MicroRNAs in Patients on Chronic Hemodialysis (MINOS Study)

Cristina Emilian, Emeline Goretti,† Fernand Prospert,‡ Dominique Pouthier,‡ Pierre Duhoux,‡ Georges Gilson,§ Yvan Devaux,† and Daniel R. Wagner*†*

Summary

Background and objectives Diagnosis of acute myocardial injury with biomarkers is difficult in patients with advanced renal failure. Circulating microRNAs are promising new biomarkers of myocardial injury. It is unknown whether levels of microRNAs are affected in patients undergoing hemodialysis.

Design, setting, participants, & measurements High-sensitivity cardiac troponin T (hsTnT) and cardiac-enriched miR-499 were measured in 41 patients with ESRD undergoing hemodialysis and 41 controls.

Results Levels of hsTnT and miR-499 were highly elevated in patients with ESRD compared with controls (>80-fold increase; $P<0.001$). Among patients with ESRD, 98% had positive hsTnT levels and 46% had positive miR-499 levels. Levels of troponins were not affected by hemodialysis. However, miR-499 levels were decreased after hemodialysis (6.5-fold decrease; $P=0.002$).

Conclusions Both miR-499 and troponins are elevated in patients with advanced renal failure. However, whereas levels of troponins are unaffected by hemodialysis, this is not the case for miR-499. Therefore, these observations mitigate the potential of miR-499 as a marker of myocardial injury in patients with ESRD.

Clin J Am Soc Nephrol 7: 619–623, 2012. doi: 10.2215/CJN.10471011

*Division of Cardiology, ‡Division of Nephrology, and §Laboratory of Biochemistry, Centre Hospitalier, Luxembourg, Luxembourg; and †Laboratory of Cardiovascular Research, Centre de Recherche Public Santé, Luxembourg, Luxembourg

Correspondence: Dr. Yvan Devaux, Laboratory of Cardiovascular Research, Centre de Recherche Public Santé, 120 route d'Arlon, L1150 Luxembourg, Luxembourg. Email yvan.devaux@crp-sante.lu

Cardiovascular disease is the main source of morbidity and mortality in patients with ESRD, independent of classic cardiovascular risk factors (1,2). The rapid diagnosis of acute myocardial injury is essential for the triage of patients presenting to the emergency department with chest pain. This diagnosis is currently based on cardiac troponin testing and electrocardiographic findings. However, cardiac troponins are chronically elevated in most patients with ESRD (1,3) and are useful for diagnosing an acute coronary syndrome in these patients only if a rise and fall are observed (2,4). This requires serial testing and substantially delays the diagnosis. These delays may affect prognosis. Therefore, there is an urgent need to find new diagnostic tools to accurately detect or rule out myocardial injury in patients with ESRD presenting with chest pain.

MicroRNAs (miRNAs) are 20-25 oligonucleotides implicated in post-transcriptional regulation of messenger RNAs (5). miRNAs are ubiquitous and essential for all cellular processes of proliferation, cell growth, differentiation, apoptosis, or oncogenesis. We and others have observed that cardiac-enriched miRNAs, such as miR-499, are elevated in the circulation after myocardial damage and correlate with cardiac troponin levels (6–9). Thus, microRNAs appear to be promising biomarkers of acute myocardial injury, although their exact role remains to be determined (10). It is unknown whether circulating levels of microRNAs are affected in patients undergoing hemodialysis. In this study, we assessed circulating levels of miR-499 and high-sensitivity cardiac troponin T (hsTnT) in patients with ESRD.

Materials and Methods

Patients

The study population consisted of 41 patients with ESRD undergoing long-term hemodialysis and 41 healthy controls. Patients were evaluated with standard 12-lead electrocardiography and transthoracic echocardiography at the end of a hemodialysis session to exclude silent ischemia. Two blood samples were collected for each patient: one before hemodialysis and one at the end of hemodialysis. The study protocol was approved by the local ethics committee, and informed consent was obtained for each patient. The 41 controls were obtained from a local cardiovascular prevention study.

Biochemical Analyses

We measured hsTnT in serum samples using the Roche high-sensitive assay performed on the Cobas e601 system. The detection limit was 0.003 μg/L, the 99th-percentile cutoff point was 0.014 μg/L, and coefficient of variation was less than 10% at 0.035 μg/L.

Plasma miRNA Determination

Total RNA was extracted from plasma and dialysate samples with the mirVana PARIS kit (Ambion,

Applied Biosystem, Lennik, Belgium). No enrichment for small RNAs was performed. Three spiked-in synthetic *Caenorhabditis elegans* miRNAs (Qiagen, Venlo, the Netherlands) that lack sequence homology with human miRNAs were added to plasma samples to correct for extraction efficiency. Potential genomic DNA was eliminated using DNase (Qiagen). RNA was reverse-transcribed with the miScript reverse transcription kit (Qiagen), and the resulting cDNA was diluted 10-fold. Quantitative PCR was performed using the miScript SYBR-green PCR kit (Qiagen) and miScript primer sets obtained from Qiagen. An internal control containing a pool of plasma from patients was used in each PCR plate to adjust for interplate variability. All tests were performed in duplicate. Expression values were normalized using the following equation:

$$2\ exp\ (mean\ Ct\ spiked\text{-}in\ controls - Ct\ target\ miRNA).$$

The values were then log-transformed. miRNAs fulfilling the following criteria were considered present: PCR duplicate with standard deviation less than 0.3, mean quantification cycle (Cq) for target miRNA less than 35, and specific peak on the melt curve. Undetectable miRNAs were arbitrarily given a value of −7.2, which is the lowest detectable value divided by 10.

Statistical Analyses

The Wilcoxon signed-rank test was used to compare two groups of paired continuous variables, and the Mann–Whitney rank-sum test was used to compare two groups of unpaired continuous variables. Correlations were assessed using the Spearman rank test. All statistical tests were two-sided. SigmaPlot software, version 11.0, was used to perform statistical analyses. A P value less than 0.05 was considered to represent a statistically significant difference.

Results

Demographic and Functional Analysis of the Study Population

A total of 82 patients were enrolled in this study. Clinical characteristics of all patients are shown in Table 1. Forty-one patients had ESRD with ongoing hemodialysis and were evaluated by electrocardiography and echocardiography. None of these 41 patients had signs of myocardial ischemia. Forty-one healthy volunteers without documented kidney or heart diseases were used as controls.

Table 1. Clinical characteristics of the study population

Characteristic	Controls (n=41)	ESRD (n=41)
Men, n (%)	41 (100)	26 (63)
Median BMI (range) (kg/m^2)	29 (22–40)	26 (17–42)
Median age (range) (yr)	57 (54–60)	67 (30–82)
Cardiovascular history, n (%)		
myocardial infarction	0 (0)	5 (12)
PTCA	0 (0)	6 (15)
CABG	0 (0)	2 (5)
pacemaker	0 (0)	0 (0)
defibrillator	0 (0)	1 (2)
hypertension	10 (24)	20 (49)
diabetes	0 (0)	7 (17)
hypercholesterolemia	9 (22)	13 (32)
tobacco	5 (12)	9 (22)
family history of CAD	–	13 (32)
Renal failure, n (%)	0 (0)	41 (100)
Hemodialysis, n (%)	0 (0)	41 (100)
Median hemodialysis duration (range) (yr)	–	5 (0.5–24)
Medication, n (%)		
aspirin	0 (0)	19 (46)
clopidogrel	0 (0)	5 (12)
β-blocker	0 (0)	21 (51)
calcium antagonists	0 (0)	7 (17)
nitrates	0 (0)	2 (5)
ACE inhibitors	0 (0)	3 (7)
ARBs	0 (0)	1 (2)
statins	0 (0)	18 (44)
fibrates	0 (0)	1 (2)
warfarin	0 (0)	7 (17)
furosemide	0 (0)	6 (15)
amiodarone	0 (0)	3 (7)

BMI, body mass index; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery bypass grafting; CAD, coronary artery disease; ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor blockers.

Plasma Levels of hsTnT and miR-499

Blood samples obtained in controls and in patients with ESRD before hemodialysis were used to determine hsTnT and miR-499 plasma levels. miR-499 expression levels were log-transformed, and a value of −7.2 (corresponding to the lowest detected value divided by 10) was assigned to undetectable expressions. A cutoff value of −6.2, corresponding to the 99th percentile of miR-499 values, was calculated for miR-499. Because of the small size of the control group, we used the method described by Hyndman and Fan (11) for this calculation. This method interpolates the position of quantiles and therefore allowed for the calculation of 99th percentiles from the cohort of 41 controls. A cutoff value of 0.014 μg/L was used for hsTnT, as reported elsewhere (12).

Patients with ESRD had highly elevated hsTnT and miR-499 levels compared with controls (elevations of 85-fold and 83-fold, respectively; Figure 1, A and B). One control had a level of hsTnT above the cutoff value of 0.014 μg/L, and 6 (15%) had a level of miR-499 above the cutoff value of −6.2. All patients with ESRD but 1 (98%) had a level of hsTnT above the cutoff value, and 19 (46%) had a level of miR-499 above the cutoff value (Figure 1C). Therefore, hsTnT and miRNAs were both elevated in patients with ESRD.

We next investigated the association between miR-499 levels and hypertension. Twenty patients with ESRD and 10 controls had hypertension. We have compared miR-499 levels in patients with ESRD and controls, with or without hypertension. No significant differences were found. Five patients with ESRD had a history of acute myocardial infarction. miR-499 levels did not differ between these patients and the patients who did not have a previous myocardial infarction. Thus, miR-499 levels in patients with ESRD are not associated with elevated BP or with a history of myocardial infarction in the present population.

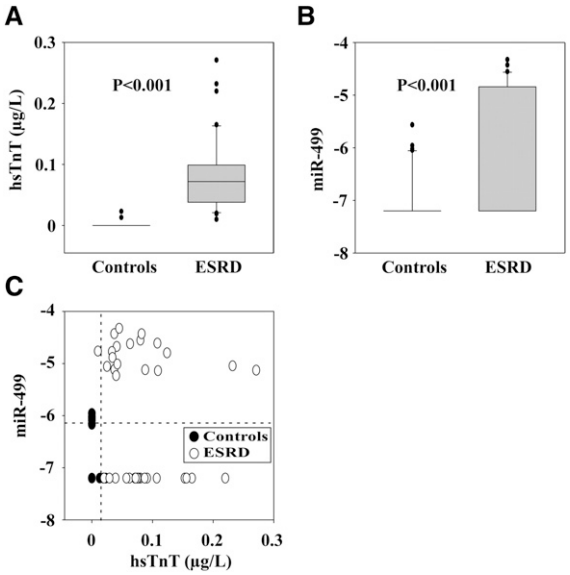

Figure 1. | Plasma levels of high-sensitivity cardiac troponin T (hsTnT) and miR-499 in 41 patients with ESRD and 41 controls. Samples were obtained before hemodialysis in patients with ESRD. (A and B) Box plots showing elevated hsTnT and miR-499 levels in patients with ESRD compared with controls. The lower boundary of the box designates the 25th percentile, the line within the box marks the median, and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box designate the 90th and 10th percentiles. *P* values are indicated. (C) Dot plot showing hsTnT and miR-499 levels for individual patients. Cutoff values of hsTnT (0.014 μg/L) and miR-499 (−6.2) are indicated by dotted lines. Log-transformed values of miRNA expression are represented.

Biomarker Levels before and after Hemodialysis

Levels of hsTnT and miR-499 were assessed in blood samples obtained before and after hemodialysis in patients with ESRD. Hemodialysis did not modify hsTnT levels, and a robust correlation was found between hsTnT levels before and after hemodialysis (Figure 2A). In contrast, levels of miR-499 were lower after hemodialysis (6.5-fold reduction; P=0.002) and miR-499 levels before and after hemodialysis were not correlated (Figure 2B). We then assessed the levels of six miRNAs, including miR-499, in dialysates from four patients. Although some miRNAs, such as miR-16 and miR-451, were present in some dialysates, miR-499 was undetectable (Table 2).

Discussion

In the present study, plasma levels of miR-499 and hsTnT were measured in patients with ESRD undergoing hemodialysis and controls. Both markers were highly elevated compared with values in controls, and miR-499 levels were decreased after hemodialysis.

Troponin levels were elevated in patients with ESRD. This observation is expected and consistent with previous reports (1,3). Circulating levels of miR-499 were also higher (>80-fold) in patients with ESRD than in controls, although echocardiographic assessment ensured that none of the patients with ESRD had signs of myocardial ischemia. We recently showed that miR-499 is highly elevated after acute myocardial infarction (13). Average levels of miR-499 were 350-fold higher in patients with acute myocardial infarction than patients with ESRD. Therefore, although miR-499 may not be the optimal marker of the presence or absence or myocardial injury in patients with

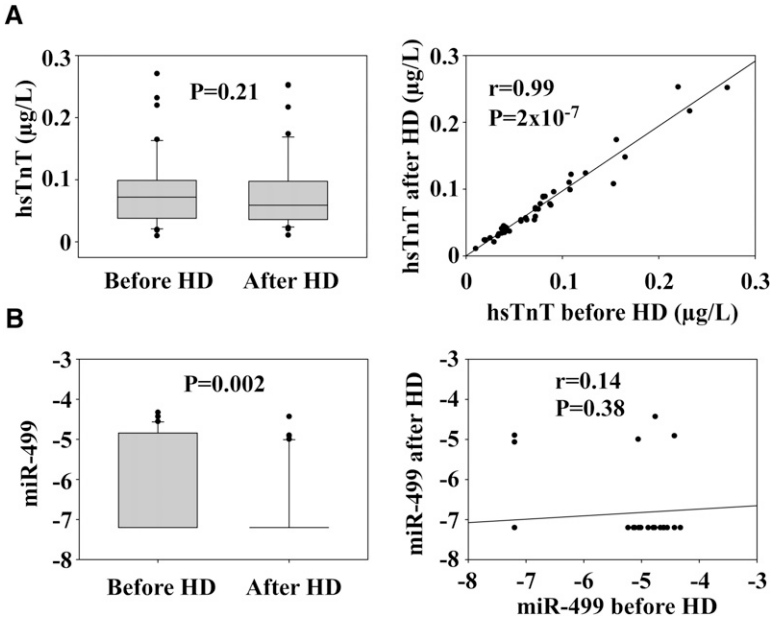

Figure 2. | Biomarker levels before and after hemodialysis. High-sensitivity cardiac troponin T hsTnT (A) and miR-499 (B) levels were measured in 41 patients with ESRD undergoing hemodialysis (HD). Blood samples were obtained before and after hemodialysis. Left panels represent box plots and right panels represent linear correlations curves. *P* values and correlation coefficients are indicated. Log-transformed values of miRNA expression are represented.

Table 2. miRNA expression in dialysates of 4 patients with ESRD

Patient	miR-16	miR-122	miR-208b	miR-223	miR-451	miR-499
1	−5.0	ND	ND	ND	ND	ND
2	−4.4	ND	ND	ND	−5.0	ND
3	−4.2	ND	ND	ND	−3.7	ND
4	ND	ND	ND	ND	ND	ND

ND, not detected.

ESRD, its clinical relevance in the diagnosis of acute myocardial infarction remains.

Of the 41 controls enrolled in this study, 6 had miR-499 levels above, although very close to, the cutoff point. The presence of miR-499 in the plasma of these controls is intriguing. None of them had a previous myocardial infarction, which excludes the possibility that miR-499 emanates from myocardial injury. Accordingly, these 6 controls had normal levels of troponins. A very recent report by Jaffe and colleagues (14) showed that diseased skeletal muscle is a potential source of circulating troponins. It would be interesting to test whether diseased muscles also secrete miR-499. However, we do not have any indication of the presence of muscular diseases in the controls enrolled in our study.

The observation of a strong correlation between troponin levels before and after hemodialysis suggests that cardiac troponins are not eliminated by hemodialysis. There is no consensus on the origin of elevated troponins in patients with ESRD. Several hypotheses have been discussed, such as a decreased renal clearance with subsequent accumulation in the circulation. This mechanism is unlikely given that troponins have a molecular weight similar to that of albumin and are therefore unlikely to be filtered at the glomerular level (2,15). Furthermore, renal transplantation and improvement in renal function are not associated with normalization of cardiac troponin levels (16). Other studies have suggested that uremic cardiomyopathy, myocardial hypertrophy, apoptosis, or small areas of silent myocardial necrosis might be involved (15). Cross-reactivity of muscular cTnT isoforms reported by McLaurin and colleagues (3) for first-generation cTnT assays is no longer demonstrated with the high-sensitivity new-generation assays (17).

In contrast to troponins, miR-499 levels were significantly decreased after hemodialysis, suggesting that miRNAs were possibly eliminated by hemodialysis. To address this possibility, we assessed the levels of miR-499, together with five other miRNAs, in dialysates. We observed that some miRNAs, such as miR-16 and miR-451, are dialyzed. However, we were unable to detect miR-499 in any dialysate. Gidlöf and colleagues recently reported that cardiac-enriched miR-1 and miR-133a, but not miR-208b and miR-499, are subjected to renal elimination and can be detected in urine after acute myocardial infarction (18). The relevance of the presence of some miRNAs in the dialysate of some patients deserves further investigation.

This study is limited by its small size. In addition, only patients with ESRD were enrolled and only a single measurement was performed. Thus, we could not investigate the association between miRNAs levels and various grades of renal dysfunction. The control group consisted only of men, but this may not have affected our data because miRNA levels are not related to gender, as previously reported (7). Finally, patients with ESRD also differed from controls in terms of age, body mass index, cardiovascular history, and risk factors; these differences temper the observation that the differences in troponin and miR-499 levels are due solely to ESRD.

In conclusion, troponin levels and miR-499 levels are both elevated in patients with ESRD. miR-499 levels are decreased after hemodialysis, whereas troponin levels remain stable. Our observations argue against the use of miR-499 as a diagnostic marker of myocardial injury in patients with ESRD.

Acknowledgments

We thank Malou Gloesener, Loredana Jacobs, Céline Jeanty, Emilie Leyder, Christelle Nicolas, and Laurent Quennery for expert technical assistance.

This study was supported by the Society for Research on Cardiovascular Diseases, the Ministry of Culture, Higher Education and Research of Luxembourg, and the National Fund of Research of Luxembourg. E.G. is receipient of a fellowship from the National Fund of Research of Luxembourg.

Disclosures

None.

References

1. Dierkes J, Domröse U, Westphal S, Ambrosch A, Bosselmann HP, Neumann KH, Luley C: Cardiac troponin T predicts mortality in patients with end-stage renal disease. *Circulation* 102: 1964–1969, 2000
2. Wang AY, Lai KN: Use of cardiac biomarkers in end-stage renal disease. *J Am Soc Nephrol* 19: 1643–1652, 2008
3. McLaurin MD, Apple FS, Voss EM, Herzog CA, Sharkey SW: Cardiac troponin I, cardiac troponin T, and creatine kinase MB in dialysis patients without ischemic heart disease: Evidence of cardiac troponin T expression in skeletal muscle. *Clin Chem* 43: 976–982, 1997
4. Hamm CW, Giannitsis E, Katus HA: Cardiac troponin elevations in patients without acute coronary syndrome. *Circulation* 106: 2871–2872, 2002
5. Ambros V: The functions of animal microRNAs. *Nature* 431: 350–355, 2004
6. Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, Nonogi H, Iwai N: Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clin Chem* 56: 1183–1185, 2010
7. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B: Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 3: 499–506, 2010

8. D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, Rubino M, Carena MC, Spazzafumo L, De Simone M, Micheli B, Biglioli P, Achilli F, Martelli F, Maggiolini S, Marenzi G, Pompilio G, Capogrossi MC: Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J* 31: 2765–2773, 2010
9. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q: Circulating microRNA: A novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J* 31: 659–666, 2010
10. Widera C, Gupta SK, Lorenzen JM, Bang C, Bauersachs J, Bethmann K, Kempf T, Wollert KC, Thum T: Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. *J Mol Cell Cardiol* 51: 872–875, 2011
11. Hyndman RJ, Fan Y: Sample quantiles in statistical packages. *Am Stat* 50: 361–365, 1996
12. Reichlin T, Hochholzer W, Bassetti S, Steuer S, Stelzig C, Hartwiger S, Biedert S, Schaub N, Buerge C, Potocki M, Noveanu M, Breidthardt T, Twerenbold R, Winkler K, Bingisser R, Mueller C: Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. *N Engl J Med* 361: 858–867, 2009
13. Devaux Y, Vausort M, Goretti E, Nazarov PV, Azuaje F, Gilson G, Corsten MF, Schroen B, Lair ML, Heymans S, Wagner DR: Use of circulating microRNAs to diagnose acute myocardial infarction [published online ahead of print January 17, 2012]. *Clin Chem* doi: 10.1373/clinchem.2011.173823
14. Jaffe AS, Vasile VC, Milone M, Saenger AK, Olson KN, Apple FS: Diseased skeletal muscle: A noncardiac source of increased circulating concentrations of cardiac troponin T. *J Am Coll Cardiol* 58: 1819–1824, 2011
15. Freda BJ, Tang WH, Van Lente F, Peacock WF, Francis GS: Cardiac troponins in renal insufficiency: Review and clinical implications. *J Am Coll Cardiol* 40: 2065–2071, 2002
16. Fredericks S, Chang R, Gregson H, Bewick M, Collinson PO, Gaze D, Carter ND, Holt DW: Circulating cardiac troponin-T in patients before and after renal transplantation. *Clin Chim Acta* 310: 199–203, 2001
17. Apple FS, Ricchiuti V, Voss EM, Anderson PA, Ney A, Odland M: Expression of cardiac troponin T isoforms in skeletal muscle of renal disease patients will not cause false-positive serum results by the second generation cardiac troponin T assay. *Eur Heart J* 19 [Suppl N]: N30–33, 1998
18. Gidlöf O, Andersson P, van der Pals J, Götberg M, Erlinge D: Cardiospecific microRNA plasma levels correlate with troponin and cardiac function in patients with ST elevation myocardial infarction, are selectively dependent on renal elimination, and can be detected in urine samples. *Cardiology* 118: 217–226, 2011

Received: October 12, 2011 **Accepted:** January 17, 2012

Published online ahead of print. Publication date available at www.cjasn.org.