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Circulating miR-126 and miR-499 reflect progression of cardiovascular disease; correlations with uric acid and ejection fraction

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ABSTRACT

Background: The aim of this study was to assess plasma levels of endothelium- and heart-associated microRNAs (miRNAs) miR-126 and miR-499, respectively, using quantitative reverse transcriptase polymerase chain reaction.
Methods: A two-step analysis was conducted on 75 patients undergoing off-pomp coronary artery bypass graft (CABG) surgery. Five biomarkers of inflammation and cardiac injury were assessed in addition to the above-mentioned miRNAs.
Results: Plasma concentrations of miRNAs were found to be significantly correlated with plasma levels of cardiac troponin I (cTnI) (miR-499, r 0.49, p~0.002; miR-126, r = 0.30, p~0.001), indicating cardiac damage. Data analysis revealed that miR-499 had higher sensitivity and specificity for cardiac injury than miR-126, which reflects more endothelial activation. Interestingly, a strong correlation was observed between both miRNAs and uric acid (UA) levels with ventricular contractility measured as ejection fraction (EF) (miR-499/EF%, r = 0.58, p~0.004; UA/EF%, r = -0.6, p~0.006; UA/miR-499, r = -0.34; UA/miR-126, r = 0.5, p~0.01).
Conclusions: In patients undergoing CABG, circulating miR-126/499 is associated with presentation of traditional risk factors and reflects post-operative response to injury. Plasma pool of miRNAs likely reflects extracellular miRNAs which are proportional to intracellular miRNA levels. Therefore, circulating levels of these miRNAs have prognostic implications in detection of higher risk of future cardiovascular events.

Keywords: CABG, Cardiac damage, miR-126, miR-499, Off-pomp, Uric acid

Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs that regulate the gene expressions involved in various cellular processes (1-3).

Vascular endothelium-enriched miR-126 has been associated with coronary artery disease (CAD), atherosclerosis and also incidence of thrombosis. Interestingly, the level of miR-126 has been observed to be increased in patients with cardiac ischemia and hypertension (1, 4-6). Furthermore, increased amounts of miR-126 are released from the platelet/endothelium to the plasma compartment during platelet activation and thrombosis and also in atherosclerosis-related complications, which is directly correlated with endothelial injury and the inflammatory response of endothelium (2, 5, 7). In the inflammatory response of endothelium and via transcriptional regulation of vascular cell adhesion molecule-1 (VCAM-1), over-expression of miR-126 and activation of interferon regulatory transcription factor 1 (IRF-1) is observed (8). Silencing of IRF-1 or miR-126 expression recapitulates the antiatherogenic regulation of VCAM-1. Plasma concentration of miR-126 has been reported to be also correlated with brain natriuretic peptide and the degree of heart failure (9). Regardless of treatment, miR-126 expression has been significantly up-regulated in hypoxia/inflammatory conditions (2, 10).

Differentially expressed miRNAs (and in particular miR-126 and -499) reflect several aspects of vulnerable CAD, such as

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inflammation, hypoxia, apoptosis, and extracellular matrix (ECM) degradation. Data indicate that the up-regulated miRNAs in the circulation of vulnerable CAD patients are derived from systemically activated/apoptotic cardiac/endothelial cells (ECs) and stimulated platelets. Platelets or activated cells produce microparticles (MPs), which are released into the extracellular space. These miRNA-containing MPs actually play roles in cardiovascular diseases by sharing their contents with plasma and transferring their miRNAs to target cells (2, 5, 6, 11-13).

Data demonstrate that circulating miR-499 is a novel, early biomarker for identifying perioperative myocardial infarction in cardiac surgery (12). For example, increased levels of cardio-enriched miR-499 reflect myocardial damage, and are associated with long-term poor prognosis due to reduced systolic function and risk of death or heart failure (9, 11, 14). Also, hypertensive individuals prone to future heart failure produce increased levels of cardiovascular-associated miRNAs (9, 11).

On the other hand, uric acid (UA) has been identified as a major risk factor associated with CAD. Lowering serum UA level improves endothelial function (15). Accordingly, chronic elevation in circulating UA will affect multiple organs either directly or via impairment of endothelial function. UA in vascular system may hamper regeneration of endothelial cells and impair nitric oxide (NO) production (15-19).

Furthermore, studies have demonstrated an inverse relationship between insulin-like growth factor 1 (IGF-1) and UA levels in adults, and suggest that UA might affect hepatic IGF-1 synthesis (18).

Higher levels of serum UA may be implicated in the development and progression of atherosclerotic cardiovascular disease. Accordingly, serum UA levels are positively associated with peripheral arterial disease (PAD), independent of smoking, body mass index, hypertension, diabetes, serum total cholesterol, serum creatinine, and other confounders (20).

There is also evidence that serum UA concentrations are closely associated with IL-6, C-reactive protein (CRP), and tumor necrosis factor-α (TNF-α). The evidence suggests the possibility that UA could contribute to systemic inflammatory conditions and play a crucial role in inflammatory responses (21). Clinical evidence demonstrates an inverse correlation between CRP levels and endothelial vascular inactivity in human subjects. This promotes myocardial infarction and cerebral infarct (22). CRP also decreases endothelial nitric oxide synthase (eNOS) activity in ECs, and inhibits endothelium-dependent NO-mediated vasodilation *in vitro* (22-25).

With the emerging role of circulating miRNAs in the diagnosis of CVD and their early appearance in circulation in comparison to classical biomarkers, the objective of the present study was to examine the release patterns of cardiovascular-specific miRNAs and to find whether miRNAs can provide early prediction of acute events after cardiac surgery.

A two-step study was conducted on 75 off-pomp coronary bypass graft (CABG) patients. In step I, we selected two cardiovascular-specific miRNAs as candidate markers, tested their release patterns and relevance to myocardial injury, and compared miRNAs' release between two time points before and after off-pump CABG surgery. In step II, some plasma biomarkers were assessed and their correlations with miRNAs and cardiac function were analyzed.

Methods

Ethics statement

The study has been approved by the Ethical Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and we adhered to the tenets of the Declaration of Helsinki. In addition, all patients provided informed written consent prior to cardiac surgery (26, 27).

Participants and study design

The study population included adult patients referred for CABG surgery to the Afshar Hospital affiliated with Shahid Sadoughi University of Medical Sciences in Yazd, Iran. Emergencies, reoperations, abnormal preoperative serum cardiac troponin I (cTnI) and combined procedures were excluded. In step I, 75 consecutive patients scheduled for off-pump CABG were enrolled. Circulating levels of miR-126 and miR-499 were detected at two time points (on day 0 before revascularization surgery, and on day 4 post-surgery) in plasma samples. We studied their release patterns and their association with myocardial damage. In step II, some plasma inflammatory biomarkers (CRP, UA), heart necrosis indicators (creatine kinase-MB, cTnI) and cardiac contractility (as ejection fraction [EF]) were also measured and compared with the miR-499 and miR-126 levels between the two time points (28, 29).

RNA extraction

Total RNA was extracted from 100 µL of plasma, using the mirVana PARIS kit (Ambion, Warrington, UK) according to the manufacturer's instructions and without enrichment for small RNAs. It was then eluted in 50 µL of nuclease-free water. Subsequently, potential genomic DNA contamination was eliminated using DNA-free kit (Ambion) (9, 11).

cDNA synthesis and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA (15 µL) was used per 20-µL reaction to generate cDNA using the miScript kit (Qiagen, Venlo, The Netherlands), which is designed to specifically detect mature microRNAs. The 20-µL reaction mix was then diluted × 4 in nuclease-free water, and 2 µL of cDNA was added per quantitative RT–PCR, using BR SYBR-green supermix for IQ (Quanta Biosciences, Amsterdam, The Netherlands) in a MyIQ iCycler (Bio-Rad, Veenendal, The Netherlands) device (9, 11).

All reactions were run in triplicate, and microRNA expressions were normalized to small RNA U6, with the similar efficiency of microRNAs. Data of quantitative RT-PCR were demonstrated by nominal CT value (normalized to U6), and fold changes were calculated by $2^{\Delta\Delta CT}$. Higher nominal CT value here means lower microRNA expression level (9, 11).

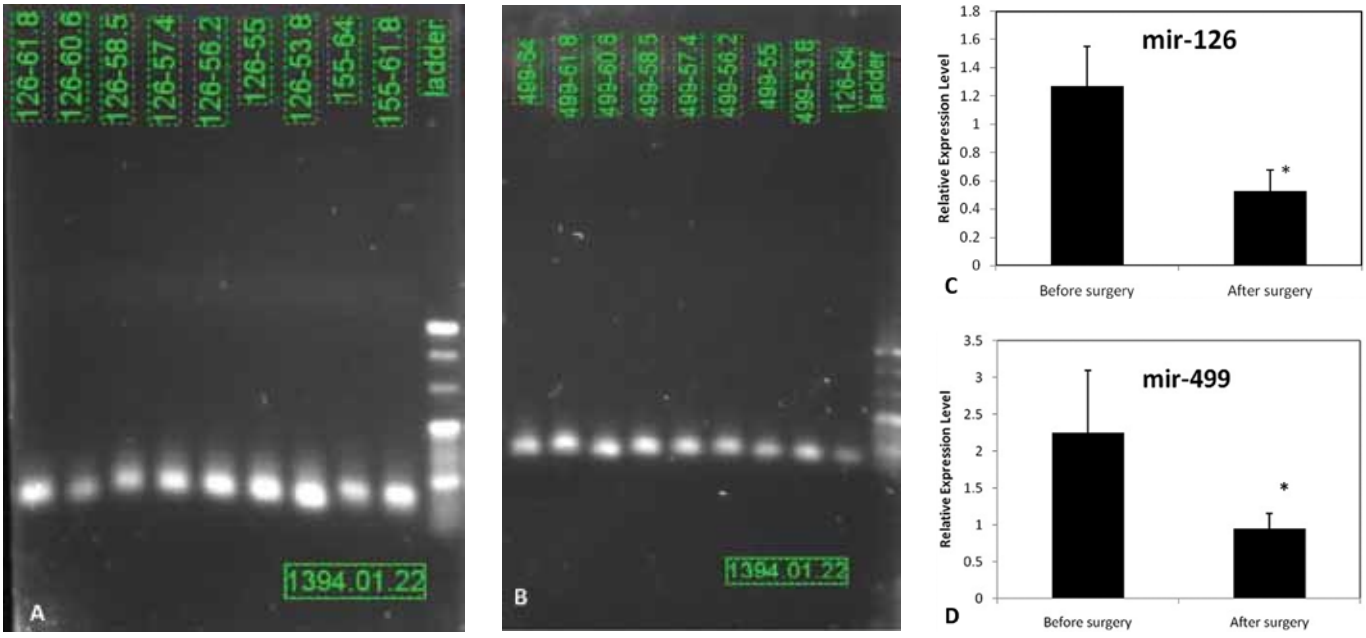

Fig. 1 - The relative expression level of miR-126 (**A**), and miR-499 (**B**), was highly reduced (**C, D**) after revascularization surgery in patients with coronary ischemia undergoing off-pomp surgery. MicroRNA levels were transformed into quantities using the formula $2^{-\Delta Ct}$. Error bars represent standard deviation of the mean (SD). *p<0.01 for significant difference between pre- and post-surgery mean values.

Myocardial biochemical markers determination

At the time points day 0 and day 4 pre-and post-operation, blood samples were taken and centrifuged for serum separation before measurement of the levels of cTnI and creatine kinase- MB (CK-MB) using a high-sensitivity cardiac enzyme-linked immunosorbent assay kit (USCN Life Science, Wuhan, CHN) (28-30).

Statistical analysis

All values are presented as mean ± standard deviation. Results of cardiac enzymes, hemodynamic parameter changes, and quantitative RT-PCR results between time intervals were analyzed by paired t-test. Spearman correlation coefficients were used to examine the relationship between miR-499 and cardiac enzymes levels. A p value of <0.05 was considered to be indicative of a statistically significant difference (30).

Results

Cardiac ischemia elevated the expression levels of miR-126 and miR-499

We assessed the relative expression of well characterized miR-126 and miR-499, from their concentrations in plasma samples of the study population using an established reliable assessment method (9). MiR-126 and miR-499 are almost specifically expressed in the ECs and the ventricle of heart, respectively. Thus, it is likely that we can identify the pathophysiological significance of up- or down-regulation of these miRNAs in plasma. Plasma concentrations of miR-126 and miR-499 were significantly different between two time points: at day 0 of pre-revascularization surgery, and at day 4 post-revascularization surgery. They were highly increased in cardiac ischemia (miR-126~1.24 ± 0.27 and miR-499~2.25 ± 0.90), which were reduced to 0.54 ± 0.15; p<0.001, and to 0.99 ± 0.20, p<0.005, respectively, with fold change expression~0.4 (Fig. 1A-D).

Plasma levels of miR-126 highly associated with those of miR-499

We evaluated the correlation between expressions of two miRNAs at the two above-mentioned time points. Plasma concentrations of miR-126 were highly and positively correlated with miR-499 before and after off-pomp CABG (Fig. 2A; r = 0.62; at pre-revascularization, and r = 0.55; at post-revascularization surgery, p<0.02).

Even more, the mean values of fold change expression of both miRNAs post-revascularization surgery were significantly correlated with each other (Fig. 2A; r = 0.69; p<0.005).

MiRNA levels specifically correlated with myocardium injury (with cTnI)

After surgery, plasma concentrations of myocardium biomarker cTnI showed a mild positive correlation with miR-126 (r = 0.30, p<0.01), while depicting a more strong positive correlation with miR-499 (r = 0.507, p<0.005). However, before surgery in ischemia conditions, miRNAs exhibited a negative correlation with cardiac injury (Fig. 2B; miR-499/CK-MB, r~-0.46; miR-126/CK-BM r~-0.26, p<0.01). No significant

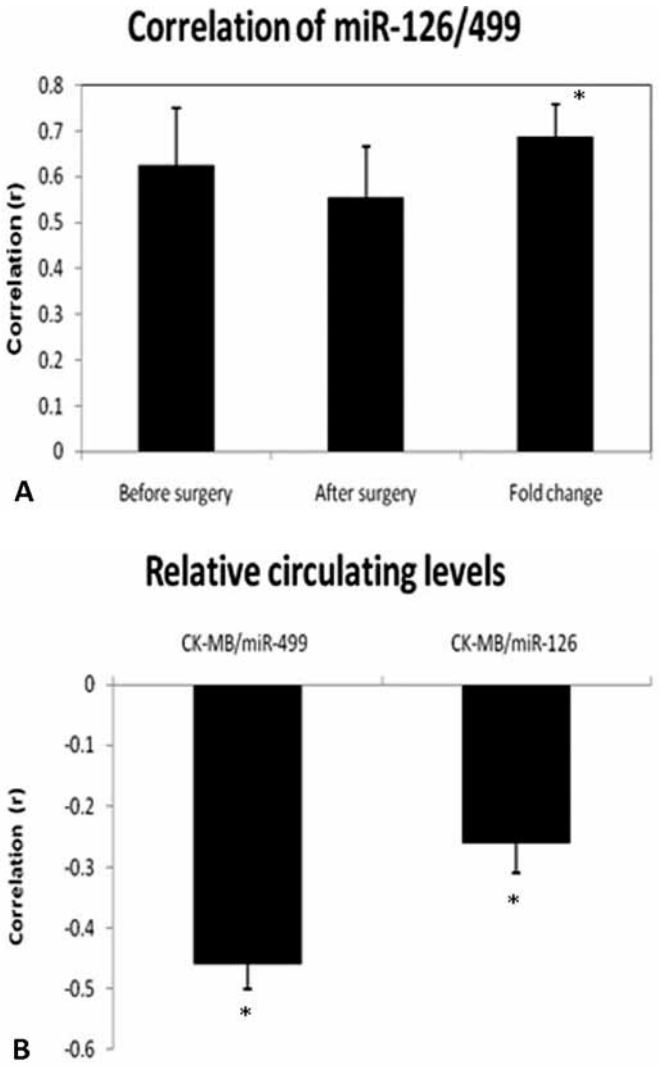

Fig. 2 - Relations between endothelial and myocardium-enriched miR-126 and -499 (**A**) and their associations with circulating levels of cardiac injury marker CK-MB (**B**). (**A**) Elevated levels of miR-499 were positively and highly associated with miR-126, whereas (**B**) both miRNAs negatively correlated to circulating levels of cardiac marker CK-MB, under ischemic conditions in patients undergoing off-pomp coronary artery bypass graft (CABG) surgery. Error bars represent standard deviation of the mean (SD). p<0.01, *p<0.001 for significant correlation between variables levels.

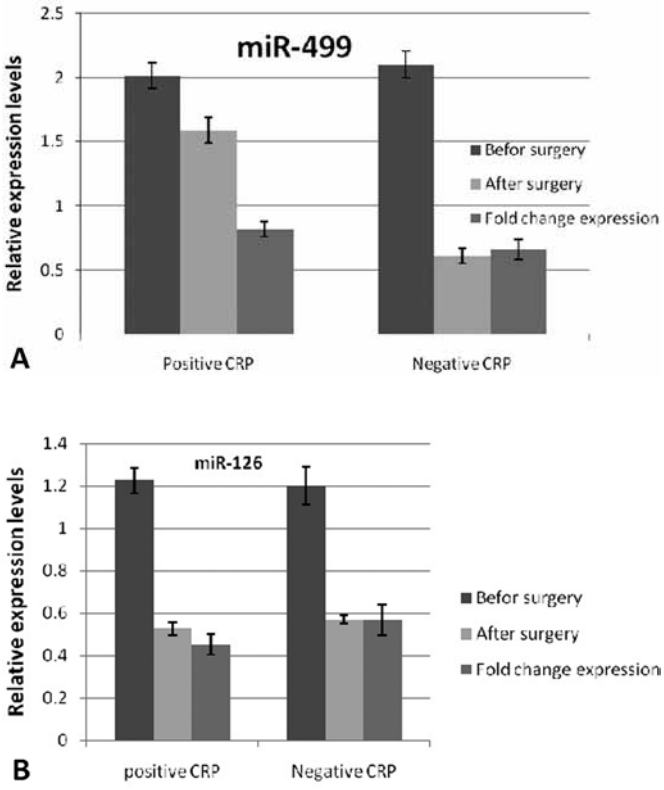

Fig. 3 - Expression profiles of miR-499 (**A**) and miR-126 (**B**) were differentially affected by C-reactive protein (CRP) in plasma of patients with coronary ischemia who were candidates for off-pomp surgery. Expression of miR-499 (**A**) versus miR-126 (**B**) was positively affected by plasma CRP in patients. Error bars represent standard deviation of the mean (SD). In general, p<0.05 for significant difference between the means, before and after revascularization surgery.

correlation was observed between the plasma concentrations of UA and cardiac markers CKs and cTnI.

Plasma CRP reduced the miR-126 expression of endothelium

We assessed and analyzed the plasma concentrations of miR-126 twice in those patients positive for CRP post-revascularization surgery. Expression of miR-126 was negatively correlated with the presence of CRP in plasma (Fig. 3A). Samples that were negative for CRP before surgery showed higher plasma concentrations of miR-126 after surgery. Figure 3A shows that miR-126 was more up-regulated in those subjects negative for CRP pre-operatively.

Expression of miR-499 was positively related to CRP

Expression of miR-499 was positively correlated with EF% in ischemia conditions before revascularization procedure. However, plasma concentrations of miR-499 were higher in those subjects positive for CRP before surgery (Fig. 3B). Thus, mild inflammatory conditions, as well as ischemia, positively influence plasma concentrations of miR-499 (Fig. 3A, B).

Expression level of miR-499 versus miR-126 negatively related to plasma levels of UA

There was a significant reduction in the plasma concentrations of UA 4 days after revascularization surgery. However, cardiac-enriched miR-499 negatively associated with UA circulating levels (r = -0.34, p<0.025) (Fig. 4), indicating that the higher UA causes the lower expression of miR-499, or vice versa. In contrast to miR-499, there was a significant positive association between endothelial-enriched mir-126 and UA (r = 0.48, p<0.01) (Fig. 4), indicating that the higher UA induces the higher expression of miR-126, or vice versa.

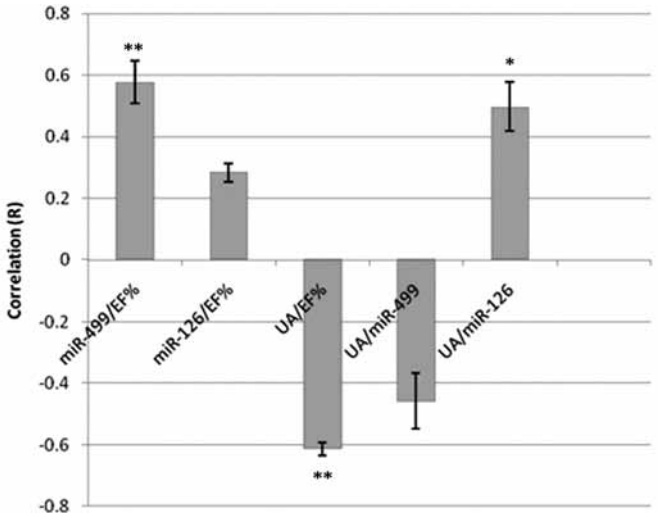

Fig. 4 - Expressions of miR-499 and -126 positively affected ventricular contractility, whereas UA levels showed strong negative association with ventricular function, in patients with ischemia undergoing off-pomp surgery. The heart ejection fraction (EF)% was strongly related to the expression levels of heart-enriched miR-499, with a poor correlation with endothelial-enriched miR-126. There was a strongly negative association between UA and the EF%, in patients undergoing off-pomp surgery. The endothelial-enriched miR-126 showed increased expression in increasing levels of UA. Expression levels of miRNAs had a positive effect on ventricle contractility, whereas UA circulating levels negatively affected ventricle function, in patients with ischemia who were candidates for off-pomp surgery. Error bars represent standard deviation of the mean (SD). $p<0.05$, *$p<0.01$, **$p<0.005$ for significant relation between variables.

Ventricular contractility is positively correlated with miR-499

In spite of a significant reduction in plasma levels of miR-499, it exhibited a strong correlation with ventricular EF (miR-499/EF%, r~0.58; miR-126/EF%, r~0.28, $p<0.01$), indicating that the higher miR-499, the higher heart contractility (Fig. 4). However, there was a highly negative correlation between plasma concentrations of UA and the EF (r~-0.6, $p<0.01$), indicating that the higher UA concentration, the lower EF% (Fig. 4).

Moreover, the plasma concentrations of UA showed a strong positive correlation with miR-126 (r~0.49, $p<0.01$).

Discussion

Tissue specificity, rapid-release dynamics and stability in blood make miRNAs promising candidates for diagnostic and prognostic utility in a wide range of disease states. Some miRNA species expressed at particularly high levels in cardiovascular system, such as miR-126 and miR-499, have been thought as candidate biomarkers in CAD (9, 11, 14). Furthermore, plasma levels of miRNAs have been observed to be markedly robust against clinical confounders including age, sex, and renal function (11, 15).

Cardio-enriched miR-499 has been reported to be higher in patients with myocardial ischemia and correlated with left ventricular EF. Increased levels of cardio-enriched miRNAs in the blood of ischemia patients have been reported to be associated with reduced systolic function, risk of death or heart failure after an acute coronary syndrome event. Circulating levels of miR-499 specifically reflect myocardial damage in cardiovascular diseases (11, 14). In particular, in patients with acute heart failure, only miR-499 was significantly elevated (two-fold), whereas no significant changes in other cardiac-enriched microRNAs have been observed in diastolic dysfunction (11).

Accordingly, under the conditions of hypoxia–ischemia, high levels of circulating miR-499 induce anti-angiogenic effects on human ECs, through specific signaling pathways including those for calcineurin and Wnt signaling, both of which reported to be involved in angiogenesis. For example, calcineurin catalytic subunit α isoform (CnAα), one of the target molecules of miR-499, is related to the progression of angiogenesis via activation of the transcriptional factor referred to as nuclear factor of activated T cells (NFAT). It was also reported that this calcineurin/NFAT pathway regulates the activation of hypoxia-inducible factor 1-α (HIF1-α), resulting in the secretion of vascular endothelial growth factor (VEGF) from cells (31). On the other side, up-regulation of miR-499 in rat BM-MSCs increases expression of cardiac-specific genes, such as NKx2.5, GATA4, MEF2C, and cTnI, and decreases ratio of phosphorylated/dephosphorylated β-catenin in the Wnt/β-catenin signaling pathway, thus activating the pathway. Activation of the Wnt/β-catenin signal pathway induces cardiac differentiation. MiR-499 also regulates myosin gene expression in human heart (32).

Moreover, down-regulation of the SK3 channel by miR-499 reveals its potential role in atrial fibrillation (AF). Atrial miR-499 is over-expressed in AF, leading to SK3 (the gene that encodes the small-conductance calcium-activated potassium channel 3) down-regulation and contributing to the electrical remodeling during AF (33). Also, high levels of miR-499 in NSTEMI patients and in those with congestive heart failure (CHF) have been reported to cause significantly higher rates of death within a year, as expected (34).

On the other hand, under pathological conditions, decreased levels of miR-499 are associated with cardiovascular remodeling through increase in levels of NMDAR1 and DNMT1. The expression of NMDAR1, DNMT1, and matrix metalloproteinase 9 is increased in parallel with increase in H3K9 acetylation, while HDAC1, miR-133a, and miR-499 are decreased in cardiomyocytes. A decrease in HDAC1 and an increase in H3K9 acetylation and DNA methylation are suggestive of chromatin remodeling. The levels of DNA methyltransferases (DNMTs) in cardiomyocytes, are supposed to be regulated by cardiac-enriched miR-499 and -133a (35).

Data also reveal that miR-126 expression levels are closely related to hypertension in humans, as they indicate a distinct expression profile associated with clinical prognostic indices of hypertensive target-organ damage (4).

Myocardial injury during ischemia/reperfusion results in release of intracellular components into the blood. Recent reports suggest that plasma levels of heart-expressed miRNAs respond to cardiac injury similar to cardiac enzymes, and several groups have reported circulating miRNA levels are increased in patients with acute myocardial infarctions.

In the present study, real-time PCR analysis revealed that the levels of miR499 and -126 decrease in the plasma of CABG patients, and especially correlate significantly with levels of cTnI (11, 12). Two possible reasons exist. Plasma miRNA profile likely reflects extracellular miRNAs and may not fully reflect intracellular miRNA levels. Further, circulating miRNAs are delivered to cells in the heart or blood vessels through microparticles (MPs) or apoptotic bodies to regulate distant genes (1-3, 5, 13, 36).

In fact, increased circulating miRNAs related to cardiovascular system probably associate with presentation of traditional cardiovascular risk factors and reflect subsequent declining vasomotion in CAD subjects. Therefore, circulating miRNA levels could be potentially implicated in the modulation of post-coronary ischemia reparative response to injury, with prognostic implications. Accordingly, CAD patients with a higher endothelial precursor cell level had a higher risk of future cardiovascular events including major adverse cardiovascular accidents (2, 4-6, 13).

Evidence favoring the role of miRNAs in the plasma of CAD patients shows that miRNA levels in MPs are shared by the plasma and are isolated from the plasma of patients. These findings indicate that MPs miRNAs regulate several key signaling pathways in vulnerable plaque pathogenesis, such as pathways involving transforming growth factor-b (TGF-b), toll-like receptor-4 (TLR-4) and hypoxia-inducible factor-1α (HIF-1 α) (6). MPs are released from various cell types, such as ECs, leucocytes, erythrocytes and platelets, but the majority of increased MPs in the plasma of CAD patients are reported to originate from ECs and platelets. Increased EC apoptosis during atherosclerosis highlights the importance of endothelial injury in atherogenesis (5, 13).

Induced perfusion of capillary network is rapidly impaired during ischemia as a consequence of EC swelling, impaired vaso-relaxation, increased leukocyte adhesion and microvascular destabilization initiated by the loss of EC-EC and EC-pericyte interactions (36, 37).

Evidence shows that miR-126 contributes to micro-vascular VCAM-1 protein expression in acute inflammation. VCAM-1 is an endothelial cell adhesion molecule controlling vascular inflammation. These data imply that miR-126 has a major role in the segmental, heterogenic response of micro-vascular endothelial cells to systemic inflammatory stimuli (7).

Also, in ischemic EC, stromal cell-derived factor 1 (SDF-1) is up-regulated in a HIF-1α-dependent fashion. SDF-1 or CXCL12 has been demonstrated to facilitate the homing of progenitor cells from the peripheral circulation to sites of vascular injury or tissue ischemia. SDF-1 is a direct target of miR-126 in the context of ischemia. Systemic silencing of miR-126 in organ ischemia is associated with elevated SDF-1 levels and mobilization of progenitor cells into the peripheral circulation, potentially in response to elevated SDF-1 expression by ECs present in the ischemic tissue (36, 37).

Accordingly, during the vascular disease and inflammatory processes in ischemia/reperfusion injury, miR-126 is originated from activated endothelial/platelets and is taken up by other cells including kidney vascular cells (1-3, 36). Indeed, systemic inhibition of platelet activation would predict a decrease in miR-126 and other platelet-derived miRNAs such as miR-16. Indeed, our results showed that the transfer from the cardiac- and endothelial/platelet to the plasma compartment of miR-499 and -126, which are abundantly present in cardiovascular system, were likewise inhibited after revascularization surgery. Consistent with our current findings, cardiovascular-related miRNAs have been reported to be down-regulated to the highest extent in treated CAD patients, and were the highest expressed vascular miRNAs, of which miR-126 and -499 were ranked top (2, 5, 6, 12, 30, 36). These data suggest that when *in vivo* cardiac/vascular disease progression is inhibited, as was the case in our off-pomp CABG study and the CAD previous studies (1, 2, 7, 9, 11, 14), the release of cardiovascular-derived miRNAs in general and miR-499 and -126 in particular, is inhibited accordingly (1, 2, 6, 9, 11, 12). In fact, the present and previous studies showed an actual positive influence of CAD treatment on circulating CAD-related miRNAs.

As in our study, miRNA levels (miR-126 and -499) specifically reduced after revascularization surgery, whilst showed a mild positive correlation with myocardium injury marker cTnI. However, no correlation was observed between the plasma concentrations of UA and cardiac marker cTnI.

There is a relationship between plasma miR-126 level and severity of narrowing in coronary arteries (3). It has been found that elevated levels of miR-126 cause an increase in nitrogen metabolites, resulting in a severe impairment of organ function after ischemia (36). Higher levels of UA, a purine metabolite, are found in patients with vascular disease and in those with major cardiovascular risk factors, such as hypertension, diabetes mellitus, obesity, insulin resistance, and renal dysfunction. UA levels are also associated with generation of free radicals, impaired endothelial function, increased platelet adhesiveness, and higher levels of inflammatory markers (17, 20). In our study also, reduced plasma levels of miR-126 versus miR-499 showed positive correlation with plasma levels of UA, indicating that the higher UA causes the higher expression of endothelial-enriched miR-126, or vice versa. In contrast to miR-126, there was a considerable negative association between cardiac-specific mir-499 and UA, indicating that the higher UA induces the lower expression of miR-499, or vice versa.

Data have indicated the levels of UA as a direct measure of atherosclerotic burden, which is associated with activated neutrophils, xanthine oxidase of endothelial cells, and damaged heart mitochondria. In addition, UA levels are strongly and independently associated with elevations of inflammatory markers, including the leukocyte and neutrophil count, C-reactive protein, and pro-inflammatory cytokines. Several of these have, in turn, been associated with a poorer outcome in patients undergoing CABG operation (20, 38-40).

Increased activation of the renin-angiotensin system and decreased bioavailability of NO are among the mechanisms responsible for higher levels of UA (41). Given the relationship between UA and endothelial function, we hypothesized that UA levels would impact the putative role of ECs and miRNA products on the outcome of CABG, and may have prognostic utility in this procedure. The current study tests this hypothesis.

In this study, we investigated heart contribution in the pool of miR-126 and miR-499 in plasma of patients undergoing revascularization surgery, and whether it is affected by

UA and CRP concentrations. Off-pomp CABG resulted in both reduced circulating levels of UA and concomitantly reduced circulating levels of endothelial/platelet-derived miR-126.

The present study confirmed that the expression levels of miR-126 would be decreased in the presence of CRP and in vascular injury. It has been reported that plasma concentrations of miR-126 were down-regulated in patients with CAD, in patients with type 2 diabetes and in heart failure (2, 4, 6, 9, 36, 42).

Herein, plasma levels of CRP negatively affect expression levels of endothelial-specific miR-126, while mild inflammatory conditions as well as ischemia, positively influence plasma concentrations of miR-126.

As circulating levels of CKs and cTnI are often assessed as biomarkers of heart injury and are both products of cardiomyocytes, thus endothelial (miR-126) and cardiac highly enriched (miR-499) are expected to reflect some aspects of cardiovascular injury in ischemia/hypoxia conditions. In this regard there are reports that atherosclerosis, diabetes, and CHF induce release of MPs or remnants of apoptotic cells, which would thereby increase the circulating levels of miR-126 and -499. Thereby, it is possible that reperfusion may induce metabolic activity, renewal of ECs and cellular integrity, which could lead to reduction in the release of miRNAs, including miR-126 and -499. On the other side, it is also likely that reperfusion and endothelial reactivation may activate uptake of circulating miRNAs and thereby reduce plasma concentrations of miRNAs.

As is evident in our results, patients undergoing off-pomp CABG showed more circulating levels of miR-126 and -499, which were reduced after revascularization. The higher levels of circulating miR-126 and -499 in plasma of patients before revascularization, may be ascribed to hypoxia/ischemia.

Also, in our patients with severe coronary ischemia, miR-499 expression levels showed a significant positive correlation ($r = 0.58$, $p<0.001$) with left ventricular EF%. Furthermore, relative expression levels of both miR-126 (from 1.24 ± 0.27 to 0.54 ± 0.15; $p<0.001$), and miR-499 (from 2.25 ± 0.90 to 0.99 ± 0.20, $p<0.005$) showed significant positive correlations ($r = 0.441$; $p<0.001$) before and after CABG. Our data reveal that miR-499 and miR-126 are closely related to plasma concentration of UA. Indeed, miR-126 levels show a distinct positive correlation with UA, in contrast to miR-499. Thus, they may be clinically useful as prognostic indices of ischemic target organ damage in CAD patients.

One explanation of these results is possible reduced level of miR-126 expression in low-grade inflammation. Increased UA reflects high rate of cell turnover, i.e., degradation of nuclear structures (purine bases) and increased xanthine oxidase activity and oxidative stress, which itself may be part of inflammatory processes or cerebrovascular stroke (43). Studies show that increased level of miR-126 is a negative regulator of VEGF-A, while decreased miR-126 level induces angiogenesis by activation of VEGF-A pathway (44).

Our results comply with those of previous studies showing an independent association between increased UA circulating levels and adverse cardiovascular outcome in high-risk coronary and hypertensive patients.

CRP appeared to be the strongest predictor for adverse cardiac outcome. To the best of our knowledge, the present study is the first to demonstrate that CRP has an impact on miR-126 and -499 expression levels, which may be used for prediction of major cardiac events occurring in patients with CAD. Serum CRP levels are associated with endothelial dysfunction, coronary artery calcification and cardiac diastolic dysfunction (23).

Also, our findings are analogous to the reported association between UA levels and other measures of subclinical atherosclerosis, including carotid arterial intima-media thickness and atherosclerotic renal artery stenosis (20).

It is now believed that UA is one of the important causal agents in endothelial dysfunction, which is characterized by reduced endothelial production of nitric oxide (NO) and expression of endothelial nitric oxide synthase (eNOS) (25). Accordingly, chronic elevation in circulating UA and CRP will affect multiple organs either directly or via impairment of endothelial function. For example, UA concentrations strongly and specifically affect major pathways of protein synthesis and degradation, including eIF4 pathway and ubiquitin-proteasome system, respectively. Furthermore, UA in vascular system may hamper regeneration of endothelial cells and impair NO production (16-20).

UA decreases NO production by reducing the interaction of eNOS and the activator calmodulin, leading to inhibition of mitochondrial electron transport chain, thereby impairing cellular respiration and reduced endothelium-dependent vasodilation and increased risk of development of hypertension in patients (15, 17).

There is evidence showing that UA can contribute to systemic inflammatory conditions and play a crucial role in oxidative stress and vascular inflammation (9, 14-17). UA increases endothelial reactive oxygen species (ROS) production, and causes mitochondrial calcium overload. Calcium influx through NCXmito triggers mitochondrial ROS production. NCXmito is one of the main transporters involved in the uptake of Ca^{2+} into mitochondria (16).

Furthermore, increased levels of UA have been linked to some metabolic disorders including insulin resistance. Evidence suggests that lower circulating levels of IGF-1 are associated with higher UA levels in adult individuals, which increases renal vascular resistance and glomerular filtration rate (18). UA levels are associated with injurious effects on the vasculature and renal tissues (19). According to our findings, decrease in circulating levels of UA would reduce levels of cardiac and vasculature-derived miR-499 and -126, respectively.

Endothelial dysfunction is also induced by CRP, which is also linked to uncoupling of eNOS due to reduction in dimerization of the enzyme, as well as inhibition of GTPCH1 and decrease in BH4 levels. CRP causes activation of NADPH oxidase, uncoupling of eNOS directly or via inhibition of GTPCH1 or oxidation of BH4, which provide evidence for a role for CRP in atherothrombosis (10, 22-24).

Since UA and CRP both decrease eNOS activity, this might explain in part the significant benefit of lowering UA and CRP for cardiovascular system. Our study demonstrates for the first time a strong and independent relationship between preoperative levels of UA and CRP with miR-499 and -126, particularly in those with EF% >45.

Also, ventricular contractility positively correlated with miR-499 expression levels versus UA concentration. In spite

of significant reduction in plasma levels of miR-499 after CABG, it exhibited a strong correlation with ventricular EF and is indicating that the higher miR-499, the higher heart contractility. Conversely, there was a highly negative correlation between plasma concentrations of UA and the EF and is indicating that the higher UA concentration, the lower EF%.

The current study confirms that elevated UA levels are a marker of cardiovascular disease, and are inversely associated with circulating levels of miR-126 and -499. High levels of UA have been associated with an increased risk of reperfusion injury and a variety of factors that determine both early and long-term outcome, such as inflammatory cell and platelet activation, increased atherosclerotic burden, and increased prevalence of major cardiovascular risk factors (40). Moreover, to confirm the myocardial origin of these miRNAs, we analyzed and confirmed the correlation of miRNA contents with usual cardiac biomarkers in the blood before and after CABG.

Finally, on potential values and benefits with other classic biomarkers, miRNAs can be proposed as clinically useful prognostic biomarkers to differentiate ischemic cardiovascular pathologies. As well, unique patterns of miRNA expression have also been implicated as biomarkers for prognosis to predict and monitor therapeutic responses in diseases. However, despite a significant simplicity in measuring these miRNAs levels in plasma and also their execution and costs of measuring, their applicability in the clinics has not yet been established.

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Disclosures

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