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Title: Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients

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Keywords: aortic stenosis, myocardial fibrosis, microRNA, plasma miR-21, TGF-β signaling

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Abstract: Background: Various human cardiovascular pathophysiological conditions associate aberrant expression of microRNAs (miRNAs) and circulating miRNAs are emerging as promising biomarkers. In mice, myocardial miR-21 overexpression is related to cardiac fibrosis elicited by pressure overload. This study was designed to determine the role of myocardial and plasmatic miR-21 in the maladaptive remodeling of the extracellular matrix induced by pressure overload in aortic stenosis (AS) patients and the clinical value of miR-21 as a biomarker for pathological myocardial fibrosis.
Methods: In left ventricular biopsies from 75 AS patients and 32 surgical controls, we quantified the myocardial transcript levels of miR-21, miR-21-targets and ECM- and TGF-β-signaling-related elements. miR-21 plasma levels were determined in 25 healthy volunteers and in AS patients. In situ hybridization of miR-21 wasperformed in myocardial sections.
Results: The myocardial and plasma levels of miR-21 were significantly higher in the AS patients compared with controls and correlated directly with the echocardiographic mean transvalvular gradients. miR-21 overexpression was confined to interstitial cells and absent in cardiomyocytes. Using bootstrap validated multiple linear regression, the variance in myocardial collagen expression was predicted by myocardial miR-21 (70% of collagen variance) or plasma miR-21 (52% of collagen variance), together with the miR-21 targets RECK and PDCD4, and effectors of TGF-β signaling.
Conclusions: Our results support the role of miR-21 as a regulator of the fibrotic process that occurs in response to pressure overload in AS patients and underscore the value of circulating miR-21 as a biomarker for myocardial fibrosis.

Suggested Reviewers:

Dear Dr Coats,

We are herewith submitting the manuscript entitled "***Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients***" to be considered for publication in the International Journal of Cardiology. The results of our study evidence that both myocardial and circulating levels of miR-21 reflect in human patients with aortic stenosis the process of extracellular matrix remodeling in the left ventricle. The study cohort is, to our knowledge, the largest ever published of prospectively collected aortic stenosis patients in whom the availability of left ventricular biopsies, harvested at valvular surgery, allowed the study of myocardial gene expression together with other clinical, imaging or serological variables. Our findings provide new mechanistic insights and reinforce the value of the plasma levels of this mi-RNA, possibly in conjunction with other biomarkers, in the clinical assessment of a phenomenon of utmost prognostic repercussion as myocardial fibrosis is.

Statistical advisorship was performed by Prof. Javier Llorca (Professor of Statistics and Epidemiology) from our university. The manuscript has been edited by qualified native English speaking editors at American Journal Experts.

All authors have read and agree to the manuscript as written. The manuscript is not under consideration elsewhere and none of the paper's contents have been published previously. The authors maintain no conflict of interest.

Thank you for your consideration. We look forward to hearing from you.

Yours sincerely
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International Journal of Cardiology

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Manuscript Title:

Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients

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This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication and is not under consideration for publication elsewhere. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission.

This research has not been submitted for publication nor has it been published in whole or in part elsewhere. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the *International Journal of Cardiology.*

The Authors of this manuscript have also certified that they comply with the Principles of Ethical Publishing in *the International Journal of Cardiology* and will cite a reference that includes this statement in their reference list. All authors agree that author list is correct in its content and order and that no modification to the author list can be made without the formal approval of the Editor-in-Chief, and all authors accept that the Editor-in-Chief's decisions over acceptance or rejection or in the event of any breach of the Principles of Ethical Publishing in the International Journal of Cardiology being discovered of retraction are final.

Santander, June 26, 2012

Prof. Andrew Coats

Editor-in-Chief

International Journal of Cardiology

Dear Prof. Coats,

I am pleased to send you the revised version of our manuscript "Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients" (Ref. No.: IJC-D-12-00582). The manuscript was modified according to your instructions and the criticisms of the reviewer have been addressed, as outlined below in detail. We hope that you will find the revised version suitable for publication in the *International Journal of Cardiology*.

Reviewers' comments:

1. Is miR-21 the only miRNA regulated? What about other miRNAs involved in fibrosis such as miR-29?

Authors' answer:

In a preliminary set of PCR experiments we studied the regulation by the pressure overload condition of several miRNAs selected on the basis of their involvement in myocardial remodeling or their relationship with TGF-beta signaling pathways. miR-133a, miR-21 and miR-29 arose as those miRNAs which featured the greatest changes in mice subjected to aortic banding and in aortic stenosis patients. miR-133a is associated in humans with aortic stenosis with a lesser degree of LV hypertrophy preoperatively and a better reverse remodeling after aortic valve replacement, but it does not predict fibrosis severity prior to or after surgery (Villar et al. Heart 2011). With regard to miR-29, we found that in the pressure overload

condition it experiences a complex sex dependent regulation that we are currently analyzing in detail.

2. Methods/microRNA expression: please correct: using specific primers for mi-R21.

Authors' answer:

This point was addressed in page 9, line 4.

3. miR-21 expression has been previously demonstrated by in situ hybridization; however, why is there such a big difference in RT-PCR data vs. miR-21 hybridization signal?

Authors' answer:

Quantitative-PCR provides the average expression values of miR-21 in the whole biopsy sample while *in situ hybridization* shows the topographic distribution of miR-21 *hybridization* signals which, in the AS patients, is concentrated in fibrotic areas. We selected for the figure a representative area of fibrotic tissue with intense accumulation of miR-21 to further support that this miRNA is expressed mostly by fibroblasts. Hence, both the PCR and *in situ hybridization* data are not necessarily incompatible.

4. Please check pri-miR-21 expression in myocardium to see whether there are also transcriptional changes.

Authors' answer:

We included additional data showing that myocardial pri-miR-21 was also up-regulated in the pressure overload condition, and that its expression correlated significantly with the expression of TGF-beta, which suggests that, in this context, the transcription of miR-21 is under the control of this cytokine (page 11, lines 9 to 12; page 13, lines 13 to 16; and page 17, lines 4 to 6).

Minor comments:

The page numbers were added.

The abbreviation list was corrected.

Thank you for your kindness. We look forward to hearing from you.

J. Francisco Nistal, M.D., Ph.D., FECTS.

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Myocardial and circulating levels of microRNA-21 reflect left ventricular fibrosis in aortic stenosis patients

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Abstract

Background: Various human cardiovascular pathophysiological conditions associate aberrant expression of microRNAs (miRNAs) and circulating miRNAs are emerging as promising biomarkers. In mice, myocardial miR-21 overexpression is related to cardiac fibrosis elicited by pressure overload. This study was designed to determine the role of myocardial and plasmatic miR-21 in the maladaptive remodeling of the extracellular matrix induced by pressure overload in aortic stenosis (AS) patients and the clinical value of miR-21 as a biomarker for pathological myocardial fibrosis.

Methods: In left ventricular biopsies from 75 AS patients and 32 surgical controls, we quantified the myocardial transcript levels of miR-21, miR-21-targets and ECM- and TGF-β-signaling-related elements. miR-21 plasma levels were determined in 25 healthy volunteers and in AS patients. In situ hybridization of miR-21 was performed in myocardial sections.

Results: The myocardial and plasma levels of miR-21 were significantly higher in the AS patients compared with controls and correlated directly with the echocardiographic mean transvalvular gradients. miR-21 overexpression was confined to interstitial cells and absent in cardiomyocytes. Using bootstrap validated multiple linear regression, the variance in myocardial collagen expression was predicted by myocardial miR-21 (70% of collagen variance) or plasma miR-21 (52% of collagen variance), together with the miR-21 targets RECK and PDCD4, and effectors of TGF-ß signaling.

Conclusions: Our results support the role of miR-21 as a regulator of the fibrotic process that occurs in response to pressure overload in AS patients and underscore the value of circulating miR-21 as a biomarker for myocardial fibrosis.

Key words: aortic stenosis, myocardial fibrosis, microRNA, plasma miR-21, TGF-ß signaling

Non-standard Abbreviations and Acronyms

AS: aortic stenosis

Col: collagen

ECM: extracellular matrix

FN: fibronectin 1

LV: left ventricle

miRNA: microRNA

PDCD4: programmed cell death 4

PTEN: phosphatase and tensin homolog

RECK: reversion-inducing-cysteine-rich protein with kazal motifs

SPRY1: sprouty homolog 1

TAK-1: TGF-β activated kinase-1

TIMP3: tissue inhibitor of metalloproteinase 3

TGF-β: transforming growth factor-β

Introduction

Degenerative aortic stenosis (AS) has become the most frequent type of valvular disease in industrialized countries (2–7% of the population older than 65 years) and its incidence is rising in parallel to the increasing life expectancy [1]. Surgical aortic valve replacement is the only therapy with confirmed survival benefit, and at present, no medical therapy can delay the inevitability of surgery. Older age is associated with a higher frequency of comorbidity, which contributes to increased operative risk and renders decision-making for intervention more complex [2]. Under pathological pressure loading, such as AS, the left ventricle (LV) experiences a remodeling process that is characterized by the hypertrophic growth of cardiomyocytes and the increased deposition of extracellular matrix (ECM) elements [3]. The development of diffuse, reactive myocardial fibrosis is a major cause of abnormal myocardial stiffness [4] which results in systolic and/or diastolic dysfunction that is proportional to the degree of ECM deposition [5-8]. The extent of myocardial fibrosis, which may not be reversible after delayed surgery, has important prognostic implications, because it is an independent predictor of perioperative mortality [9] and a key determinant of long-term functional outcome following aortic valve replacement [5,10,11].

MicroRNAs (miRNAs) are non-coding small RNAs that modulate the expression of target genes by repressing translation and/or inducing mRNA degradation. The aberrant expression of miRNAs is associated with multiple pathological processes, including processes that affect the cardiovascular system [12-15]. To date, few studies have analyzed the differential expression profiling of miRNAs in failing human hearts of different etiologies in comparison with control patients [16-18]. The distinctive pattern of

miRNA expression changes between heart diseases has led to the suggestion that miRNAs may have etiological implications and that their expression profiles could be useful as potential biomarkers for diagnosing patients and predicting the prognosis and response to therapy [19]. One particular miRNA that is consistently deregulated under various cardiovascular pathological conditions is miR-21 [20]. Through loss-of-function and gain-of-function approaches, a key regulatory role for miR-21 has been evidenced in the pathological remodeling of the myocardium induced by hemodynamic stress [21-23]. The up-regulation of miR-21 in response to cardiac injury protects fibroblasts against apoptosis, which results in LV interstitial fibrosis and hemodynamic dysfunction [22]. The relationship of miR-21 up-regulation with the remodeling of ECM has also been demonstrated in mouse models of ischemia/reperfusion and acute myocardial infarction [24-26]. miR-21 was postulated to repress the transcription of myocardial mRNA targets including sprouty homolog 1 (SPRY1) [22], phosphatase and tensin homolog (PTEN) [23] and programmed cell death 4 (PDCD4) [27,28]. In addition, some miR-21 targets validated in extracardiac tissues [29], such as reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and the tissue inhibitor of metalloproteinase 3 (TIMP3), play key roles in the control of the ECM homeostasis [30,31] which deserve further attention in the scenario of human pressure overload.

Transforming growth factor-β1 (TGF-β1) is a crucial player in the LV remodeling response to hemodynamic stress [3] both in rodent models [32,33] and in AS patients [5,8,34]. The involvement of miR-21 in the molecular mechanism underlying the effects of TGF-β1 has become a key focus of recent research. Smads, which are the canonical effectors of TGF-β signaling, regulate the transcription of primary miR-21 and its post-

transcriptional processing by the DROSHA complex [35]. In TGF-β-dependent events, miR-21 is closely linked to the abnormal an excessive deposition of ECM [20,36], and several reports determined that miR-21 acts downstream of TGF-β to modulate the genesis and progression of pathological fibrotic processes in the liver [37], kidneys [38,39] and lungs [40]. Overall, these findings led us to hypothesize that miR-21 could be an actor in the pressure overload-induced ECM remodeling process dependent on TGF-β signaling in AS patients.

Recent reports indicate the presence of circulating miRNAs in microvesicles or protein–miRNA-complexes that can be consistently quantified in human plasma and other body fluids [41]. Several studies reported altered plasma levels of various miRNAs in cardiovascular diseases [42], including acute myocardial infarction, stable coronary artery disease, acute and chronic heart failure and myocarditis [43]. Therefore, the altered circulating levels of selected miRNAs may reflect specific cardiovascular pathologies and could be useful biomarkers for cardiovascular diseases. However, there are no data available on the possible function of circulating/extracellular miRNAs as endocrine/paracrine signaling molecules that contribute to cardiovascular disease pathogenesis.

Thus, the aim of this study was to investigate the following: (a) the role of both myocardial and plasmatic miR-21 in the maladaptive ECM remodeling induced by pressure overload in AS patients; (b) the association between validated miR-21 targets and TGF-β signaling effectors; and (c) the potential clinical value of miR-21 as a disease biomarker for pathological myocardial fibrosis progression.

Methods

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [44].

Study populations

The clinical and demographic characteristics of the AS and control groups are shown in Table 1. The study was performed using LV myocardial intraoperative biopsies and plasma samples obtained from a cohort of 75 consecutive patients diagnosed with isolated severe AS and undergoing aortic valve replacement surgery in the University Hospital Marqués de Valdecilla in Santander, Spain. Patients with aortic or mitral regurgitation greater than mild or with major coronary stenosis greater than 50%, previous cardiac operations, malignancies or poor renal or hepatic function were deemed ineligible for the study. The control group for comparing the myocardial gene expression was a cohort of 32 surgical patients with pathologies (atrial septal defect: n=20, aortic aneurysm: n=6, mitral stenosis: n=3, left atrial myxoma: n=2, pulmonary valve fibroelastoma: n=1) that did not include pressure or volume overload, coronary heart disease or cardiomyopathies. The reference values for the circulating miR-21 plasma levels were obtained from a cohort of 25 healthy volunteers who were age-matched with the AS patients and lacked previous cardiac disease. The study followed the Declaration of Helsinki guidelines for investigation in humans. The institutional ethics and clinical research committee of the University Hospital Valdecilla approved the study and subjects gave written informed consent.

Echocardiography

A two-dimensional transthoracic echocardiogram was performed (Philips-Hewlett Packard, IE 33) preoperatively. Images were digitalized and analyzed off-line (Xcelera software, Philips). Internal LV end-diastolic and end-systolic diameters (LVEDD and LVESD respectively) and interventricular septum thickness (IVST) and posterior wall thickness (PWT) were measured according to the American Society of Echocardiography guidelines, using bidimensional or M-mode images depending on the quality and angulation between the ultrasound beam and the LV. LV ejection fraction (LVEF) was calculated using Quiñones formula and LV mass (LVM) was estimated according to the Devereux formula and indexed to patient height in meters to the 2.7th power. Peak and mean transvalvular aortic gradients were obtained using the modified Bernoulli equation.

Myocardial and plasma sampling

Myocardial tru-cut needle subepicardial biopsies (4-10 mg) were collected from the lateral LV wall during surgery and were immediately either frozen in liquid nitrogen or fixed in paraformaldehyde. Peripheral venous blood samples were collected from healthy volunteers and AS patients; in the latter group, the samples were drawn 24 h preoperatively. To minimize platelet degranulation, the blood was drawn from an antecubital vein without tourniquet, using a syringe with a wide-gauge needle and the blood was then gently transferred to a collection tube containing EDTA. Within 15 minutes of collection, the plasma was separated by centrifugation at 1000×g for 30 min; plasma aliquots were stored at -80°C until analysis.

Determination of mRNA and microRNA expression by quantitative reverse transcription PCR

Total RNA was isolated from the LV using TRIzol (Invitrogen), according to the manufacturer's protocol. The mRNA was reverse transcribed using random primers with an RT-PCR kit (Fermentas) and the tissue miRNAs were reversed transcribed using specific primers for miR-21 and RNU6B (TaqMan assays, Applied Biosystems). The cDNA products were amplified using quantitative PCR (q-PCR) and an MX-3000P Stratagene thermocycler using specific TaqMan assays (Applied Biosystems) for the following: (a) miR-21; (b) the remodeling-related genes TGF-β1, Smad2, Smad3, Smad4, TAK-1, collagen I α1 (Col I), collagen III α1 (Col III), fibronectin-1 (FN), and (c) the miR-21 target genes PTEN, PDCD4, RECK, TIMP3 and SPRY1.

The expression levels of myocardial genes were normalized to the housekeeping gene, ribosomal 18S, which was measured in parallel for each sample e. The myocardial miR-21 expression was normalized to the endogenous control, RNU6B. No differences in the threshold cycles of RNU6B between the AS patients (27.1±0.8) and controls (27.3± 0.7) were found.

Total RNA was isolated from the plasma samples using TRIzol. The isolation efficiency of the plasma miRNAs was assessed using spiked-in Caenorhabditis elegans miRNA (cel-miR-39) (Qiagen) lacking sequence homology to human miRNAs, as described previously [45]. The oligonucleotides were spiked into the samples during the RNA isolation after incubating the plasma with the denaturing solution. The plasma miRNAs were reverse transcribed using specific primers for mi-R21 and cel-miR-39 (Applied Biosystems), and the plasma miR-21 levels were normalized to cel-miR-39. To assure that the isolation efficiency was homogeneous between the samples, the

extraction procedure was repeated, if necessary, until the cel-miR-39 cycle threshold fell within a range of 23.0±1.0.

miR-21 in situ hybridization

A digoxigenin (DIG)-double-labeled specific miR-21 probe (5´-DIG/TCA ACA TCA GTC TGA TAA GCT A/DIG-3´) and a scramble miRNA probe (5´-DIG/GTG TAA CAC GTC TAT ACG CCC A/DIG-3´) as a negative control were purchased from Exiqon (Vedbaek, Denmark). The procedure for the in situ hybridization was conducted in accordance with the manufacturer's instruction using 4 μm sections of the paraformaldehyde-fixed, paraffin-embedded LV samples [46]. Digital photographs of the LV sections were captured using a camera (Axiocam MRc5, Zeiss) attached to a microscope (Leica DM IRB).

Statistics

The data sets were assessed for normality with the Kolmogorov-Smirnov test. Continuous variables were compared using two-tailed Student's t-test when Gaussian and the Mann-Whitney U test when non-Gaussian. miR-21 was measurable in all patients and was treated as a continuous variable. Variables that were not normally distributed were transformed to their natural logarithm for the regression analyses. Correlations between the expression levels of miR-21 and cardiac remodeling genes were performed using Pearson's correlation coefficient. Multiple linear regression analysis was used to identify the predictors of the myocardial collagen I expression variance. Independent variables introduced in the regression equation were assessed for multicollinearity and eliminated when appropriate. Models were validated using the bootstrap method with 2,000 replications. The threshold for statistical significance was

p<0.05. The following statistical packages were used: GraphPad Prism 5.03, PASW

Statistics 18 (SPSS Inc, Chicago, IL) and Stata 10 (StataCorp LP, College Station, TX).

Results

The preoperative echocardiographic, clinical and demographic data of the patient

cohorts are shown in Table 1. Patient demographic and clinical characteristics were

presented as means $\pm$ standard deviation.

Myocardial and circulating levels of miR-21 were higher in the AS patients than in

the control patients and correlated directly with the transvalvular gradients

Patients with AS featured higher myocardial expression levels of primary

transcripts (pri-miR-21) as well as mature miR-21 (Fig 1A) compared with a group of

surgical controls with no pressure or volume overload and normal left ventricular mass

[pri-miR-21(surgical controls: 8.9±2.4 vs. AS:15.0±9.9; p<0.05 Mann-Whitney test);

miR-21 (surgical controls: 45.3±5.7 vs. AS: 80.3±7.7; p<0.05 Mann-Whitney test)]. Since

AS patients were significantly older than surgical controls (Table 1), we ruled out an

influence of age on miR-21 expression by comparing subgroups of age-matched

individuals from both cohorts (38 AS patients aged 63.0±10.5 years vs. 15 surgical

controls aged 59.8±9.0 years). As shown in Fig 1B, the difference of miR-21 expression

between the subgroups of age-matched patients was similar to the observed in the full

cohorts.

Using in situ hybridization (Fig 1 C), we detected intense miR-21 signals in the LV

myocardium from the AS patients. The signal was restricted to the cells located in the

interstitial space, presumably fibroblasts, whereas no miR-21 signals were detected in the cardiomyocytes. In contrast, there was no miR-21signal or only a very weak hybridization signal in the LV myocardia from the control patients.

The myocardial miR-21 expression levels displayed no significant differences related to sex (women: 92.2±12.7; men: 77.0±9.2), hypertension (normotensive: 93.9±13.2; hypertensive: 75.7±8.9), obesity (BMI<30: 81.65±9.9; BMI>30: 73.6±7.8) and diabetes mellitus (non-diabetic: 87.4±9.4; diabetic: 73.4±10.6). The myocardial levels of miR-21 did not correlate significantly with the LV mass index (R=0.19), the body mass index (R=-0.08) or the age (R=-0.01).

The circulating concentrations of miR-21 (Fig 1D) were significantly higher in the AS patients than in the age-matched healthy volunteers of similar demographic characteristics and also higher than in surgical controls [AS patients (20.1±3.7) vs. healthy individuals (3.9±0.6): p<0.01; and AS patients vs. surgical controls (8.8±1.8): p<0.05)]. The difference between healthy and surgical controls did not reach statistical significance. There was a direct and positive correlation between the myocardial expression and plasma levels of miR-21 (Fig 1E), suggesting that the myocardium is among the relevant sources that contribute to the circulating miR-21.

Both, the myocardial and plasma levels of miR-21 maintained significant positive correlations with the mean transvalvular aortic gradients (Figs 1 F and G), which suggests a relationship between the level of the LV hemodynamic burden and miR-21 up-regulation.

Sex (women: 23.5±5.9; men: 17.8±4.8), hypertension (normotensive: 19.0±5.5; hypertensive: 20.4±5.2); obesity (BMI<30: 20.1±4.5; BMI>30: 23.4±7.7) and diabetes

mellitus (non-diabetic: 21.0±4.5; diabetic: 17.4±4.2) did not have a significant effect on the circulating miR-21 levels. LV mass index (R=0.01), body mass index (R=0.13) and age (R=-0.11) did not correlate significantly with the plasma levels of miR-21.

Myocardial and circulating levels of miR-21 correlated positively with the myocardial expression levels of genes encoding ECM remodeling-related elements and TGF-β1

The relative degree of myocardial fibrosis displayed by our cohort of AS patients was assessed by quantifying the LV expression levels of several ECM genes. The expression levels of collagen I, collagen III and fibronectin were significantly higher in the AS patients than in the surgical controls (Table 2). The myocardial and circulating levels of miR-21 were positively correlated with the LV myocardial expression levels of genes encoding collagen I and fibronectin (Table 3). The expression levels of genes encoding TGF-β1 and its effectors are shown in Table 2. The LV transcript levels of TGF-β1 (Table 3) sustained significant positive correlations with the myocardial expressions of both pri-miR-21 (R=0.69; p<0.01) and mature miR-21 (R=0.51; p<0.001), as well as with the plasma levels of miR-21 (R=0.50; p<0.001).

Myocardial expression levels of miR-21 together with TGF-β effectors and miR-21 target mRNAs predicted the variance of myocardial collagen I mRNA expression levels

The bootstrap validated multiple linear regression analysis identified the myocardial expression levels of miR-21, Smad2 and TAK-1 as independent, significantly, positive predictors of collagen I expression variance. The resulting equation was the following: Collagen I = 0.08 + 0.014 (miR-21) + 0.87 (Smad2) + 1.21 (TAK-1).

The adjusted R2 (0.42; p<0.0001) indicated that the 42% of variance in myocardial collagen I can be predicted from this model.

In a second step, we assessed whether introducing the myocardial expression levels of miR-21 targets, such as SPRY1, PTEN, TIMP3, RECK and PDCD4, in the regression model as independent variables would improve the prediction power of the equation. Table 2 shows the expression levels of the miR-21 targets in AS patients and surgical controls. As shown in Table 4, PDCD4 was a significant negative predictor of collagen I expression, whereas RECK, miR-21, Smad2 and TAK-1 appeared positively related to fibrosis [Collagen I = -0.41 + 0.02 (myocardial miR-21) + 8.73 (RECK) – 0.77 (PCDC4) + 0.78 (Smad2) + 1.09 (TAK-1)]. The predictive strength of the resulting equation (adjusted R2: 0.70; p<0.0001) was clearly improved with these changes. In this model, myocardial miR-21 expression was the relatively largest contributor to collagen I variance, as indicated by its highest standardized beta coefficient (beta=0.58, Table 4).

Circulating levels of miR-21 together with TGF-β effectors and miR-21 target mRNAs predicted the variance of myocardial collagen I mRNA expression levels

We reproduced the same analysis substituting the miR-21 myocardial expression levels with the miR-21 plasma levels. The circulating miR-21, myocardial Smad2 and TAK-1 levels constituted significant positive predictors of the collagen I expression variance. The resulting equation (Collagen I = 0.33 + 0.019 (miR-21) + 0.79 (Smad2) + 1.55 (TAK-1)] was similar to the one presented above but with a lower predictive strength (adjusted R2: 0.33; p<0.0001).

Following the introduction of SPRY1, PTEN, TIMP3, RECK and PDCD4 expressions as independent variables in the regression model (Table 5), PDCD4 still

constituted a significant negative predictor of collagen I expression and RECK, miR-21, and TAK-1 appeared positively related to fibrosis (Collagen I =-0.06 + 0.024 (plasma miR-21) + 9.80 (RECK) – 0.52 (PCDC4) + 1.57 (TAK-1)]. The inclusion of these two miR-21 targets still improved the predictive strength of the resulting equation (adjusted R2: 0.52; p<0.0001).

Age, sex, body mass index, hypertension or diabetes mellitus did not improve the predictive power of the model and revealed no significant relationships with myocardial collagen I mRNA.

Myocardial expression and circulating levels of miR-21 lacked of significant value for LV mass prediction

There was no correlation between myocardial or plasma levels of miR-21 and LV mass or its related echocardiographic parameters. Further, multivariate analysis failed to find a significant value of miR-21, in combination with other clinical and gene expression parameters, to predict crude or indexed LV mass.

Discussion

Abundant evidence derived from basic research suggests a key role for several miRNAs, including miR-21, in the pathophysiology of many cardiovascular pathological processes. However, a direct assessment of their involvement in human heart diseases has rarely been addressed [19]. In the present study, which was performed in one of the largest series of intraoperative LV myocardial biopsies from AS patients, we provided evidence supporting a significant role for miR-21 in the pathological remodeling of the myocardium in the scenario of human aortic valve stenosis. We found that myocardial

expression of miR-21 was significantly higher in AS patients than in surgical controls, as previously reported in mice subjected to aortic arch constriction [22]. The miR-21 levels were proportional to the mean aortic transvalvular gradients; that is, to the degree of hemodynamic overload that the LV of the AS patients had to bear. This result suggests a link between the severity of the pathology and miR-21 up-regulation in the myocardium. Ikeda et al reported that miR-21 is not among the miRNAs whose expression is deregulated in the LV, as based on a cohort of 13 AS patients [47]. This divergent finding may be accounted for by differences in the area where the biopsies were harvested or by the different technologies used for the miRNA determination.

Although, miR-21 null mice display no alterations in the LV remodeling responses to different stressors [48], several studies in rodent experimental models support a profibrotic effect mediated by miR-21 in the myocardium under pressure overload [21] and in other pathological conditions, including ischemia/reperfusion injury and acute myocardial infarction [24]. Herein, the miR-21 overexpression was confined to the interstitial cells within the ECM, which is in agreement with a predominant presence of miR-21 in the cardiac fibroblasts as described previously [22-24]. This topographic distribution and the positive correlation between the myocardial expression levels of miR-21 and the mRNAs that encode fibrillar proteins, such as collagen I and fibronectin, strongly support the hypothesis that miR-21 can act as an intracellular mediator with an active role in the maladaptive remodeling of the myocardial ECM that is triggered by pressure overload in AS patients.

TGF-β is one of the most important promoters of the excessive and abnormal deposition of ECM proteins in the myocardium under biomechanical stress [3]. In a

previous report [8] and in the present study, the AS patients exhibited myocardial overexpression of TGF-β1, which was accompanied by a proportional up-regulation of fibrosis-related mRNAs. We observed that the myocardial transcript levels of TGF-β1 also maintained positive relationships with the expression levels of pri-miR21 and mature miR-21, which agree with the regulatory roles of TGF-β signaling on miR-21 transcription [20] and post-transcriptional maturation [35,49]. In addition, miR-21 is also critically involved in the mechanisms underlying the profibrogenic effect of TGF-β1: it regulates the TGF-β1-induced transdifferentiation of cultured fibroblasts into myofibroblasts [36], and the process of endothelial-to-mesenchymal transition, which is dependent on TGF-β1, both in vitro and in the pressure overloaded murine heart [23]. Furthermore, miR-21 contributes to the genesis and progression of fibrotic processes in animal models by acting downstream of TGF-β1 [37-40]. Our results showing a coordinated expression of miR-21 and the mRNAs encoding TGF-β1 and its signaling effectors in the myocardium of AS patients, strongly suggest that these molecules may constitute a cluster of functionally interacting elements whose expression is tightly coordinated to regulate the remodeling of the ECM under conditions of pressure overload. This idea is further supported by our multiple regression analysis which showed that the myocardial mRNA expression levels of Smad2 and TAK-1, together with miR-21 predicted 42% of the variance of the collagen I expression levels, whereas miR-21 alone predicted only 24%.

In a further step, we assessed whether the expression of a series of miR-21 target mRNAs (SPRY, PTEN, TIMP3, RECK and PDCD4) contributed to predicting myocardial fibrosis more than the set of variables composed of miR-21, Smad2 and

TAK-1. The inclusion of PDCD4 and RECK as independent variables in the multiple

regression analysis led to a robust improvement in the predictive strength of the final

model that accounted for 70% of the variance in the expression of collagen I. PDCD4

constituted a negative predictor of collagen I expression, whereas RECK, miR-21,

Smad2 and TAK-1 expression levels appeared to be positively related to fibrosis in the

resulting equation. The repression of PDCD4 by miR-21 enhances the TGF-β1-

stimulated transdifferentiation of cultured fibroblasts into myofibroblasts [36]. In addition,

the protective effect of miR-21 against ischemia-induced cardiac cell death is mediated,

at least in part, by targeting PDCD4.27-29 In our study, the behavior of PDCD4 as a

negative predictor of fibrosis and its inverse relationship with miR-21 indicate that it

performed, as expected, as a miR-21 target involved in the remodeling of the ECM in AS

patients.

The membrane-anchored metalloproteinase (MMP) regulator RECK behaved as

a positive predictor of collagen I expression in our multiple regression model. This result

is in agreement with the reported profibrogenic effects exerted by RECK in pancreatic

stellate cells [50]. Furthermore, in patients with atrial fibrillation, pathological atrial

fibrosis is associated with increased levels of RECK and results in severe alterations in

the turnover of collagens I and III [51]. Mutant mice lacking RECK display an elevated

MMP activity that is accompanied by a greatly reduced amount of type I collagen and

important defects in collagen fibrils [28]. RECK is a target of repression by miR-21 in

cancer cells [29]. Herein, however, RECK did not behave as would be expected for a

miR-21 target because, similar to miR-21, RECK positively predicted collagen

expression, and its expression levels did not correlate inversely with miR-21.

In our study, the lack of predictive power of collagen I expression by SPRY, PTEN and TIMP3 suggests that these proteins are not involved in the pathophysiological imbalance between pro- and anti-fibrotic forces in severe human AS. Species-related differences and limitations in the adequacy of experimental models for the clinical diseases may account for the dissimilarities observed in the human scenario.

The regulatory role of miR-21 in cardiomyocyte hypertrophy is a controversial matter at present. Whereas some studies find that miR-21 promotes the hypertrophic growth of cultured neonatal cardiomyocytes [21], others report that miR-21 represses the hypertrophic growth of myocytes [52]. Studies in vivo support that, under myocardial biomechanical stress such as pressure overload, the proliferation of fibroblasts, which are the major source of miR-21 in the heart, would be responsible for miR-21 enrichment, fibrosis development and subsequent indirect effects on cardiomyocytes [22]. In our study, there was no correlation between the myocardial expression of miR-21 and the LV mass. Further, multivariate analysis failed to find a significant value of miR-21, in combination with other clinical and gene expression parameters, to predict LV mass. Our results suggest a limited pathophysiological relevance of miR-21 in the development of myocardial hypertrophy under pressure overload in AS patients. On the other hand, we reported previously that myocardial miR-133a is negatively correlated with LV mass in the same cohort of AS patients, and its expression levels predict regression of LV hypertrophy after aortic valve replacement [15].

The preoperative circulating levels of miR-21 were higher in the AS patients when compared to cohorts of healthy individuals and surgical controls with pathologies that did not include pressure overload. The plasma levels of miR-21 directly correlated with the

myocardial expression levels of miR-21 and also maintained a direct linear relationship with the mean aortic transvalvular gradients. A remarkable observation is that myocardial fibrosis can be influenced by circulating miR-21, as suggested by the direct correlation between the preoperative plasma levels of miR-21 and the myocardial expression levels of collagen I and fibronectin. Furthermore, substitution of myocardial miR-21 mRNA levels by circulating miR-21 in the multiple regression analysis and inclusion of RECK, PDCD4 and TAK-1 led to a model that predicts 52% of the myocardial collagen I variance. The biological significance of circulating miRNAs is unclear, and to our knowledge, there is currently no evidence demonstrating a functional role for plasma miRNAs via distant gene modulation in humans. Our present results strongly suggest, for the first time, that circulating miR-21 may function as a paracrine signal to influence the remodeling of the myocardial ECM. The potential sources of miR-21 in the plasma may be multiple because miR-21 is almost ubiquitously expressed; however, the direct correlation observed between the myocardial expression and plasma levels of miR-21 suggests that myocardial cells are among the relevant contributors to the levels of circulating miR-21.

Specific signatures of circulating miRNAs may serve as novel biomarkers for diagnostic use and/or prognostic stratification [53]. Our results strongly suggest that the plasma levels of miR-21 could constitute a clinically relevant measurable characteristic of AS patients that provide information on the extent of myocardial fibrosis. Although miR-21 is not cardiac specific and its predictive strength on myocardial fibrosis-related gene expression is moderate, these facts do not exclude the use of miR-21 as a biomarker but suggest its use in conjunction with other clinical, echocardiographic or

serological markers to construct a myocardial fibrosis risk profile. Furthermore, the reliable assessment of the time progression of myocardial fibrosis using such a tool may add criteria for earlier valve replacement surgery, avoiding the dismal prognosis that is associated with severe fibrosis and help in the follow-up of changes promoted by pharmacological or surgical treatments.

Disclosures

None

References

1. Vahanian A, Baumgartner H, Bax J, Butchart E, Dion R, Filippatos G, Flachskampf F, Hall R, Iung B, Kasprzak J, Nataf P, Tornos P, Torracca L, Wenink A. Guidelines on the management of valvular heart disease: the task force on the management of valvular heart disease of the European Society of Cardiology. Eur Heart J 2007; 28:230-268.

2. Faggiano P, Frattini S, Zilioli V, Rossi A, Nistri S, Dini FL, Lorusso R, Tomasi C, Cas LD. Prevalence of comorbidities and associated cardiac diseases in patients with valve aortic stenosis. Potential implications for the decision-making process. Int J Cardiol 2011; Mar 2.

3. Yetkin E, Waltenberger J. Molecular and cellular mechanisms of aortic stenosis. Int J Cardiol 2009;135:4-13.

4. Yamamoto K, Masuyama T, Sakata Y, Nishikawa N, Mano T Yoshida J, Mano T, Yoshida J, Miwa T, Sugawara M, Yamaguchi,Y, Ookawara T, Suzuki K, Hori M. Myocardial stiffness is determined by ventricular fibrosis, but not by compensatory or excessive hypertrophy in hypertensive heart. Cardiovasc Res 2002; 55:76–82.

5. Yarbrough WM, Mukherjee R, Ikonomidis JS, Zile MR, Spinale FG. Myocardial remodeling with aortic stenosis and after aortic valve replacement: Mechanisms and future prognostic implications.J Thorac Cardiovasc Surg 2011; Jul 13.

6. Polyakova V, Loeffler I, Hein S, Miyagawa S, Piotrowska I, Dammer S, Risteli J, Schaper J, Kostin S.Fibrosis in endstage human heart failure: severe changes in collagen metabolism and MMP/TIMP profiles. Int J Cardiol 201; 151:18-33.

7. Heymans S, Schroen B, Vermeersch P, Milting H, Gao F, Kassner A, Gillijns H, Herijgers P, Flameng W, Carmeliet P, van de Werf F, Pinto YM, Janssens S. Increased cardiac expression of tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 is related to cardiac fibrosis and dysfunction in the chronic pressure-overloaded human heart. Circulation. 2005;112:1136-1144.

8. Villar AV, Llano M, Cobo M, Expósito V, Merino R, Martín-Durán R, Hurlé MA, Nistal JF. Gender differences of echocardiographic and gene expression patterns in human pressure overload left ventricular hypertrophy. J Mol Cell Cardiol 2009; 46:526–535.

9. Herrmann S, Störk S, Niemann M, Lange V, Strotmann JM, Frantz S, Beer M, Gattenlöhner S, Voelker W, Ertl G, Weidemann F. Low-gradient aortic valve stenosis myocardial fibrosis and its influence on function and outcome. J Am Coll Cardiol 2011; 58:402-412.

10. Dweck MR, Joshi S, Murigu T, Alpendurada F, Jabbour A, Melina G, Banya W, Gulati A, Roussin I, Raza S, Prasad NA, Wage R, Quarto C, Angeloni E, Refice S, Sheppard M, Cook SA, Kilner PJ, Pennell DJ, Newby DE, Mohiaddin RH, Pepper J, Prasad SK. Midwall fibrosis is an independent predictor of mortality in patients with aortic stenosis. J Am Coll Cardiol 2011; 58:1271-1279.

11. Lund LH, Kristensen O, Baandrup U, Hansen OK, Nielsen TT, Emmertsen K, Jensen FT, Flo C, Rasmussen BS, Pilegaard HK. Myocardial structure as a

determinant of pre- and postoperative ventricular function and long-term prognosis after valve replacement for aortic stenosis. Eur Heart J 1998; 19:1099-1108.

12. Weidemann F, Herrmann S, Störk S, Niemann M, Frantz S, Lange V, Beer M, Gattenloehner S, Voelker W, Ertl G, Strotmann JM. Impact of myocardial fibrosis in patients with symptomatic severe aortic stenosis. Circulation 2009; 120:577-584.

13. Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. Circulation 2010; 121:1022-1032.

14. Tijsen AJ, Pinto YM, Creemers EE. Non-cardiomyocyte microRNAs in Heart Failure. Cardiovasc Res 2011; Dec 16.

15. Villar AV, Merino D, Wenner M, Llano M, Cobo M, Montalvo C, García R, Martín-Durán R, Hurlé JM, Hurlé MA, Nistal JF. Myocardial gene expression of microRNA-133a and myosin heavy and light chains, in conjunction with clinical parameters, predict regression of left ventricular hypertrophy after valve replacement in patients with aortic stenosis. Heart. 2011; 97:1132-1137.

16. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A 2006 ;103:18255-18260.

17. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J.

MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 2007; 116:258–267.

18. Naga-Prasad SV, Duan ZH, Gupta MK, Surampudi VS, Volinia S, Calin GA, Liu CG, Kotwal A, Moravec CS, Starling RC, Perez DM, Sen S, Wu Q, Plow EF, Croce CM, Karnik S. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. J Biol Chem 2009; 284:27487-27499.

19. Ikeda S, Pu WT. Expression and function of microRNAs in heart disease. Curr Drug Targets 2010; 11:913-925.

20. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol 2011; 8:5.

21. Cheng Y, Ji R, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? Am J Pathol 2007; 170:1831-1840.

22. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 2008; 456:980-984.

23. Kumarswamy R, Volkmann I, Jazbutyte V, Dangwal S, Park DH, Thum T. Transforming growth factor-β-induced endothelial-to-mesenchymal transition is partly mediated by microRNA-21. Arterioscler Thromb Vasc Biol 2011; Nov 17.

24. Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 2009; 82:21-29.

25. Haghikia A, Hilfiker-Kleiner D. MiRNA-21: a key to controlling the cardiac fibroblast compartment? Cardiovasc Res 2009; 82:1-3.

26. Zhu H, Fan GC. Role of microRNAs in the reperfused myocardium towards post-infarct remodelling. Cardiovasc Res 2011; Nov 29.

27. Cheng Y, Zhu P, Yang J, Liu X, Dong S, Wang X, Chun B, Zhuang J, Zhang C.Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4. Cardiovasc Res 2010; 87:431-439.

28. Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C. MicroRNA-21 protects against theH(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. J Mol Cell Cardiol 2009; 47:5-14.

29. Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS, Krichevsky AM. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008 ;28:5369-5380.

30. Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C, Noda M. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. Cell 2001; 107:789-800.

31. Kassiri Z, Defamie V, Hariri M, Oudit GY, Anthwal S, Dawood F, Liu P, Khokha R. Simultaneous transforming growth factor beta-tumor necrosis factor activation and cross-talk cause aberrant remodeling response and myocardial fibrosis in Timp3-deficient heart. J Biol Chem 2009; 284:29893-29904.

32. Bujak M, Frangogiannis NG. The role of TGF-β signaling in myocardial infarction and cardiac remodeling. Cardiovascular Research 2007; 74:184–195.

33. Koitabashi N, Danner T, Zaiman AL, Pinto YM, Rowell J, Mankowski J, Zhang D, Nakamura T, Takimoto E, Kass DA. Pivotal role of cardiomyocyte TGF-β signaling in the murine pathological response to sustained pressure overload. J Clin Invest 2011; 121:2301-2312.

34. Villar AV, Cobo M, Llano M, Montalvo C, González-Vílchez F, Martín-Durán R, Hurlé MA, Nistal JF. Plasma levels of transforming growth factor-beta1 reflect left ventricular remodeling in aortic stenosis. PLoS One 2009; 4:e8476.

35. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 2008; 454:56-61.

36. Yao Q, Cao S, Li C, Mengesha A, Kong B, Wei M. Micro-RNA-21 regulates TGF-β-induced myofibroblast differentiation by targeting PDCD4 in tumor-stroma interaction. Int J Cancer 2011; 128:1783-1792.

37. Marquez RT, Bandyopadhyay S, Wendlandt EB, Keck K, Hoffer BA, Icardi MS, Christensen RN, Schmidt WN, McCaffrey AP. Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. Lab Invest 2010; 90:1727-1736.

38. Zarjou A, Yang S, Abraham E, Agarwal A, Liu G. Identification of a microRNA signature in renal fibrosis: role of miR-21. Am J Physiol Renal Physiol 2011; 301:F793-F801.

39. Zhong X, Chung AC, Chen HY, Meng XM, Lan HY. Smad3-mediated upregulation of miR-21 promotes renal fibrosis. J Am Soc Nephrol 2011; 22:1668-1681.

40. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, Abraham E. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med 2010; 207:1589-1597.

41. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids-the mix of hormones and biomarkers. Nat Rev Clin Oncol 2011; 8:467-477.

42. Xu J, Zhao J, Evan G, Xiao C, Cheng Y, Xiao J. Circulating microRNAs: novel biomarkers for cardiovascular diseases. J Mol Med (Berl). 2011 Dec 8.

43. Zhu H, Fan GC. Extracellular/circulating microRNAs and their potential role in cardiovascular disease. Am J Cardiovasc Dis 2011; 1:138-149.

44. Shewan LG and Coats AJ. Ethics in the authorship and publishing of scientific articles. Int J Cardiol 2010; 144:1-2.

45. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods 2010; 50:298-301.

46. Jørgensen S, Baker A, Møller S, Nielsen BS. Robust one-day in situ hybridization protocol for detection of microRNAs in paraffin samples using LNA probes. Methods 2010; 52:375-381

47. Ikeda S, Kong SW, Lu J, Bisping E, Zhang H, Allen PD, Golub TR, Pieske B, Pu WT. Altered microRNA expression in human heart disease. Physiol Genomics. 2007; 31:367-373.

48. Patrick DM, Montgomery RL, Qi X, Obad S, Kauppinen S, Hill JA, van Rooij E, Olson EN. Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. J Clin Invest 2010; 120:3912-3916

49. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. Mol Cell 2010; 39:373-384.

50. Lee H, Lim C, Lee J, Kim N, Bang S, Lee H, Min B, Park G, Noda M, Stetler-Stevenson WG, Oh J. TGF-beta signaling preserves RECK expression in activated pancreatic stellate cells. J Cell Biochem 2008; 104:1065-1074.

51. Polyakova V, Miyagawa S, Szalay Z, Risteli J, Kostin S. Atrial extracellular matrix remodelling in patients with atrial fibrillation. J Cell Mol Med 2008; 12:189-208.

52. Tatsuguchi M, Seok HY, Callis TE, Thomson JM, Chen JF, Newman M, Rojas M, Hammond SM, Wang DZ. Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy. J Mol Cell Cardiol 2007; 42:1137-1141.

53. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res 2011; Nov 9.

Figure 1. (**A**) The relative expression level (RE) of miR-21 in the myocardium from AS patients was significantly higher than in surgical controls. (**B)** The myocardial overexpression of miR-21 in the subgroup of aged-matched patients (red symbols) was similar to the full cohort (black symbols). **(C)** miR-21 expression (*in situ* hybridization using digoxigenin-labeled riboprobes). Compared with controls, the AS patients displaye strong overexpression of miR-21 that is localized in cells within the extracellular matrix. (**D**) The plasma levels of miR-21 were higher in AS patients in comparisson with either surgical controls or age-matched healthy volunteers. The circulating levels of miR-21 were not significantly different in the surgical controls than in the healthy volunteers (Mann-Whitney test). Linear regression analysis showed the positive correlation between myocardial (**E**) and plasma (**F**) expression levels of miR-21 (log-transformed) with the mean transvalvular gradients. (**G**) In AS patients, there was a positive correlation between LV myocardial and circulating expression levels of miR-21 (log-transformed). A and B: *p<0.05 vs. surgical controls. C: **p<0.01 vs. healthy volunteers; #p<0.05 vs. surgical controls.

Table 1. Clinical characteristics of the study subjects.

Variable	Aortic stenosis	Surgical controls	Healthy volunteers
Number	75	32	25
Age (yrs)	70.2±11.0*	53.6±15.5	70.8±7.1
Male/Female (n)	42/33	10/23	12/13
Systolic blood pressure, mm Hg	119.4±19.9	120.1±18.0	131.0±22.1
Diastolic blood pressure, mm Hg	66.2±11.9	69.8±10.4	75.3±11.8
LV mass index (g/m$^{2.7}$)	77.0±19.8***	39.8±14.0	49.7±14.5
Posterior wall thiknesses (mm)	12.7±2.2***	8.6±1.3	9.0±1.6
LV ejection fraction	62.2±11.5	61.9±8.2	62.0±8.5
Body Mass Index, kg/m^2	28.9±4.1	28.2±4.9	27.4±3.3
Body Mass Index ≥ 30, n (%)	25 (33)	10 (31)	5 (20)
Systemic hypertension, n (%)	41 (55)	14 (44)	5 (20)
Diabetes Mellitus, n (%)	17 (23)	2 (6)	0 (0)
Atrial fibrillation or flutter, n (%)	13 (17)	4 (13)	1 (4)
ACE inhibitors, n (%)	16 (21)	5 (16)	1 (4)
AT-II receptor antagonists, n (%)	9 (12)	4 (13)	1 (4)
Diuretics, n (%)	34 (45)	9 (28)	1 (4)
Calcium antagonists, n (%)	12 (16)	3 (9)	2 (8)
β-Blockers, n (%)	11 (15)	10 (31)	3 (12)
Statins, n (%)	23 (31)	2 (6)	6 (24)

Patient demographic and clinical characteristics are presented as means ± standard deviation. *p<0.05, ***p<0.001 AS patients vs. surgical controls (Mann Whitney U test). ACE = Angiotensin converting enzyme; AT-II = Angiotensin II

Table 2. Myocardial expression levels of genes encoding the extracellular matrix proteins collagen I, collagen III and fibronectin, TGF-β1 and its effectors Smad2, Smad3, Smad4 and TAK-1, and the mir-21 targets PTEN, TIMP3, PCDC4, SPRY1 and RECK. AS patients vs. controls: $*p<0.05$, $***p<0.001$ (Student's t test or Mann Whitney U test).

	Controls	AS patients
Collagen I	2.2±0.2	4.4±0.2***
Collagen III	5.4±0.4	7.0±0.6*
Fibronectin	5.7±0.8	9.0±0.6*
TGF-β1	1.7±0.1	2.4±0.1*
Smad2	1.4±0.2	1.5±0.2
Smad3	1.7±0.2	1.5±0.09
Smad4	4.1±0.2	3.8±0.2
TAK-1	1.2±0.01	1.3±0.02
PTEN	1.7±0.1	1.3±0.1*
TIMP3	4.7±0.3	3.4±0.1***
PCDC4	3.2±0.3	3.1±0.2
SPRY1	0.04±0.002	0.03±0.004
RECK	0.37±0.03	0.32±0.02

Table 3. Pearson's correlation coefficient values (R) obtained from the linear regression analyses correlating the myocardial and plasma levels of miR-21 with the myocardial mRNA expression levels of collagen I (Col I), fibronectin 1 (FN) and TGF-β1 in the AS patients.

	Col I	FN	TGF-β1
Myocardial miR-21	0. 49***	0.53***	0.51***
Plasma miR-21	0. 41***	0.54***	0.50***

Table 4. Myocardial miR-21 is a significant predictor of the myocardial collagen I expression variance in the AS patients. A) Model calculated using bootstrap validated multiple linear regression analysis; **B**) goodness of fit and **C**) significant predictors of the myocardial expression levels of collagen I.

A)   Collagen I = -0.41 + 0.02 (myocardial miR-21) + 8.73 (RECK) – 0.77 (PCDC4) + 0.78 (Smad2) + 1.09 (TAK-1)

B)   Adjusted R^2: 0.70; $p < 0.0001$

C)

Variable	Coeff. B	Std. Error	Beta	p value
Myocardial miR-21	0.02	0.003	0.58	<0.0001
RECK	8.73	1.42	0.55	<0.0001
PCDC4	-0.77	0.16	-0.45	<0.0001
Smad2	0.78	0.33	0.27	<0.02
TAK-1	1.09	0.55	0.19	<0.05

Table 5. Circulating miR-21 is a significant predictor of the myocardial collagen I expression variance in the AS patients. A) Model calculated using bootstrap validated multiple linear regression analysis; **B**) goodness of fit; and **C**) significant predictors of the myocardial expression levels of collagen I.

A)   Collagen I =-0.06 + 0.024 (plasma miR-21) + 9.80 (RECK) – 0.52 (PCDC4) + 1.57 (TAK-1)

B)   Adjusted R^2: 0.52; $p < 0.0001$

C)

Variable	Coeff. B	Std. Error	Beta	p value
RECK	9.80	1.56	0.62	<0.0001
PCDC4	-0.52	0.19	-0.30	<0.008
TAK-1	1.57	0.53	0.33	<0.004
Plasma miR-21	0.024	0.008	0.27	<0.006

Figure 1