Circulating Levels of miR-133a Predict the Regression Potential of Left Ventricular Hypertrophy After Valve Replacement Surgery in Patients With Aortic Stenosis

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Background—Myocardial microRNA-133a (miR-133a) is directly related to reverse remodeling after pressure overload release in aortic stenosis patients. Herein, we assessed the significance of plasma miR-133a as an accessible biomarker with prognostic value in predicting the reversibility potential of LV hypertrophy after aortic valve replacement (AVR) in these patients.

Methods and Results—The expressions of miR-133a and its targets were measured in LV biopsies from 74 aortic stenosis patients. Circulating miR-133a was measured in peripheral and coronary sinus blood. LV mass reduction was determined echocardiographically. Myocardial and plasma levels of miR-133a correlated directly (r=0.46, P<0.001) supporting the myocardium as a relevant source of plasma miR-133a. Accordingly, a significant gradient of miR-133a was found between coronary and systemic venous blood. The preoperative plasma level of miR-133a was higher in the patients who normalized LV mass 1 year after AVR than in those exhibiting residual hypertrophy. Logistic regression analysis identified plasma miR-133a as a positive predictor of the hypertrophy reversibility after surgery. The discrimination of the model yielded an area under the receiver operator characteristic curve of 0.89 (P<0.001). Multiple linear regression analysis revealed plasma miR-133a and its myocardial target Wolf-Hirschhorn syndrome candidate 2/Negative elongation factor A as opposite predictors of the LV mass loss (g) after AVR.

Conclusions—Preoperative plasma levels of miR-133a reflect their myocardial expression and predict the regression potential of LV hypertrophy after AVR. The value of this bedside information for the surgical timing, particularly in asymptomatic aortic stenosis patients, deserves confirmation in further clinical studies. (J Am Heart Assoc. 2013;2:e000211 doi: 10.1161/JAHA.113.000211)

Key Words: aortic stenosis • microRNA • miR-133a • myocardial hypertrophy • myocardial reverse remodeling
and long-term rates of morbid events after aortic valve replacement remains to be determined. However, in line with the preoperative epidemiological evidence, a lack of reverse remodeling after valve replacement in patients with AS has been identified as one of the postoperative threats to a positive long-term outcome for these patients, and it also results in both decreased short- and long-term survival and poorer clinical status.\textsuperscript{5-7} Preoperative monitoring of a variable that is able to predict the potential for regression of the myocardial hypertrophy could help both in the timing of the valve replacement surgery and in the improvement of postoperative outcomes.

MicroRNAs (miRNAs) are single stranded, short length (21 to 23 nucleotides), noncoding RNAs that act as negative posttranscriptional modulators of target mRNAs.\textsuperscript{8} miRNAs play a major regulatory role in a myriad of mechanisms in developmental biology, physiology, and pathology of almost every organ including the cardiovascular system.\textsuperscript{9-12} One further characteristic of miRNAs that make them particularly attractive in clinical medicine is their presence in human body fluids incorporated in microvesicles and protein–miRNA complexes.\textsuperscript{13} Circulating miRNAs display a marked stability and can be detected with high sensitivity and specificity in plasma and serum. These characteristics raise novel questions concerning the potential merits of circulating miRNAs as bedside biomarkers for therapeutic stratification and for diagnostic or prognostic purposes.\textsuperscript{12-15}

miR-133a is a muscle-specific miRNA that, in the heart, is exclusively expressed by cardiomyocytes.\textsuperscript{16} Several studies have reported that downregulation of miR-133a promotes rodent and human cardiomyocyte hypertrophy\textsuperscript{17,18} and that, conversely, the forced expression of miR-133a promotes cardiac hypotrophy in transgenic mice and inhibits agonist-induced hypertrophy in cultured cardiac myocytes.\textsuperscript{19-21} Targets of miR-133a, which may be responsible for its antihypertrophic effect, include Wolf-Hirschhorn syndrome candidate 2/Negative elongation factor A (WHSC2/NELFA), Inositol 1,4,5′-triphosphate receptor II (IP3RII), Calcineurin, Serum response factor (SRF), Ras homologue gene family member A (RhoA), Cell division control protein 42 (Cdc42), and Nuclear factor of activated T cells calcineurin-dependent 4 (Nfatc4).\textsuperscript{17,19,22,23}

In patients with pure severe AS, we have previously shown that the LV myocardial expression of miR-133a at surgery, together with additional remodeling-related genes and clinical parameters, predicts the amount and completeness of LV reverse remodeling 1 year after AVR.\textsuperscript{24} Taking advantage of the presence of this miRNA in plasma, we hypothesized that preoperative circulating miR-133a level might estimate the potential for postoperative LV mass (LVM) absolute loss and/or the normalization of the LVM in AS patients, with a predictive power similar to its myocardial expression. The involvement of major miR-133a targets related with the control of cardiomyocyte trophic state was assessed.

Methods

We prospectively studied 74 consecutive patients (42 men and 32 women) with pure severe AS who were undergoing AVR surgery in the University Hospital Marqués de Valdecilla in Santander, Spain. Demographic and clinical characteristics of the patients and their preoperative pharmacological treatments are shown in Table 1. Patients with aortic or mitral regurgitation greater than mild, major coronary stenosis >50%, previous cardiac operations, malignancies, or poor renal or hepatic function were ineligible for the study. The study cohort represents the average population of patients with pure aortic stenosis undergoing conventional open surgical treatment in Western Europe. The study followed the Declaration of Helsinki guidelines for biomedical research involving human subjects. The institutional Ethics and Clinical Research Committee of the Hospital Universitario Marqués de Valdecilla approved the study, and all patients gave written informed consent.

Echocardiography

A 2-dimensional transthoracic echocardiogram (Philips-Hewlett Packard, IE 33), together with clinical and blood analytical evaluation, was performed preoperatively and 1 year after surgery. Internal LV end-diastolic and end-systolic diameters and wall thicknesses were measured according to the

| Table 1. Clinical Characteristics of the Patients |
|-----------------------------------------------|
| Variable                                       | n=74 |
| Age (y±SD)                                     | 70.4±11.1 |
| Male/female, n                                 | 42/32 |
| Systolic blood pressure, mm Hg                 | 119.8±19.9 |
| Diastolic blood pressure, mm Hg                | 67.1±11.4 |
| Body mass index, kg/m²                         | 29.1±4.2 |
| Body mass index ≥30, n (%)                     | 25 (34) |
| Systemic hypertension, n (%)                   | 40 (54) |
| Diabetes mellitus, n (%)                       | 17 (23) |
| Atrial fibrillation or flutter, n (%)          | 10 (14) |
| ACE inhibitors, n (%)                          | 14 (19) |
| Angiotensin II receptor antagonists, n (%)     | 9 (12) |
| Diuretics, n (%)                               | 31 (42) |
| Calcium antagonists, n (%)                     | 14 (19) |
| β-Blockers, n (%)                              | 9 (12) |
American Society of Echocardiography guidelines, using bidimensional or M-mode images depending on the image quality and the angulation between the ultrasound beam and the LV. LV ejection fraction was calculated using the Quinones formula and indexed to patient height in meters to the 2.7th power. The threshold value defining LV hypertrophy was, irrespective of gender, a LVM index (LVMI) of ≥51 g/m².7,25

Myocardial Tissue and Plasma Sampling
In all patients (n=74), LV subepicardial biopsies (4 to 10 mg) were taken from the lateral wall with a Tru-cut needle during the surgical procedure and snap frozen in liquid nitrogen.

To determine the plasma levels of miR-133a, a blood sample was drawn 24 hours before surgery from an antecubital vein without tourniquet, using a syringe with a wide-gauge needle. The specific cardiac contribution to total miR-133a was assessed using coronary sinus blood samples withdrawn from 29 patients during the insertion of a cannula for retrograde cardioplegia, and prior to the initiation of extracorporeal circulation. Simultaneously, a right atrial blood sample was also taken to measure the systemic (including muscular and cardiac contributions) circulating miR-133a. Blood was harvested into 10 mL EDTA-containing tubes and centrifuged at 1389 g for 30 minutes at room temperature within 1 hour after collection. Plasma samples were frozen and stored at −80°C.

Quantification of mRNA and miRNA Expressions
Total RNA, including the small RNA fraction, was extracted from myocardial biopsies using TRIzol reagent (Invitrogen). Reverse transcription was performed using random primers for mRNA (Fermentas); tissue miR-133a and RNU6B were reverse transcribed with specific primers (Applied Biosystems). Real-time PCR was conducted in an MX-3000P thermocycler (Stratagene) using specific TaqMan assays (Applied Biosystems). Mature miR-133a levels were normalized to the expression levels of RNU6B. We determined the myocardial transcript levels of a number of miR-133a target genes (SRF, RhoA, WHSC2/NELFA, Cdc42, Nfatc4) that were previously validated and found to be associated with the pathophysiology of cardiovascular disorders, including myocardial hypertrophy.17,19,20,22 The expression levels of the myocardial genes were normalized to the housekeeping gene 18S ribosomal RNA, which was measured in parallel for each sample.

Circulating RNA was isolated from plasma samples (100 μL) using TRIzol reagent. Twenty-five femtomoles of a Caenorhabditis elegans oligonucleotide (cel-miR-39) were added to the samples after TRIzol addition as a spike-in control.13 Reverse transcription was performed using specific miR-133a and cel-miR-39 primers and a Taqman microRNA transcription kit (Applied Biosystems). Plasma miR-133a levels were normalized to cel-miR-39. To ensure that the isolation efficiency was consistent between the samples, the extraction procedure was repeated, if necessary, until the qPCR threshold for cel-miR-39 fell within the range of 23.0±1.0 cycles.

Statistics
GraphPad Prism 5.01, PASW Statistics 18 (SPSS, Inc) and Stata 10 (StataCorp LP) packages were used. The data sets were assessed with the D’Agostino and Pearson omnibus normality test. Continuous variables were expressed as the mean±SD if Gaussian and as median (25th and 75th IQR) if non-Gaussian. Variables that were not normally distributed were transformed to their natural logarithm. To assess the relationships between myocardial and plasma levels of miR-133a, linear regression and Pearson's correlation analyses were performed. Differences between coronary sinus and peripheral venous miR-133a levels within patients were assessed by the Wilcoxon test for paired samples. A multiple linear regression analysis was used to identify predictors of LVM regression 1 year after AVR. The variables introduced into the regression equation were assessed for multicollinearity and excluded when appropriate. Predictors of post-operative LVM normalization were identified with a forward stepwise logistic regression analysis, and the Hosmer-Lemeshow test was used to evaluate goodness of fit of the model. A post-hoc assessment of the regression model was performed with the bootstrapping method, with 2000 iterations. The receiver operator characteristic (ROC) curve was calculated to assess the capability of the model to discriminate patients who would normalize LVM 1 year after AVR from those who would maintain residual hypertrophy. The threshold for statistical significance was P<0.05.

Results
Seventy-four patients were enrolled in this study. The clinical and demographic characteristics of the study population are shown in Table 1.

Preoperative Circulating miR-133a in AS Patients
The preoperative levels of circulating miR-133a (Figure 1A) were significantly higher in the cohort of AS patients who normalized LVM (LVMI <51 g/m².7) after pressure overload release (n=30; median=113.2, interquartile range=80.9 to 523.3) compared with those who maintained residual hypertrophy (LVMI >51 g/m².7) (n=44; median=71.3,
interquartile range = 29.3 to 150.7) \( (P=0.012, \text{ Mann–Whitney U test}) \).

**Cardiac Contribution to the Levels of Circulating miR-133a**

We initially sought to assess the relationship between the preoperative levels of circulating miR-133a and its myocardial expression. We found a direct, positive and significant correlation between both variables (Figure 1B), suggesting that the myocardium is among the sources that contribute to the levels of circulating miR-133a. To further investigate this possibility, we assessed the existence of a miR-133a concentration gradient between the coronary and the systemic venous blood in 29 AS patients. Plasma levels of miR-133a in the coronary sinus (median = 140.4, interquartile range = 63.4 to 193.3) were significantly higher than those in the right atrium (median = 72.3, interquartile range = 29.3 to 113.2) (Figure 2A), indicating a net contribution of the heart to the systemic levels of miR-133a. Accordingly, coronary sinus miR-133a levels exhibited significant positive correlations with both the myocardial expression levels (Figure 2B) and the systemic venous levels of miR-133a (Figure 2C).

**Preoperative Circulating miR-133a as a Predictor of Postoperative LV Hypertrophy Regression**

Stepwise multiple linear regression models were used to assess the value of preoperative miR-133a plasma level together with several clinical variables (age, sex, preoperative LVM, body mass index [BMI], hypertension, diabetes mellitus) for estimating the extent of LVM regression 1 year after AVR in AS patients. The variables entered in the regression equation were evaluated for multicollinearity and proved independent of each other. The validity of the predictive regression model was assessed for accuracy by bootstrapping. The models developed are shown in Table 2. Among the variables tested, the preoperative plasma levels of miR-133a together with LVM arose as significant positive predictors of the LVM absolute reduction after surgery, whereas BMI appeared to be a significant negative predictor (Table 2, model 1). The adjusted \( R^2 \) (0.49; \( P<0.0001 \)) indicated that 49% of the variance in LVM reduction 1-year after AVR can be estimated from this model.

In a step further, we assessed whether entering the myocardial mRNA expression levels of several validated miR-133a targets (WHSC2/NELFA, SRF, RhoA, Cdc42, Nfatc4)
as independent variables in the equation would improve the predictive power of the resulting equation. As shown in Table 2 (model 2), WHSC2/NELFA constituted, together with the BMI, a significant negative predictor of 1-year postoperative LVM reduction. The bootstrapping validated regression model was as follows: \[DLVM = C_0^{16.8} + 0.51 \times \text{LVM} + 12.9 \times \text{plasma miR-133a}/C_0^{10.1} \times \text{WHSCD2}/C_0^{2.7} \times \text{BMI}.\] The adjusted \(R^2 (0.61; P < 0.0001)\) indicated that inclusion of WHSC2/NELFA improved substantially the predictive power of the model. On the other hand, SRF, RhoA, Cdc42 or Nfatc4 did not constitute significant predictors of LVM evolution after surgery.

We next evaluated by logistic regression analysis the value of circulating miR-133a levels, in conjunction with the same clinical parameters, to predict LVM normalization (LVMI < 51 g/m^2.7) 1 year after AVR. As shown in Table 3, preoperative circulating miR-133a was identified as positive predictor of LVMI normalization, whereas BMI, LVM, and hypertension were negatively associated with the probability of LVM normalization. Bootstrapping validation of the logistic regression model revealed a sensitivity of 87.9% and a specificity of 76.9%. The accuracy of this model to discriminate the patients who maintained residual LV hypertrophy from those who normalized the LVM 1 year after surgery was determined by the ROC curve analysis. As shown in Figure 3, the prognostic accuracy of the model yielded an area under the ROC curve of 0.89 (CI 95% 0.81 to 0.97, \(P < 0.001\)). When the same analysis was performed using preoperative miR-133a circulating level as the only independent predictor, the area under the ROC curve was 0.64 (CI 95% 0.49 to 0.79, \(P=0.035\)).

### Discussion

Based on intraoperative LV biopsies harvested at the time of AVR, we have previously shown in patients with pure severe

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### Table 2. Significant Preoperative Predictors of Quantitative LVM Loss 1 Year After Surgery in AS Patients Undergoing Aortic Valve Replacement

| Predictors of LVM Loss (Grams) 1 Year After Surgery in AS Patients (Multiple Linear Regression Analysis) | Unstandardized Coefficient | Standardized Coefficient \(\beta\) | \(P\) Value |
|---|---|---|---|
| **MODEL 1** | | | |
| Preoperative LVM, g | 0.45 | 0.07 | 0.64 | 0.0001 |
| Preop. plasma miR-133a (RE vs cel-miR-39) | 10.02 | 3.32 | 0.29 | 0.004 |
| Body mass index (1 point) | –2.20 | 1.11 | –0.19 | 0.055 |
| **MODEL 2** | | | |
| Preop. LVM, g | 0.51 | 0.07 | 0.64 | 0.0001 |
| Preop. plasma miR-133a (RE vs cel-miR-39) | 12.94 | 3.15 | 0.39 | 0.0001 |
| Body mass index (1 point) | –2.74 | 1.13 | –0.22 | 0.02 |
| Myocardial WHSCD2 (RE vs 18S rRNA) | –10.10 | 2.87 | –0.33 | 0.001 |

Two multiple linear regression models were calculated using a bootstrap validated analysis: One excluding WHSCD2 expression (model 1: adjusted \(R^2: 0.49; P<0.0001\)) and one including WHSCD2 expression (model 2: adjusted \(R^2: 0.61; P<0.0001\)). RE: Relative expression normalized to spiked-in Caenorhabditis elegans oligonucleotide (cel-miR-39) for circulating miR-133a, or to the ribosomal subunit 18S for myocardial WHSCD2. LVM indicates left ventricular mass; AS, aortic stenosis.

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### Table 3. Significant Preoperative Predictors of LVMI Normalization 1 Year After Surgery in AS Patients Undergoing Aortic Valve Replacement

| Predictors of LVMI Normalisation 1-Year After Surgery in AS Patients (Logistic Regression Analysis) | Coefficient B | SE | \(P\) Value | Odds Ratio (95% CI) |
|---|---|---|---|---|
| Preop. plasma miR-133a (RE vs cel-miR-39) | 0.81 | 0.29 | 0.006 | 2.25 (1.27 to 3.99) |
| Body mass index (1 point) | –0.31 | 0.12 | 0.013 | 0.73 (0.58 to 0.97) |
| Preop. LVM, g | –0.02 | 0.007 | 0.003 | 0.98 (0.96 to 0.99) |
| Hypertension (present or absent) | –2.01 | 0.80 | 0.013 | 0.13 (0.03 to 0.65) |

The goodness of fit of the logistic regression analysis (\(x^2=2.63;\) significance=0.96) was assessed using the Hosmer-Lemeshow test. RE: Relative expression of circulating miR-133a normalized to spiked-in Caenorhabditis elegans oligonucleotide (cel-miR-39). LVMI indicates left ventricular mass index; AS, aortic stenosis; SE, standard error of the mean.
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AS that the myocardial expression of miR-133a predicts the amount and completeness of LV reverse remodeling 1 year after AVR. This predictive information, albeit important, is of little clinical utility as it cannot be obtained easily in everyday practice. Finding bedside biomarkers that help in estimating the postoperative reversibility of LV hypertrophy in potential surgical candidate AS patients is warranted. In the present study, which was performed using the same cohort of AS patients, we show that miR-133a is released by the myocardium into the circulation in the pressure overload situation and, most importantly, that the preoperative plasma levels of miR-133a can predict the reversibility of LV hypertrophy after AVR. Thus, patients who normalized the LVM 1 year after surgery had significantly higher preoperative plasma levels of circulating miR-133a compared with patients who exhibited residual hypertrophy at this time mark. Moreover, bootstrapping-validated multiple linear regression and logistic regression analyses indicate that the preoperative level of circulating miR-133a constitutes a significant positive predictor for both absolute LVM reduction and LVM normalization 1 year after valve replacement. The present study supports the notion that preoperative circulating miR-133a represents a potential biomarker for the prognosis of postoperative LVM regression, with a predictive power similar to its myocardial expression.

The muscle-specific miR-133a is among the most abundant miRNAs in the heart. It is critically involved in the control of cardiomyocyte proliferation during embryonic heart development in the mouse. Recent reports indicate that miR-133, together with miR-1 and a few cardiac transcription factors, play important roles in generating cardiomyocytes from embryonic stem cells and in reprogramming human fibroblasts to cardiac-like myocytes. Additionally, the regenerative capacity of the adult zebrafish heart in response to major cardiac injury is under the repressive control of miR-133. Down-regulation of miR-133 is related to the hypertrophic growth of cultured cardiomyocytes. Thus, it appears that miR-133 plays a key role in the control of the trophic state of the heart under normal conditions and that, when miR-133 is down-regulated (such as in the pressure overload condition), the transcriptional derepression of genes encoding proteins that regulate cardiac structure likely contributes to the adverse remodeling response.

Is the heart a significant source of circulating miR-133a? miR-133 is preferentially expressed in striated muscle and, therefore, apart from the heart it must also be released into the blood by the skeletal muscle. In the present study, linear regression analysis identified a significant, positive correlation between the myocardial expression of miR-133a and its levels in the circulating blood. A paired comparison of miR-133a levels in coronary sinus and peripheral venous blood identified a significant concentration gradient across the coronary circulation. A transcoronary concentration gradient of muscle-enriched miRNAs, including miR-133a, has been previously reported to be suggestive of a release into the coronary circulation during myocardial injury. Taken together, these data support a substantial contribution of the heart to the systemic plasma levels of miR-133a and reinforce its value as a biomarker for pathological cardiac processes. In this regard, it is interesting to note that plasma levels of miR-133 have been reported recently to be helpful in the diagnosis and prognosis of coronary artery disease and acute myocardial infarction, and of heart failure.

In addition to miR-133a, the preoperative LVM also arose as a strong, positive, significant predictor of absolute LVM regression in our predictive equation. These data further support our own and others’ findings suggesting that a greater excess of preoperative LVM is associated with a quantitatively greater mass loss after palliation of the pressure overload condition but also with a less overall probability of mass normalization at the 1-year mark.

Obesity and hypertension are known to be risk factors for the development of LV hypertrophy and both have a negative impact on postoperative LV reverse remodeling after AVR. Accordingly, herein both obesity and hypertension arose as negative predictors of postoperative LVM normalization. The logistic regression model indicates that, in this
particular cohort of patients and with the variables analyzed, the reduction in 1 unit of BMI would increase the probability of LVM normalization by 27% (OR: 0.73, 95% CI: 0.58 to 0.94), regardless of the values of the other predictors. Conversely, hypertension (OR: 0.13, 95% CI: 0.03 to 0.65) would be associated with a 7.7-fold lower likelihood of LVM normalization. Overall, these findings underscore the need for ancillary therapeutic interventions acting on these factors prior to and after surgical treatment of AS patients.

One of the main difficulties for the treatment of AS patients is the proper timing of the AVR in such a way as to achieve an adequate equilibrium between surgical risk and prospects of survival and good functional status. Surgical indication for AVR in asymptomatic patients is a particularly controversial issue that has, in the past, led to the inclusion and subsequent withdrawal of some indications in successive versions of the intersocietal guidelines for the management of patients with valvular heart disease. In this regard, evolution towards irreversible hypertrophy should favor earlier surgery in asymptomatic AS patients with excessive or rapidly growing LVM for the sake of postoperative LVM normalization. The discovery of biomarkers that are informative, prior to surgery, with regard to the postoperative prognosis could represent a significant advance for the identification of patients who would benefit most from earlier surgery.

Aside from the merit of circulating miRNAs as clinically relevant biomarkers, today, thanks to clinical profiling and gain-and-loss of function experimental studies, it is widely recognized that miRNAs play a major role in many physiological and pathophysiological processes. The development of miRNA-based therapeutics has sprouted as a logical next step and, despite the recent discovery of miRNAs, several candidates have already progressed into clinical development. The therapeutic aim is the normalization of miRNA expression, by either silencing with anti-miRs those miRNAs that become inappropriately overexpressed, or by replacing with miRNA mimics those that become downregulated.

Cardiac miR-133a downregulation has been reported to be involved in the pathogenesis of myocardial hypertrophy in rodent models of cardiac disease and in AS patients. Overexpression of this miRNA attenuates hypertrophy in mice through repression of cardiomyocyte targets from hypertrophy-related signaling cascades. Conversely, derepression of these targets by miR-133a loss-of-function plays a key role in the cardiomyocyte growth response. Collectively, these studies suggest that miR-1333-based therapies directed at manipulating the trophic state of the heart could be a reality in the near future. miR-133a mimics would synergize with conventional medical and surgical measures to achieve reverse remodeling of the overloaded LV. On the other end of the spectrum, the induction of therapeutic hypertrophy with anti-miR-133a in patients with advanced heart failure under mechanical circulatory support might be of help as part of a bridge to recovery strategy.

Conclusion

Our present findings support the notion that the level of circulating miR-133a, in conjunction with clinical parameters, could help to create an individual preoperative risk profile disclosing the potential for regression of LV myocardial hypertrophy in AS patients. This information could help the clinician not only to make decisions about the timing of AVR surgery for asymptomatic patients but also in the reinforcement of adjuvant measures (weight loss, arterial pressure control, etc) aimed at improving the postoperative outcome in all AS patients. Our results support a role for WHSC2/NELFA in the miR-133a-dependent regulation of myocardial trophic state.

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Disclosures

None.

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