# miR-92a: A Novel Potential Biomarker of Rapid Aortic Valve Calcification

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*Background and aim of the study:* **The study aim was to compare the tissular expression of microRNAs (miRs) in bicuspid and tricuspid valves, and to evaluate their use as potential novel biomarkers of aortic valve calcification in bicuspid valves.**
*Methods:* **A prospective single-center observational study was conducted on stenotic bicuspid and tricuspid human aortic valves. According to their potential role in valve vascular and valvular calcification, a decision was taken to include miR-92a, miR-141, and miR-223 in this analysis. A real-time quantitative polymerase chain reaction was used to measure the expression of each miR, using U6 and Cel-miR-39 as endogenous and exogenous gene controls, respectively.**
*Results:* **Among a total of 47 human calcified aortic valves collected, 30 (63.8%) were tricuspid valves. The mean preoperative transvalvular gradient was 50.8 mmHg (range: 37-89 mmHg), with no significant difference between bicuspid and tricuspid valves (50 mmHg versus 51.2 mmHg; p = 0.729). The mean aortic valve area was 0.79 cm$^2$ (range: 0.33-1.3 cm$^2$), again with no significant difference between the two groups (p = 0.34). The level of miR-92a expression was twofold higher in bicuspid valves compared to tricuspid valves (0.38 versus 0.17; p = 0.016), but no significant difference in miR-141 and miR-223 expression was observed between the two groups (p = 0.68 and p = 0.35, respectively). A positive correlation was observed between miR-92a expression and mean preoperative transvalvular gradient (r = 0.3257, p = 0.04).**
*Conclusion:* **miR-92a is overexpressed in calcified bicuspid aortic valves, and may serve as a potential biomarker of rapid aortic valve calcification. Further studies based on these results may be designed to correlate the relative expression of miR-92a in the serum with its tissular expression in AS.**

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Aortic stenosis (AS) is the most common form of valvular heart disease in the elderly (1). It occurs in a normal tricuspid aortic valve (TAV), in a degenerative setting, especially in patients aged >65 years, but may also occur earlier during adulthood, in congenitally abnormal aortic valves, such as bicuspid aortic valves (BAVs), which occur in 1-2% of the overall population (2), suggesting an accelerated calcification process in the case of bicuspid valves. Calcific AS is the result of a complex molecular and cellular pathogenic process, involving shear stress, inflammation, osteogenesis, and fibroblastic differentiation (3). The diagnosis and follow up of calcific AS is mainly clinical, assisted by the use of transthoracic echocardiography (TTE). Aortic valve replacement (AVR) or trans-arterial valve implantation (TAVI) are the two main treatments for this disease (4). Many ongoing or recently conducted studies have focused on the potential role of some biomarkers in the follow up and treatment of AS (5).

MicroRNAs (miRs) are short sequences of non-coding ribonucleic acid (RNA) that regulate the genetic expression of more than half of the human genome, either by degradation or translational repression of their target messenger RNA (mRNA) (6). Their involvement in all main biological processes accounts for the large number of disorders where specific miR expression is altered.

The role of miRs in calcified AS has not been extensively studied, although some miRs (miR-26a,

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miR-30b, miR-92a, miR-141 and miR-195) have been shown to be involved in various processes leading to valve calcification (7-10). miR-30b has been described as a regulator of aortic valve calcification and cellular apoptosis by targeting Runx2, Smad 1, and Caspase-3 mRNAs (7), while miR-141 is involved in the cellular regulation of bone morphogenetic protein-2 (BMP-2) (9). miR-92a has been reported to play a major role in the regulation of angiogenesis and endothelial proliferation (10). Finally, miR-223 overexpression, acting on vascular smooth cells, has been shown to be involved in active vascular calcification, especially in the context of chronic kidney disease (11,12). Hence, it was hypothesized that miR-223 might be involved in aortic valve calcification, with a direct action on endothelial valvular tissue.

In the light of recent discoveries concerning the role of miRs in many forms of heart disease, the present study was a rarely accomplished investigation on the tissular expression of miRs in different human aortic valves, in order to consider the difference between bicuspid and tricuspid valves. The aim was to verify results previously obtained at the authors' institution, and to determine which miRs were different between these two types of calcified valve.

## Clinical material and methods

### Study population

A total of 47 consecutive patients treated at the Heart, Chest, and Vascular Surgery Center of the authors' institution for severe AS was included in this prospective study. Severe AS was defined according to the guidelines of the American Heart Association (AHA) and the European Society of Cardiology (ESC) by echocardiographic criteria such as aortic valve area (AVA) <1 $cm^2$, mean transvalvular gradient >40 mmHg, and maximum velocity >4 m/s (4). Patients undergoing AVR for infective aortic endocarditis were excluded from this study due to a need to examine the explanted valve for bacteriological assessment. Bicuspid aortic valves (BAVs) or TAVs were identified on TTE, with confirmation during the surgical procedure. Preoperative patient characteristics were collected prospectively.

Due to the non-interventional nature of the study, its design was approved by the local ethics committee (No. ID-RCB: 2016-A00488-43), and the need for patient consent was over-rules, provided that appropriate information had been given to each participant.

### Aortic valve collection

All patients were operated on according to the local surgical protocol. With the patient under general anesthesia and cardiopulmonary bypass, the calcified aortic valves were explanted and replaced with mechanical or biological prostheses. All patients were initially monitored in a critical care unit before being transferred to the standard cardiac surgery department. All were discharged on or about the 10th postoperative day.

The explanted calcified BAVs or TAVs were sent to the local laboratory where they were stored at -80°C until further use. Tricuspid valve cusps were identified as the left coronary cusp (LC), right coronary cusp (RC), and non-coronary cusp (NC), while bicuspid valve cusps were identified as the coronary cusp (CC) and non-coronary cusp (NC).

### RNA extraction

Total RNA, including miRs, was extracted from each cusp of the calcified aortic valve using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were consistently treated in the same manner in terms of freezing and thawing so as to reduce the risk of potentially confounding methodological issues. Each calcified cusp was ground using a polytron homogenizer and lysed in a denaturing lysis solution, which stabilized the RNA and inactivated RNases. *Caenorhabditis elegans* miR-39 (syn-cel-miR-39 miScript miRNA Mimic; Qiagen, Hilden, Germany) was spiked (5 nmol/l) into the RNA sample and used as an exogenous control for data analysis, as described previously (13). The lysate was then extracted once with acid-phenol-chloroform to remove most of the other cellular components. Ethanol was added to wash the sample, which was then passed through a filter cartridge containing a glass-fiber filter to immobilize the RNAs. The filter was washed three times with RNAse-free water, and the RNA was finally eluted with a low-ionic strength solution. All RNA samples were further subjected to DNase I digestion to prevent genomic DNA contamination, and stored at -20°C until use.

### miR expression

Total RNA diluted to 1:10 (5 μl) was reverse-transcribed using miR-specific stem-loop reverse transcription primers (50 nmol/l) and reagents (dNTPs, reverse transcription buffer, RNase inhibitor, reverse transcriptase, and RNase/DNase-free $H_2O$) from the Taqman® miRNA Reverse Transcription kit (Applied Biosystems). The small nucleolar RNA U6 (Applied Biosystems) was used as an internal control for all samples. Reactions were carried out in 96-well plates for 30 min at +16°C, 30 min at +42°C, 5 min at +85°C, and then stored at +4°C.

Real-time quantitative polymerase chain reaction (RT-qPCR) was subsequently performed using TaqMan® miR Assay kits (Applied Biosystems) to quantify miR expression. Reactions were incubated in a 96-well optical plate at +95°C for 10 min, followed by 40 cycles at +95°C for 15 s and +60°C for 1 min. Reactions were run on a Bio-Rad® CFX Connect Real-Time System (Applied Biosystems) in triplicate for the assessment of technical variability.

**Data analysis**

Relative quantification of miR expression was calculated using the $2^{-\Delta Ct}$ method, and values were normalized to an exogenous control, cel-miR-39 ($2^{\wedge-([CtmiR] - [CtC39])}$), and to an endogenous control, U6 ($2^{\wedge-([CtmiR] - [Ct U6])}$). Results were represented as histograms reflecting the fold changes of expression levels of the selected miRs.

**Statistical analysis**

Appropriate tests were used to evaluate the normal distribution of the data acquired. Results for continuous variables were expressed as mean ± SEM and compared with the Mann-Whitney *U*-test or Student's *t*-test, as appropriate. Categorical variables were expressed as percentages and compared with the chi-square test. A logistic regression analysis was performed. Spearman rank correlation or Pearson correlation were used to compare levels of miRs with cardiovascular risk factors and preoperative echocardiographic assessment. A p-value <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS v.21 for Windows (IBM) software.

## Results

Among 47 patients with a calcified aortic valve in the study cohort, 30 (63.8%) had a TAV. The preoperative characteristics of patients and AS are listed in Table I. Patients in the BAV group were significantly younger than those in the TAV group (68.9 versus 73.4 years; p = 0.049). The mean preoperative transvalvular gradient was not significantly different between the two groups (50 ± 10.2 mmHg versus 51.2 ± 11.2 mmHg; p = 0.729). No difference was noted between the diameters of the implanted prostheses in the two groups (p = 0.18).

*Table I: Baseline characteristics of the two groups: bicuspid valves and tricuspid valves.*

| Parameter | Bicuspid valve | Tricuspid valve | p-value |
|---|---|---|---|
| No. of patients | 17 | 30 | |
| Age (years) | 68.9 ± 8.3 | 73.4 ± 7.7 | 0.04 |
| Height (m) | 1.7 ± 6.3 | 1.68 ± 7.6 | 0.498 |
| Weight (kg) | 87.9 ± 12.5 | 78.4 ± 11.7 | 0.012 |
| BMI (kg/m2) | 30.3 ± 4.2 | 27.5 ± 4.0 | 0,024 |
| Mean gradient (mmHg) | 50 ± 10.2 | 51.2 ± 11.2 | 0,729 |
| Aortic valve area (cm2) | 0.84 ± 0.3 | 0.77 ± 0.1 | 0.342 |
| Max. velocity (m/s) | 4.3 ± 0.2 | 4.4 ± 0.6 | 0.540 |
| Sodium (mmol/l) | 140 ± 1.5 | 140.0 ± 3.0 | 0.854 |
| Potassium (mmol/l) | 4.2 ± 0.3 | 4.1 ± 0.4 | 0.598 |
| Creatinine (mmol/l) | 80.8 ± 17.9 | 83.4 ± 19.2 | 0.680 |
| Creatinine clearance (ml/min) | 85.3 ± 21.9 | 76.5 ± 18.8 | 0.203 |
| Calcium (mmol/l) | 2.3 ± 0.1 | 2.3 ± 0.1 | 0.796 |
| Phosphorus (mmol/1) | 1.0 ± 0.2 | 1.0 ± 0.2 | 0.391 |
| Albumin (g/l) | 39.0 ± 3.5 | 39.1 ± 5.3 | 0.937 |
| Total cholesterol (mmol/l) | 1.7 ± 0.3 | 1.7 ± 0.4 | 0.992 |
| Triglycerides (mmol/l) | 1.5 ± 0.7 | 1.1 ± 0.3 | 0.044 |

Values are expressed as mean ± SD.

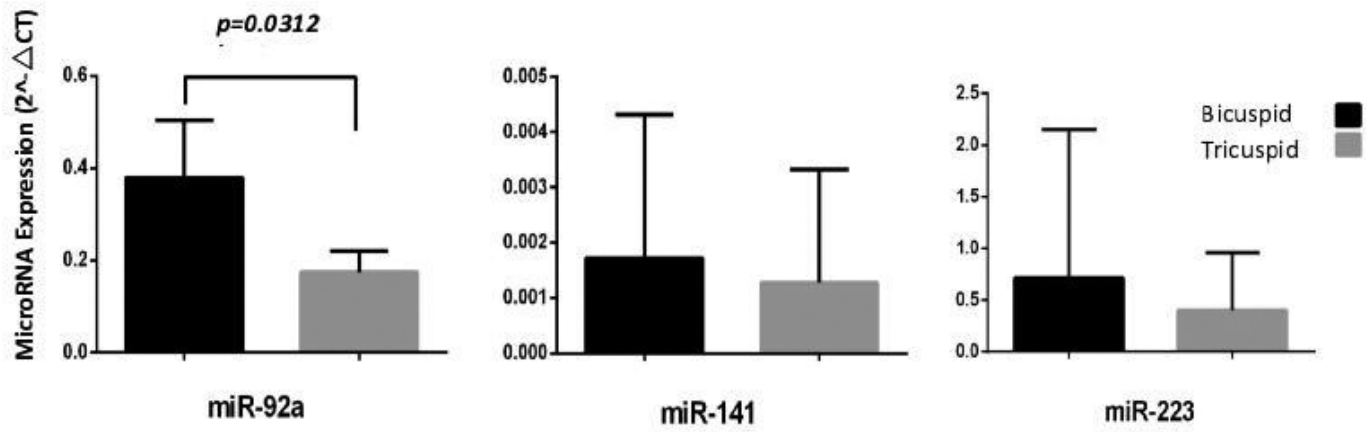


*Figure 1: Comparison of the relative expression of tissue miR-92a in bicuspid and tricuspid aortic valves.*

A preliminary analysis was achieved on the first 19 valves to verify the expression of miRs in different cusps, and to select the most appropriate miRs for the remainder of the study. A comparable level of expression was found for all six miRs between different cusps of the same valve in both the BAV group (n = 8; p = 0.38) and TAV group (n = 11; p = 0.9). A significant difference was observed between bicuspid and tricuspid valves for the expression of miR-92a, miR-141 and miR-223. miR-26a, miR-30b and miR-195 expressions were not significantly different between the two groups (Table II) and were not subsequently studied.

The decision was then taken to continue the study on a larger cohort by analyzing the expression of miR-92a, miR-141 and miR-223, compared to the U6 endogenous control expression, on one cusp of each valve.

Analysis of the 47 aortic valves showed miR-92a expression to be twofold higher in BAVs than in TAVs (0.38 versus 0.17; p = 0.0312) (Fig. 1), but no significant difference in mean gradient was observed between the two groups. In contrast, differences in miR-141 and miR-223 expression observed in the preliminary study were not confirmed in this large cohort (p = 0.684 and p = 0.35, respectively).

A significant positive correlation was observed between the overall relative expression of miR-92a and the mean preoperative transvalvular gradient (r = 0.3257, p = 0.04) (Fig. 2). In the BAV group, miR-92a was correlated positively with the serum calcium level (r = 0.5365, p = 0.03), whereas miR-223 was inversely correlated with serum calcium level (r = -0.4247, p = 0.03).

*Table II: Preliminary assessment of the expression of various miRs in bicuspid valves and tricuspid valves.*

| Parameter/miR | Bicuspid valves | Tricuspid valves | p-value |
|---|---|---|---|
| No. of samples | 8 | 11 | |
| miR-26a | 2.13 | 1.99 | 0.608 |
| miR-30b | 0.87 | 0.86 | 0.22 |
| miR-92a | 0.85 | 0.30 | 0.0006 |
| miR-141 | 0.06 | 0.03 | 0.005 |
| miR-195 | 1.42 | 1.52 | 0.94 |

## Discussion

Aortic stenosis is currently the most common form of degenerative valvular heart disease in adults (14). Its growing incidence has recently led to the search for novel biomarkers that would allow the early management and follow up of this disease, before the development of permanent left ventricular complications.

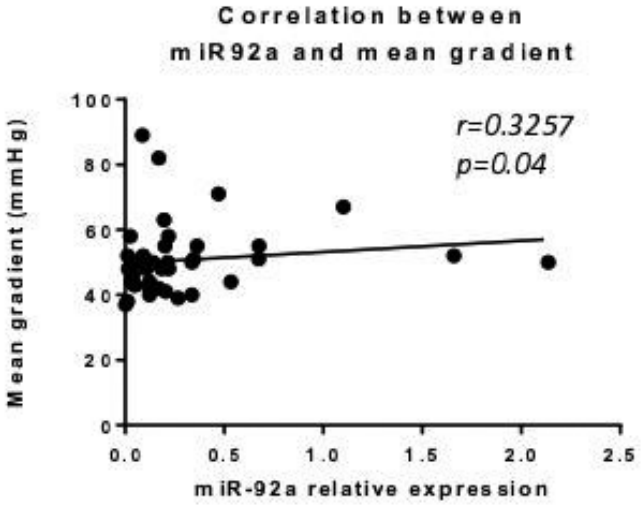


*Figure 2: Correlation between the relative expression of miR-92a and mean transvalvular gradient.*

Bicuspid aortic valve is the most common congenital anomaly of the human heart, and is responsible for a greater mortality and morbidity than the sum of all other forms of congenital heart disease (15). Age is a factor in valve calcification, and patients with BAV are subject to an earlier development of AS compared to those with TAV. The present cohort followed the statistics of the overall populations, with BAV patients treated earlier than TAV patients, with a much more severe degree of AS, defined here by the mean gradient (which did not differ between the two groups).

It is reasonable to hypothesize that miRs would be differentially expressed between the normal tricuspid valve and the bicuspid valve, which is a model of accelerated calcification. Hence, miR profile levels between the two tissues were assessed to identify specific miRs as potential calcification biomarkers of aortic calcification. This may improve the follow up of aortic valve calcification and might be used to monitor the disease in combination with echocardiographic assessments of AS, to guide early treatment for those patients with a biological profile predictive of rapid calcification.

In one study conducted on a large cohort of patients with calcified AS associated with coronary artery disease (CAD), a serious difference was demonstrated for certain miRs between AS with or without associated CAD (16), but did not provide any clear conclusions regarding the value of circulating miR as a biomarker of calcific AS. A large number of cohort studies have investigated potential biomarkers of ventricular remodeling, such as soluble ST2, BNP or B-type natriuretic peptide (NT-pro-BNP) (17) or lipoprotein-associated phospholipase-2 (Lp-PLA2) (18). Another study on the role of Galectin-3 as a potential determinant and prognostic factor for outcome in AS failed to demonstrate the expected result (19).

MiRs have the advantage of being stable markers that can be easily assayed in human biological fluids and tissues (6). In the present pilot study, miRs were selected based on available literature (as cited above), and it was confirmed that they could be detected in human BAV and TAV. The major finding was that the level of expression of miR-92a was significantly different between bicuspid and tricuspid valves, and tended to be twofold higher in bicuspid valves. These results supported the role of miRs in this process, and may suggest the existence of a genetic control by miRs, though the present findings need to be confirmed in further studies.

Interestingly, miR-92a, which is highly expressed in endothelial cells, is a known marker of vascular endothelial dysfunction (10). miR-92a may be expressed in the various cell types of heart valves, and may have important roles in the process of BAV formation. miR-92a is typically an atherosclerotic factor, suppressing transcription factors such as Krüppel-like factor (KLF) -2 and -4 in endothelial cells, thereby reducing nitric oxide production (20).

Furthermore, miR-92a acts as an endothelial pro-inflammatory factor by enhancing the NF-kB signaling pathway. It enhances endothelial activation and reduces atherosclerotic plaque stability, and negatively regulates angiogenesis and endothelial regeneration (10), both of which are factors potentially involved in the aortic valve calcification process. This miR has been shown as a valuable biomarker in other cardiovascular diseases (21) and in cancer (22). An increase in miR-92a expression only is certainly not related to the calcification process. Other factors, related specifically to bicuspid valves or more general situations, such as inflammation, could play a role. In the light of these results, the next step of the present research should consist of a translational study correlating miR-92a expression in serum and tissue, assessing its value as a biomarker of this disease. This would be especially important in view that the expression of miR-92a in the serum and pericardial fluid has been investigated in a small cohort of patients with AS (23).

The pressure gradient in AS is due to obstruction caused by a loss of normal aortic valve opening in the calcified valve, reducing the effective orifice area (EOA) and causing a subvalvular acceleration of blood flow. The higher the calcification degree of the valve, the higher the pressure gradient would be, as the EOA would be reduced in highly calcified valves. The positive correlation between mean transvalvular gradient and miR-92a expression should be investigated in patients presenting with varying degrees of severity of AS, in order to confirm its role in the calcification process. Moreover, miR-92a expression should be measured following the treatment of AS to determine if it is decreased after removal of the calcified valve (as in AVR), or whether it remains unchanged (as in percutaneous treatment by TAVI) when the native calcified valve is kept in place.

The overexpression of miR-92a can also reflect the excessive angiogenesis that occurs in BAV, and which accelerates the calcification process. This increased expression may be a physiological response of the endothelial cells to such angiogenic activity. Further histological studies comparing angiogenic activity between bicuspid and tricuspid calcified valves may provide an answer to this question.

Another important finding is that the various cusps of the same valve, whether bicuspid or tricuspid, presented the same levels of expression of the six miRs studied, with no significant differences being observed between LCs, RCs and NCs in tricuspid valves, or between CCs and NCs in bicuspid valves.

Currently, RT-qPCR is widely considered as a reliable method for quantifying miR expression, provided that a reference gene is used to normalize miR gene expression. This reference gene should ideally not be altered by pathophysiological conditions. Small nucleolar RNA U6 is commonly used as a 'house-keeping' small RNA and as a reference gene in miR quantification, although the stability of U6 is becoming increasingly controversial.

In addition to the pathophysiological implications, the present study had major clinical implications. The mean prevalence of BAV is about 2% (24), with surgery recommended in patients with severe AS (AHA and ESC guidelines) to prevent cardiac complications that are fully reversible after AVR, even in elderly patients (25). If the conclusions of the present study are confirmed with an effective biomarker of rapid calcification of the aortic valve, the laboratory diagnosis of a rapid progression of AS would allow the treatment of patients at risk before the development of left ventricular complications. In lower-risk patients - even when the hemodynamic profile does not indicate the presence of severe AS (e.g., in patients with low-gradient, low-flow severe AS (26) - the biomarker profile would potentially predict rapid aortic valve calcification, regardless of the echocardiographic assessment.

Finally, the positive correlation between miR-92a expression in the BAV group and serum calcium level indicates that miR-92a is a good potential indicator of the degree of calcification of these valves. Further studies of the correlation with echocardiographic or computed tomography calcific score would be interesting, but this was not performed in the present cohort due to an absence of systemic radiological or echocardiographic assessments of the aortic calcific score in patients undergoing surgery. Future in-vivo interventional studies involving specific miRNA modulators should confirm the exact mechanistic role of these miRs in the pathogenesis of BAV.

*In conclusion*, miR-92a is significantly overexpressed in calcified BAVs compared to TAVs. The expression level of miR-92a is correlated with the severity of AS, as defined by the preoperative mean transvalvular gradient. Further large-scale studies should be conducted to investigate the pathophysiological mechanisms of calcification related to this miR.

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