in the study. Patients with Calcium Score (CACS) = 0 and uninjured coronaries were considered as HS (n = 29), the remaining patients (n = 66) represented the CHD group. Obstructive CHD (n = 36) was defined by the presence of a stenosis equal or greater than 50% in one or more of the major coronary arteries detected by CCT, while no obstructive CHD (n = 59) was

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characterized by a coronary stenosis degree less than 50%.[7] Age, gender, body mass index (BMI), blood pressure levels, history of previous myocardial infarction, cardiovascular risk factors, medical history, and the presence of symptoms were recorded. Dyslipidemia, diabetes, and hypertension were defined according to the current guidelines.[8–10] Blood pressure was measured after ≥5 minutes rest. Morise risk score, based upon symptoms, age, gender, and cardiovascular risk factors, was calculated and then patient population was stratified into low, intermediate, and high pretest probability.[11] Framingham risk score, considering age, gender, risk factors, blood pressure, LDL, and HDL cholesterol, was computed to estimate the presence of obstructive CHD.[12]

CCT was performed with a third-generation dual source multidetector computed tomography scanner (Somatom Force, Siemens Healthcare AG, Forchheim, Germany). A prospectively ECG-triggered high pitch spiral acquisition (FLASH) without contrast medium was performed for calcium score evaluation (slice thickness of 3 mm, increment of 3 mm, small FOV). Afterwards, patients underwent angiographic Cardiac CT scans with IV contrast material (50 mL@5mL/s of iodinated contrast agent − Iomeprol 400 mg I/ml − Iomeron 400, Bracco, Milan, Italy, followed by 50 mL@5mL/s of saline flush); scans were performed with retrospective ECG gating and with prospective ECG-tube current modulation (window 25% to 75% of the R-R interval). Images were analyzed on an offline dedicated workstation (Syngo.Via VB10B, Siemens) where multiplanar reformations, maximum intensity projections, curved multiplanar reformations and 3D volume rendering images were generated. All scans were analyzed by 2 experienced radiologists and after independent evaluations a consensus interpretation was achieved.

Peripheral venous blood samples were collected into EDTA Vacutainer tubes after a 12 hours fasting in the same day of CCT, before imaging execution. All tubes were centrifuged within an hour from collection at 1900×g for 10 minutes at 4°C to separate plasma and cellular components. Cell- and platelet-free plasma was obtained by a further centrifugation at 16.000×g for 10 minutes at 4°C. Hemolysis was assessed as previously reported[13] and hemolyzed samples were excluded from the experimental workflow.

From the consultation of the bioinfarmatic database mirTarBase,[14] PhenomiR,[15] MiriAD,[16] and miR2disease[17] we selected a set of miRNAs related to atherosclerosis and CHD. The complete list of miRNAs analyzed is reported in Supplementary Table 1. Total RNA was isolated from 200 $\mu$L of EDTA plasma of HS and CHD patients using miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer instructions using a 3.5 $\mu$L per sample of $1.6 \times 10^8$ copies/$\mu$L of a synthetic *C. elegans* miRNA (cel-miR-39-3p) (Qiagen). Total RNA was reverse transcribed using miScript II RT kit (Qiagen) according to the manufacturer's instructions in 10 $\mu$L RT reaction. The cDNA products were preamplificated with miScript PreAMP PCR Kit (Qiagen) according to the manufacturer's instructions using a miScript PreAMP Custom Primer Mix (Qiagen) in 25 $\mu$L final reaction volume. Following cDNA preamplification the levels of the 42 selected miRNAs were analyzed by qRT-PCR using Custom miScript miRNA 384 PCR Arrays (Qiagen) and 2x QuantiTect SYBR Green PCR Master Mix (Qiagen) on CFX384 Touch Real-Time PCR Detection System (BioRad Laboratories, Ltd). Melt curve analysis was performed to verify a single product species. Each sample was analyzed in triplicate and data expressed as mean ± standard error. The determination of the more stable and powerful miRNA for data normalization was performed as previously reported.[18] In our experimental conditions, cel-miR-39-3p was characterized by high stability, and therefore used as reference gene for data normalization. Relative miRNA expression was assessed using the $2^{-\Delta\Delta Ct}$ calculation method.

Statistical analysis was performed using R Core Team (version 3.03 Austria, Vienna). Continuous variables were expressed as mean ± standard deviation or standard error. Data were tested for normality through the Shapiro-Wilk test and for homoscedasticity through the Levene test. For comparison between 2 groups, *t* test was used if gaussianity was met; otherwise the Mann-Whitney U test was chosen. Categorical variables were expressed as percentage and were compared using the Chi-Square test or the Fisher's exact test. We performed a preliminary power calculation analysis for multivariate regression using G*Power software, obtaining an estimated total sample size of 89 with an effect size equal to 0.15. Molecular markers were tested in a univariate logistic regression analysis; then, significant variables were included in a multivariate logistic regression analysis (stepwise forward model) adjusted for the traditional cardiovascular risk factors and baseline clinical features.[19] Receiver operating characteristic (ROC) curves were subsequently generated using CHD and obstructive CHD as the events. Areas under the curve (AUC) were compared for each single molecular variable and the multivariate model. All tests were 2-tailed and a $p < 0.05$ was considered for statistical significance.

## Results

The baseline characteristics of subjects enrolled are summarized in Table 1. Subgroup analysis comparing dyslipidemia treated versus untreated patients and male versus female gender showed no significant differences for the expression of the selected circulating microRNAs.

We prospectively investigated by qRT-PCR the circulating expression levels of miRNAs in a group of CHD patients ($n = 66$) compared with a group of HS ($n = 29$) undergoing to CCT (Figure 1). Molecular analysis showed a significant upregulation of let-7c-5p ($p = 0.032$), miR-765 ($p = 0.015$), miR-483-5p ($p = 0.043$), miR-31-5p ($p = 0.010$), and miR-206 ($p = 0.011$) in CHD group as compared to HS (Figure 1). Univariate logistic regression analysis revealed that miR-765, miR-31-5p, and miR-206 were significantly associated to the presence of CHD (Table 2). The multivariate regression analysis showed that male gender and high levels of miR-765, miR-31-5p, and miR-206 were still independently associated with CHD (Table 2). ROC curve analysis of the multivariate models provided a good performance on predicting CHD. MiR-765 showed an AUC = 0.756 ($p < 0.001$), the AUC of miR-31-5p was 0.758 ($p < 0.001$) and miR-206 showed AUC of 0.720 ($p < 0.001$). Morise pretest probability was not statistically significant between HS and CHD patients ($p = 0.153$)

Table 1
Baseline characteristics of coronary heart disease patients and healthy subjects

| Variables | CHD (n = 66) | HS (n = 29) | p Value |
|---|---|---|---|
| Age (years)* | 65.86 ± 10.0 | 61.83 ± 7.7 | 0.150 |
| Men | 52 (79%) | 13 (45%) | **0.001** |
| Symptoms | | | |
| Asymptomatic | 53 (80%) | 17 (59%) | 0.102 |
| Typical angina | 6 (9%) | 7 (24%) | |
| Atypical angina | 6 (9.1 %) | 5 (17%) | |
| Nonanginal | 1 (1%) | 0 (0 %) | |
| Pretest probability | | | |
| Low | 7 (11%) | 0 (0%) | 0.129 |
| Intermediate | 51 (77 %) | 23 (79%) | |
| High | 8 (12%) | 6 (21%) | |
| Body mass index (kg/m$^2$)* | 27.82 ± 4.62 | 27.90 ± 4.69 | 0.940 |
| Familiarity | 37 (56%) | 19 (65%) | 0.388 |
| Current smoker | 23 (35%) | 9 (31%) | 0.717 |
| Hypertension | 46 (70%) | 18 (62%) | 0.465 |
| Diabetes | 11 (17%) | 1 (3%) | 0.147 |
| Dyslipidemia | 38 (58%) | 13 (45%) | 0.251 |
| Physical activity | 24 (36%) | 8 (28%) | 0.405 |
| Anti-hypertensive therapy | 45 (68%) | 18 (62%) | 0.562 |
| Dyslipidemia treatment | 33 (50%) | 8 (28%) | **0.042** |

CHD = coronary artery disease; BMI = body mass index; HS = healthy subjects.

Bold values were considered statistically significant with a p <0.05.

Hypertension was defined as systolic/diastolic blood pressure ≥140/90 mm Hg, or in treatment with antihypertensive drugs. Dyslipidemia was defined as low-density lipoprotein level ≥140 mg/dl, or current treatment with statins and/or lipid-lowering agents. Physical activity was evaluated according to the WHO guidelines for adults 18 to 65 aged; specifically the performance of at least 150 minutes of moderate-intensity aerobical physical activity per week or at least 75 minutes of vigorous-intensity aerobic physical activity throughout the week. None of the recruited subjects had physical disabilities.

* Data are represented as mean ± SD.

as well as binary logistic regression for CHD using Morise score as dependent variable (p = 0.084). Multivariate logistic regression including Morise score and differential expressed miRNAs showed that the combination of miR-765 and Morise score was a significant predictor of CHD (p = 0.004 and p = 0.036, respectively). Furthermore, high circulating levels of miR-31-5p (p = 0.009) and miR-206 (p = 0.009) with Morise score (p = 0.050, and p = 0.042, respectively) were significant predictors of CHD presence. ROC curve analysis revealed a good performance to detect CHD for the three miRNAs in combination with Morise clinical score. In detail, circulating miR-765 expression and Morise score showed an AUC of 0.713 (p = 0.001), miR-31-5p expression levels and Morise score provided an AUC of 0.697 (p = 0.002) while the AUC for miR-206 and Morise score was 0.705 (p = 0.002). A binary logistic regression for Framingham score and CHD showed statistical significance in our study population (p = 0.019). ROC curve analysis showed an AUC of 0.661 (p = 0.013). Multivariate logistic regression including Framingham risk score and differential expressed miRNAs showed that the combination of miR-765 and Framingham score was a significant predictor of CHD (p = 0.024 and p = 0.063, respectively). Furthermore, high circulating levels of miR-31-5p (p = 0.026) and miR-206 (p = 0.038) with Framingham score (p = 0.031 and p = 0.039, respectively) were significant predictors of CHD presence. ROC curve analysis revealed a good performance to predict CHD for the 3 miRNAs in combination with Framingham clinical risk score. Circulating miR-765 expression and Framingham score showed an AUC of 0.717 (p = 0.001), miR-31-5p expression levels and Framingham score provided an AUC of 0.717 (p = 0.001) while the AUC for miR-206 and Framingham score was 0.714 (p = 0.001).

To better discriminate clinically relevant obstructive CHD, we grouped the 29 HS with the no obstructive CHD patients, as previously reported.[17] Molecular analysis showed that higher levels of let-7c-5p (p = 0.040), miR-765 (p = 0.013), and miR-483-5p (p = 0.038) were found also in subjects with obstructive CHD (n = 36) as compared with patients with no CHD or CHD <50% (n = 59) detected by CCT (Figure 2). In addition, also miR-93-5p and miR-433-3p showed significant high expression levels in patients with critical coronary stenosis versus patients with no CHD or CHD <50% (p = 0.017 for both) (Figure 2). The univariate regression analysis showed that high plasmatic expression of miR-765, miR-483-5p, miR-93-5p, and miR-433-3p were independently associated with obstructive CHD (Table 3). The multivariate regression models, adjusted for cardiovascular risk factors, baseline features, and clinical characteristics, showed that high circulating levels of miR-765, miR-93-5p, and miR-433-3p were predictors of obstructive CHD independently by male gender (Table 3). Furthermore, ROC curve analysis of the multivariate models revealed a good performance on predicting CHD severity. In detail, circulating miR-765 expression showed an AUC of 0.762 (p <0.001) (Figure 3), miR-93-5p expression levels provided an AUC of 0.770 (p <0.001) (Figure 3) while the AUC for was miR-433-3p was 0.771 (p <0.001) (Figure 3). Morise pretest probability was not statistically significant in obstructive CHD patients (p = 0.076) as well as binary logistic regression analysis for Morise score and obstructive CHD showed a border significance (p = 0.051) in our study population. Multivariate logistic regression combining Morise score and differential expressed miRNAs in obstructive CHD patients showed that high circulating levels of miR-765 (p = 0.012), miR-433-3p (p = 0.040), and miR-93-5p (p = 0.034) with Morise score (p = 0.030, p = 0.042, and p = 0.063, respectively) were able to significantly predict CHD severity. Furthermore, ROC curve analysis revealed a good performance to discriminate obstructive CHD for all the 3 miRNAs. In particular, circulating miR-765 expression and Morise score showed an AUC of 0.694 (p = 0.002), miR-93-5p expression levels and Morise score provided an AUC of 0.691 (p = 0.002) while the AUC for miR-433-3p in combination with Morise score was 0.679 (p = 0.003). Logistic regression analysis for Framingham risk score and obstructive CHD showed no significance (p = 0.196) in our study population.

## Discussion

The present study established that high levels of circulating miRNAs were able to discriminate patients with CHD (let-7c-5p, miR-765, miR-483-5p, miR-31-5p, and miR-206) and, in particular, with obstructive CHD (miR-765, miR-93-5p, and

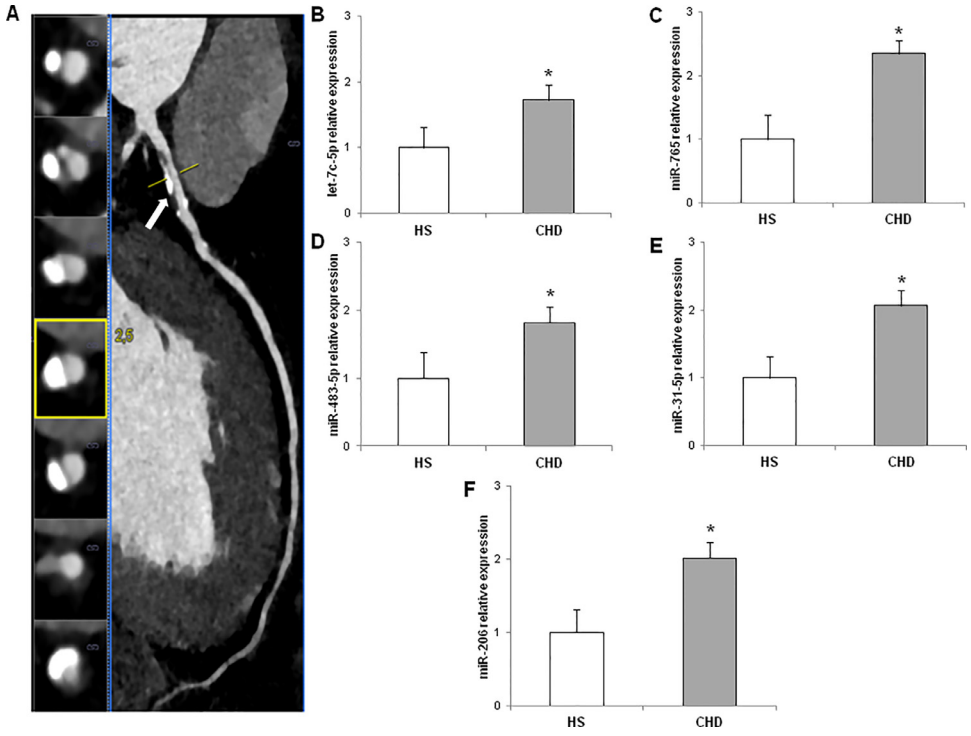


Figure 1. Circulating microRNA expression detected by qRT-PCR in CHD patients and HS undergoing CCT. (*A*) Curved multiplanar reconstruction of a left anterior descending artery in a patient affected by CHD. The white arrow indicates a stenosis <50% caused by a mixed plaque (both calcified and noncalcified). The left part of the panel represents coronary cross-sectional views of stenosis. (*B*) Let-7c-5p relative expression in plasma of CHD patients as compared to HS. (*C*) MiR-765 relative expression in plasma of CHD patients as compared to HS. (*D*) MiR-483-5p relative expression in plasma of CHD patients as compared to HS. (*E*) MiR-31-5p relative expression in plasma of CHD patients as compared to HS. (*F*) MiR-206 relative expression in plasma of CHD patients as compared to HS. MiRNA levels were normalized to the spiked-in cel-miR-39-3p. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The values are expressed as the mean ± SEM. *p <0.05. CCT = cardiac computed tomography; CHD = coronary heart disease; HS = healthy subjects; miRNA = microRNA.

Table 2
Univariate and multivariate logistic regression* analysis to predict coronary heart disease

| Variable | CHD (n = 66) | |
|---|---|---|
| | OR (95% CI) | p Value |
| Univariate analysis | | |
| let-7c | 0.790 (0.617−1.011) | 0.061 |
| miR-765 | 0.689 (0.526−0.902) | **0.007** |
| miR-483-5p | 0.801 (0.637−1.006) | 0.056 |
| miR-31-5p | 0.720 (0.555−0.935) | **0.014** |
| miR-206 | 0.723 (0.556−0.940) | **0.015** |
| Multivariate analysis | | |
| Male | 4.639 (1.702−12.650) | 0.003 |
| miR-765 | 0.728 (0.547−0.970) | 0.030 |
| Male | 5.021 (1.817−13.875) | 0.002 |
| miR-31-5p | 0.732 (0.556−0.963) | 0.026 |
| Male | 5.104 (1.850−14.085) | 0.002 |
| miR-206 | 0.716 (0.540−0.950) | 0.021 |

For logistic regression analysis Δct values of each miRNA were considered.

Bold values were considered statistically significant with a $p < 0.05$.

CHD = coronary heart disease; CI = confidence interval; OR = odds ratio.

* Multivariate analysis corrected for male gender.

miR-433-3p). Furthermore, pretest probability risk scores (Framingham and Morise scores) widely used for patient stratification were not able to detect CHD and discriminate obstructive CHD while the addition of circulating miRNA quantification provided significant results. Our findings suggest that these molecular markers may be useful for not only the prediction but also the improvement of the diagnostic accuracy. On the other hand the new generations of CT scanners with larger detector arrays, more detector rows, and dual-source systems offer high-quality CCT images with less noise and artifacts with lower radiation doses compared with earlier technologies. These observation give rise to the need for more precise and less invasive diagnostic tools in order to promote the so-called precision medicine.

Both in vitro and in vivo studies have recognized the impact of miR-31-5p as modulator of atherosclerotic process enhancement regulating endothelial progenitor cell activities and macrophage proliferation.[20] Here, we reported that miR-31-5p was upregulated in CHD detected by CCT, suggesting its involvement in development of coronary atherosclerotic disease. The exact mechanisms for the altered expression of miR-765 in CVDs are still unknown. A study by Ali et al reported that circulating levels of miR-765 were upregulated in geriatric CHD patients detected by invasive coronary angiography.[21] Our results confirmed the previous findings[21]; in addition CHD diagnosis was performed with less invasive CCT suggesting that miR-765 could be used as potential noninvasive biomarker for clinical diagnosis of CHD and obstructive CHD with a good discriminatory power. MiR-206 was identified a muscle specific miRNA but studies indicated also an important role in CVDs such heart failure with an increase of its

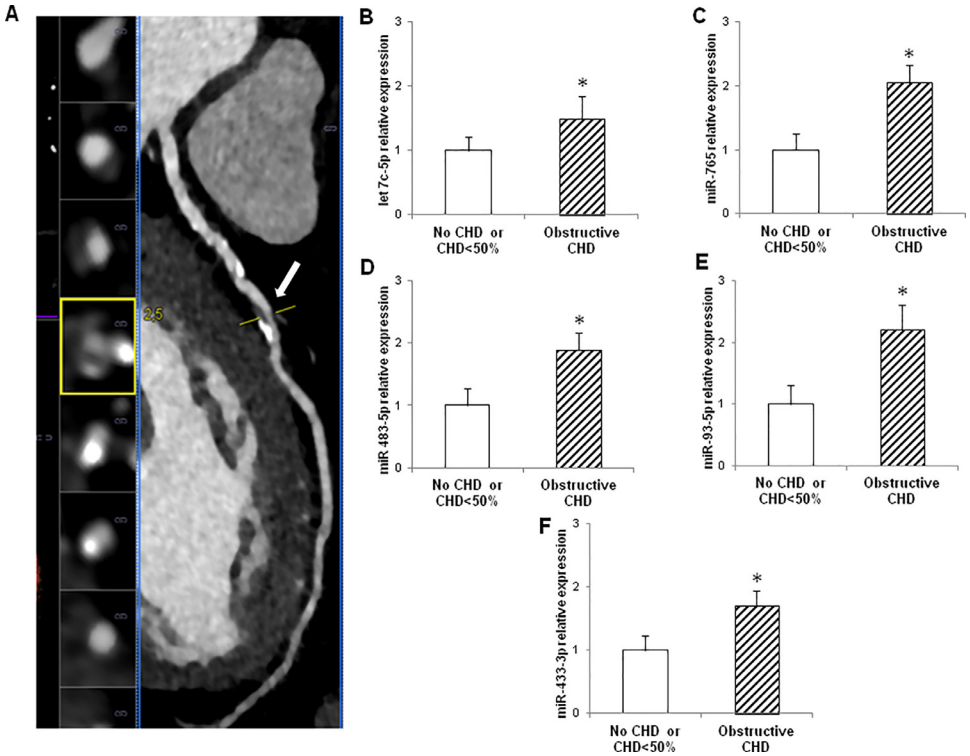


Figure 2. Circulating microRNA expression detected by qRT-PCR in obstructive and no obstructive CHD patients undergoing CCT. (*A*) Curved multiplanar reconstruction of a left anterior descending artery in a patient affected by obstructive CHD. The white arrow indicates a stenosis >50% caused by a mixed plaque (both calcified and noncalcified). The left part of the panel represents coronary cross-sectional views of stenosis. (*B*) Let-7c-5p relative expression in plasma of obstructive CHD patients as compared to no CHD or CHD <50% patients. (*C*) MiR-765 relative expression in plasma o of obstructive CHD patients as compared to no CHD or CHD<50% patients. (*D*) MiR-483-5p relative expression in plasma of obstructive CHD patients as compared to no CHD or CHD<50% patients. (*E*) MiR-93-5p relative expression in plasma of obstructive CHD patients as compared to no CHD or CHD <50% patients. (*F*) MiR-433-3p relative expression in plasma of obstructive CHD patients as compared to no CHD or CHD <50% patients. MiRNA levels were normalized to the spiked-in cel-miR-39-3p. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The values are expressed as the mean ± SEM. *p < 0.05. CCT = cardiac computed tomography; CHD = coronary heart disease; miRNA = microRNA.

Table 3
Univariate and multivariate logistic regression* analysis to predict obstructive coronary heart disease

| Variables | Obstructive CHD (n = 36) | |
|---|---|---|
| | OR (95% CI) | p Value |
| Univariate analysis | | |
| let-7c | 0.823 (0.643−1.054) | 0.123 |
| miR-765 | 0.725 (0.553−0.950) | **0.020** |
| miR-483-5p | 0.758 (0.589−0.975) | **0.031** |
| miR-93-5p | 0.810 (0.671−0.978) | **0.028** |
| miR-433-3p | 0.764 (0.585−0.998) | **0.049** |
| Multivariate analysis | | |
| Male | 9.000 (2.431−33.307) | 0.001 |
| miR-765 | 0.739 (0.558−0.980) | 0.035 |
| Male | 9.246 (2.506−34.118) | 0.001 |
| miR-93-5p | 0.812 (0.664−0.991) | 0.041 |
| Male | 9.432 (2.527−35.212) | 0.001 |
| miR-433-3p | 0.752 (0.555−1.021) | 0.043 |

For logistic regression analysis Δct values of each miRNA were considered.

Bold values were considered statistically significant with a p <0.05.

CHD = coronary heart disease; CI = confidence interval; OR = odds ratio.

* Multivariate analysis corrected for male gender.

expression levels in damaged tissues.[22] Our results are in line with Zhou et al which reported an upregulation of miR-206 in the plasma of CHD patients, diagnosed by invasive coronary angiography, versus control subjects.[23]

A previous study provided evidence of miR-93-5p involvement in coronary atherosclerosis. In detail, He et al demonstrated an overexpression of these miRNA in serum of patients suffering CHD. Authors also showed the proatherogenetic role of miR-93-5p for its capability to target 3′UTR of ATP-binding cassette transporter 1 (ABCA1) gene, the major responsible of cellular cholesterol efflux.[24] Furthermore, Sullivan et al[25] reported that miR-93-5p levels were significantly increased in stable CHD versus control group. Our data confirmed these findings for CHD[25]; in addition, we reported an high diagnostic power of miR-93-5p for obstructive CHD detected by CCT.

MiR-483-5p was found to be involved in angiogenesis regulation, a clue step in atherosclerosis and CHD. Indeed, a recent study by Li et al, investigated a set of miRNAs with the aim to find possible biomarkers for the early diagnosis of coronary plaque rupture in CHD patients who underwent PCI. MiR-483-5p were upregulated after plaque rupture until 1 hour of cardiac catheterization showing the highest discriminatory power in the early diagnosis of plaque rupture

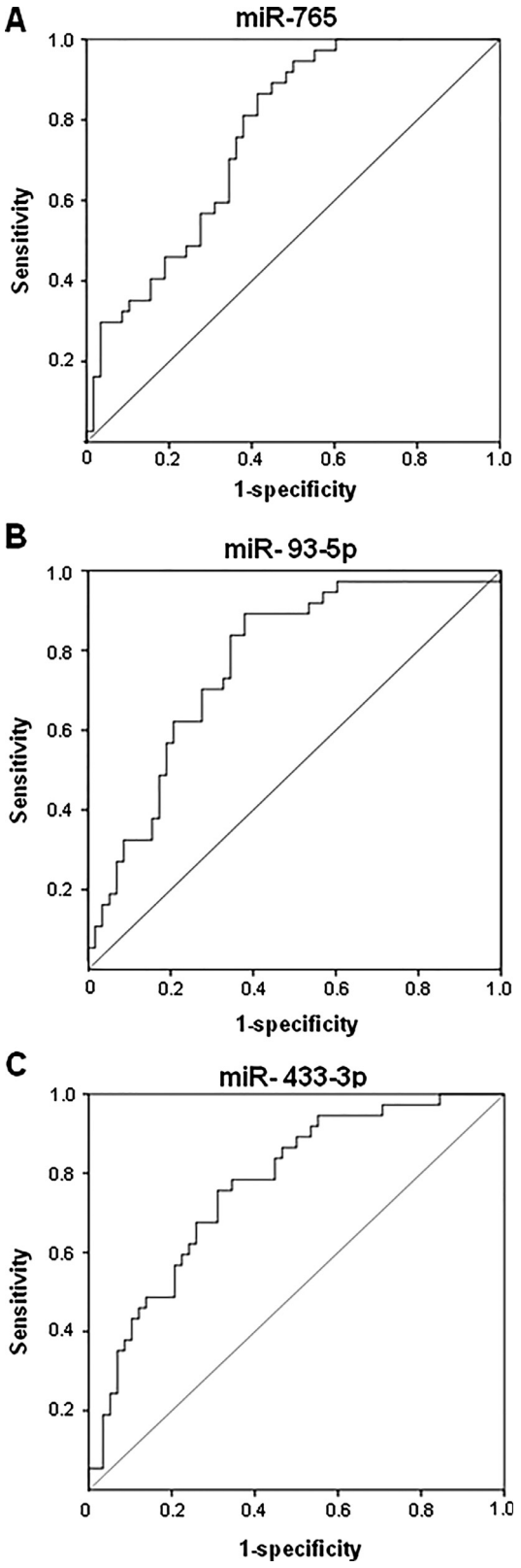


Figure 3. ROC curves analysis generated from the multivariate model for the presence of obstructive CHD. (*A*) The area under the curve (AUC) is 0.762 (p <0.001) for miR-765. (*B*) AUC is equal to 0.770 (p <0.001) for miR-93-5p. (*C*) For miR-433-3p AUC is 0.771 (p <0.001). CHD = coronary heart disease; ROC = receiver operating characteristic.

for 0.5 hour.[26] Our data confirmed the involvement of miR-483-5p in CHD, however for the first time we reported a differential expression of miR-483-5p in patients with obstructive disease and therefore with an increased risk of acute event recurrence. MiR-433-3p was also overexpressed in stable angina (SA) patients and unstable angina (UA) patients as compared with controls, but miRNA expression levels were not able to discriminate SA from UA patients.[27] Otherwise our results provided a strong evidence for miR-433 up as a biomarker of obstructive CHD with a significant discriminatory power in the study population. Interestingly, very recent studies extended the concept that other epigenetic sensitive biomarkers represented by certain methylated genes could be related to human atherosclerosis and CHD as well as other cardiovascular diseases.[28–30]

The present study has some limitations. A genome-wide approach is a better strategy to identify more circulating miRNAs associated to CHD presence and severity. Our analysis was performed considering only a small number of miRNAs already recognized to be related to atherogenesis. This study was performed in a small cohort of subjects and therefore larger cohort studies are needed to investigate potential confounders, verify our findings, as well as the sensitivity and specificity of these miRNAs and to confirm association with clinical outcomes. Furthermore, we used a limited amount of plasma samples. Indeed, due to ethical reasons, usually in clinical studies there are small amounts of starting material. A further valuable step could investigate the association of such biomarkers with myocardial ischemia detected by CCT to assess the relationship between hemodynamically significant stenosis and circulating biomarkers in CHD patients.

The costs to perform miRNA analysis have also to be taken into account, although the high sensitivity, specificity, and reproducibility of qRT-PCR. If the biomarkers quantification for the evaluation of CHD will be confirmed, this could change dramatically the screening of a high number of subjects. The identification of novel noninvasive biomarkers will be useful to stratify subjects and improve the diagnostic approach. The integration of imaging and molecular data could introduce epigenetics as noninvasive marker for diagnosis and prognosis of coronary atherosclerotic lesion progression and staging. Multiple biomarkers could be developed and combined with other clinical, serum and imaging variables in an integrated approach with the aim to generate predictive "multimarker CHD scores." In addition, noninvasive predictive evaluation of patients at risk for CHD may guide interventions designed specifically to reduce the individual vascular risk, thus avoiding additional invasive procedures and providing reduction of health expenditure. Thus, this novel integrated epigenetic/imaging approach could have an important clinical impact; in fact, the detection of circulating plasma miRNAs may represent a potential approach for rapid and noninvasive diagnostic/prognostic screening in patients with suspected CHD.

## Conflict of interest

The authors have no conflicts of interest to declare.

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## Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.amjcard.2019.04.016.

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