



Contents lists available at ScienceDirect

Indian Heart Journal

journal homepage: www.elsevier.com/locate/ihj

Research Brief

Diagnostic accuracy of MicroRNA 208b level with respect to different types of atrial fibrillation

Abraham Paul, MD, DM ^{a,*}, Praveen G. Pai, MD, DM ^a, Prasanth S. Ariyannur, MD ^b, Reenu Anne Joy, M.E. ^b^a Cardiology, Amrita Institute of Medical Sciences and Research Centre, Kochi, India^b Biochemistry & Molecular Biology, Amrita Institute of Medical Sciences and Research Centre, Kochi, India

ARTICLE INFO

Article history:

Received 11 May 2020

Received in revised form

4 May 2021

Accepted 27 June 2021

Available online 1 July 2021

Keywords:

microRNA

miRNA 208b

Atrial fibrillation

ABSTRACT

MicroRNAs (miRNA) are prerequisite for cardiovascular functions. miRNA miR-208 b is a cardio-specific miRNA with tissue (atrial) levels elevated in atrial fibrillation (AFib) and blood levels significantly elevated in myocardial infarction. We calculated serum levels of miR-208 b in paroxysmal and persistent AFib, embolic cerebrovascular accident patients with AFib as possible etiology and controls. There was a statistically significant change of miR-208 b levels in paroxysmal ($p = 0.044$) and persistent ($p = 0.040$) AFib patients, but not for embolic CVA patients. miR-208 b could serve as a new serum marker for paroxysmal AFib.

© 2021 Cardiological Society of India. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Atrial fibrillation (AFib) is the most common cardiac arrhythmia, with an estimated age-adjusted prevalence of 25.7 per 1000 person-years in the Framingham Heart Study.¹ For both men and women, prevalence and incidence of AFib were disproportionately higher in developed nations compared with developing nations.² This was explained by the increasing frequency of diagnosis and reporting of the AFib by the physicians as well as due to the rising age and cardiovascular co-morbidities in the subject population. In India, AFib patients are younger and Rheumatic Heart Disease (RHD) is still the most frequent aetiology.³ The ionic basis for AFib drivers^{4,5} and the structural and autonomic remodeling of atria by microRNAs have been well described.^{6–8} These changes render the atria more arrhythmogenic, furthering a paroxysmal AFib to become persistent and permanent.^{4,9} ECG and 24hr Holter are the cornerstones of AFib diagnosis, although routine 24hr Holter can completely miss paroxysms.^{10,11}

MiR-1, miR-133a, miR-208a/b, and miR-499 are cardiac specific.^{12,13} In another study, Mir-208 b and Mir-21 were found

elevated in atrial tissue samples in AFib patients.¹⁴ Canon et al¹⁵ identified miRNA 208a/b as the most abundantly increased miRNA in atrial tissue samples of AFib. MiR-208 b is expressed during cardiogenesis while miR-208a is expressed in adult hearts. Pathological cardiac remodelling is associated with the induction of a foetal gene expression pattern with re-expression of MYH7 and, therefore, miR-208 b. Ionic basis for AFib has been well described with Mir-208 (CACNA1C and CACNB2 were found to be direct targets of mir208a/b). Overexpression of miR208b (not mir208a) resulted in reduced SERCA2 protein and mRNA expression, important for shifting Ca into sarcoplasmic reticulum from cytosol. This calcium overload plays a role in AF perpetuation, structural and electrical remodelling with hypertrophy and fibrosis. Sox5 and Sox6 are negative factors of Myh7 transcription. Overexpression of miR-208a and miR-208 b suppressed the expression of Sox5 and Sox6, respectively. Atrial tissue of AF patients showed a drastic increase of MYH7 protein levels, suggesting that the increased expression of miR-208a/b in AF contributes to high MYH7 protein levels via inhibiting the expression of Sox5/6. Given that the healthy adult heart mainly expresses miR-208a and not miR-208 b, miR-208a may initially target Thrap1 in AF pathophysiology. This in turn promotes MYH7 and simultaneously miR-208 b transcription, which targets Sox5/6 and ultimately reinforces MYH7 transcription. Runwei Ma et al¹⁶ studied serum miRNA in paroxysmal atrial fibrillation, and found mir499 and 208 to be significantly elevated,

* Corresponding author.

E-mail addresses: abrahampluspaul@gmail.com (A. Paul), Praveen.pai@gmail.com (P.G. Pai), p.ariyannur@gmail.com (P.S. Ariyannur), reenuaj@aims.amrita.edu (R.A. Joy).

although no distinction was made between isoforms a and b of Mir208.

We hypothesized the cardio-specific microRNA miR-208 b, elevated in serum in myocardial infarction and in atrial tissue in AFib,^{14–16} as a potential serum marker for AFib. Role on miR-208 b in AFib by ionic and structural mechanisms, as well as pathological remodelling, has been described. The sole study on miR-208 in paroxysmal AFib did not differentiate between a and b isoforms.¹⁶ The primary objective of this study was to determine the diagnostic accuracy of serum miR-208 b levels in paroxysmal and persistent AFib, and cryptogenic stroke. The secondary objectives were to evaluate miR-208 b as a potential marker for paroxysmal AFib in otherwise healthy subjects, assist in identification of AFib in cryptogenic stroke patients and estimate the ideal cut-off for miR-208 b through ROC curve.

2. Methods

This is a single centre, comparative performance evaluation of two modalities, blood levels of miR-208 b were tested against ECG for detection of AFib, done over a period of two years. Study was approved by the Institutional Ethical Board (IRB-AIMS-2019-035), and informed consent was taken from study participants.

2.1. Inclusion and exclusion criteria

This study included patients with paroxysmal AFib, persistent AFib (as defined by Gallagher et al⁹), cryptogenic stroke with possible arrhythmic cardiac aetiology and healthy subjects. Exclusion criteria comprises acute inflammatory conditions, recent (<1 month) myocardial infarction or heart failure, liver dysfunction (Child B, C), rheumatic heart disease and structural heart diseases.¹⁷

2.2. Sample size

Previous publication by Ma et al.¹⁶ showed that in paroxysmal AFib, sensitivity of mir208 with respect to ECG was 81.3%. With 95% confidence and 20% precision, minimum sample size was calculated to 15, per group. Assuming the sensitivity will be either same or more than that in other groups, minimum sample size of 25 were collected in each of the four groups (1 - paroxysmal AFib, 2 - persistent AFib, 3 - cryptogenic (embolic) CVA and 4 - Control).

2.3. miRNA extraction and qRT-PCR

Whole blood was collected in EDTA vacutainer from both test and control subjects. Total miRNA was extracted using miRNeasy kit (Qiagen, USA), according to the manufacturer's instructions. Target specific cDNA was synthesized using miRCURY LNA RT Kit (Qiagen, USA) following the manufacturer's instructions.^{18,19} Quantitative real-time polymerase chain reaction (qRT-PCT) was performed using miRCURY LNA SYBR Green PCR Kit (Qiagen, USA) with primer sets (Qiagen, USA) specific for miRNAs: miR-208 b (MIR208A, miRBase accession number: MIMAT0004960) and the internal control (IC) miRNA miR-17 (MIR17, miRBase accession number: MI0000071) according to previous methods^{20,21} on cobas z480 Real-Time PCR System (Roche Molecular Diagnostics, Germany). Previous studies have shown that miR-17 is stable and abundant in plasma and have less variations in serum levels during myocardial damage.²⁰ 4 µl of diluted cDNA (1:80) was used for qRT-PCR in 10 µl duplicate assays. The PCR conditions were followed according to the manufacturer's instructions^(18, 19) (Qiagen, Germany) for 45 cycles. Cycle threshold (Ct) of miR-208 b (Ct_{miR208b}) and miR-17 (Ct_{miR17}) were determined. 26 samples that did not show amplification of IC were excluded from the study and 74 samples were

taken up for further analysis. Ct-values were normalized against miR-17 ($\Delta Ct = Ct_{miR208b} - Ct_{miR17}$) and used for statistical analysis. As conducted previously,²⁰ the cut-off cycle threshold (Ct) for target miR-208 b was set to 40 cycles, so that the ΔCt (delCt) is in the log linear phase and Ct-values ≥ 40 were considered as non-specific background fluorescence. For the determination of ΔCt for samples with amplification beyond the cut-off value, the Ct value was assigned as 40.²⁰ A higher ΔCt means miR-17 was detected at lower Ct than miR-208 b, and thus denotes lower miR-208 b levels.

2.4. Statistical analysis

From a previous publication by Ma et al.¹⁶ it was found that in paroxysmal AFib, sensitivity of mir208 with respect to ECG was 81.3%. With 95% confidence and 20% precision, minimum sample size comes to 15, per group. Assuming the sensitivity will be either same or more than that in other groups, minimum sample size of 25 were taken in each group. Statistical analysis was performed using IBM SPSS version 20.0 software. Chi-square test was used to study the statistical significance of the association of all demographic and clinical parameters between groups. Kruskal Wallis test was used to compare the mean values of age and delCt (ΔCt) between Groups. ROC curve was used to find the ideal cut-off for ΔCt to predict AFib in stroke patients. Pearson's correlation coefficient was used between ΔCt and other clinical parameters. A *p* value of <0.05 was considered to be statistically significant.

3. Results

Baseline characteristics were well matched between groups (Table 1). In our study, there were 19 patients in group 1, 20 in group 2, 18 in group 3, and 17 in group 4. ΔCt was lower in group 1 and 2, and higher in group 3 and 4. This translates to higher miRNA 208-b levels in group 1 and 2 and lower in 3 and 4. Kruskal–Wallis Test showed a significant difference in distribution of mean value of ΔCt between the groups (*p* = 0.015). ΔCt did not show significant difference between groups with respect to age, sex or ECHO parameters except in total wall thickness (TWT) where there was a tendency towards negative correlation (*r* = -0.261) (*p* = 0.025).

Pairwise comparison of mean ΔCt between study groups showed significant difference between persistent AFib (*p* = 0.04, *r* = -0.4) and paroxysmal AFib (*p* = 0.044, *r* = -0.42) compared to controls (Fig. 1). See Supplementary table 1 for the raw data. Statistically significant elevation of serum miRNA 208 b levels were detected in paroxysmal and persistent AFib. In our study with miR-17 as a baseline, a ΔCt of 4.18 was found to be significant for paroxysmal AFib, while it did not reach statistical significance in persistent AFib. Four people from the control group and three from the embolic CVA group developed AFib episodes on 6 months follow-up. The delCt of 6 of these patents were lesser than 4.18, the proposed cut off in our study, with 1 patient having a value 4.87 (control).

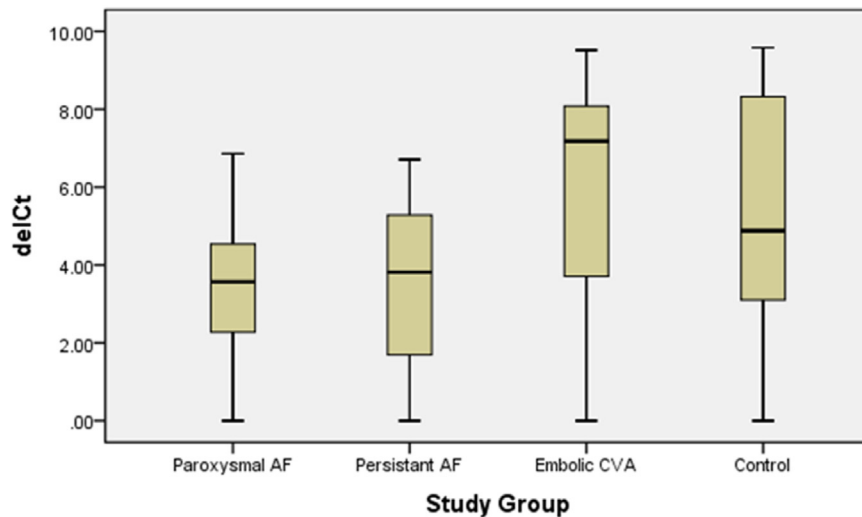
An ROC analysis for identification of ideal cut-off for both paroxysmal and persistent AFib showed a ΔCt value of 4.18, with sensitivity of 68% and 60%, and specificity of 64.7% and 64.1%, respectively (Fig. 2). The *p* value for this cut-off was significant for paroxysmal AFib (*p* = 0.047), but insignificant for persistent AFib (*p* = 0.134).

4. Discussion

This study explored the potential role serum miRNA miR-208 b plays in diagnosing atrial fibrillation. We calculated the ΔCt between miR-208 b and miR-17 for statistical analysis. ΔCt was lower in paroxysmal and persistent AFib patients, statistically significant

Table 1
Baseline characteristics of the study population.

Characteristic	Paroxysmal AFib (n = 19)	Persistent AFib (n = 20)	Cryptogenic CVA (n = 18)	Controls (n = 17)	p-value
Mean age	69.84	64.60	60.56	64.94	0.052
Men	11 (57.9%)	12 (60%)	12 (66.7%)	13 (76.5%)	0.394
Diabetics	10 (52.6%)	7 (35%)	8 (44.4%)	9 (52.9%)	0.510
Hypertensives	12 (63.2%)	7 (35%)	10 (55.6%)	12 (70.6%)	0.151
CAD	10 (52.6%)	4 (20%)	4 (22.2%)	13 (76.5%)	0.003



Pairs	P value	Pearson correlation
Paroxysmal AF-Persistent AF	0.992	0.19
Persistent AF-Control	0.040	-0.40
Persistent AF-Cryptogenic CVA	0.012	0.34
Paroxysmal AF-Control	0.044	-0.42
Paroxysmal AF-Cryptogenic CVA	0.013	0.18
Control-Cryptogenic CVA	0.668	-0.16

Fig. 1. Pairwise comparison shows significant difference in ΔC_t between Paroxysmal AFib (0.044) and Persistent AFib (0.040) compared to controls. Upper panel depicts mean and standard deviation of different groups.

from controls. This translates to higher miR-208 b levels in these patients, which is consistent with prior studies, with tissue levels of miR-208 b levels higher in patients with paroxysmal and permanent AFib as compared to controls. The ROC was significant for both paroxysmal AFib and persistent AFib, however the cut-off calculated was significant for paroxysmal AFib not for persistent AFib. A possible explanation for this could be the greater standard deviation for persistent AFib group as compared to paroxysmal AFib.

Cryptogenic CVA is mostly attributed to unrecognized embolic events, and in the absence of carotid or vertebral vessel disease, a cardiac source of embolus is most likely. In the absence of other significant structural heart disease, paroxysmal AFib is the most common implicated aetiology. We proposed that miR-208 b could be used as a blood marker for an otherwise undiagnosed AFib. The current study did not show a significant relationship in miRNA levels between cryptogenic CVA group and control ($p = 0.668$). Possible inferences for this could be that –

- The paroxysms of AFib were too few to cause any structural or electrical remodelling significant enough to lead to detectable biomarkers in blood

- The proposed marker is not an ideal candidate for its diagnosis.¹⁶

However, on 6-month follow-up, only three out of the 17 patients in this group developed AFib. On follow-up (6-months) of patients without ECG/Holter evidence of AFib on initial study inclusion (group 3, 4), subjects who subsequently developed AFib had a greater baseline miRNA 208-b levels.

5. Limitations

Studies in AFib profiling miRNA in tissue and blood have previously shown differing results.^{22–25} Till date, to the best of our knowledge, only one study has investigated miRNA levels in blood and detected elevated levels of miR-208. However, the study did not specify the subtype of miR-208 (a or b) responsible for this change.¹⁶ Differing levels in other miRNA studies have been implicated to be due to increased uptake from peripheral circulation to disease tissues.²⁴ In our study, ΔC_t for persistent AFib group varied from 1.22 to 5.7. Normalization of ΔC_t across the different samples could not be identified in the study due to wide variance of the internal control (miR-17) between different samples. Due to

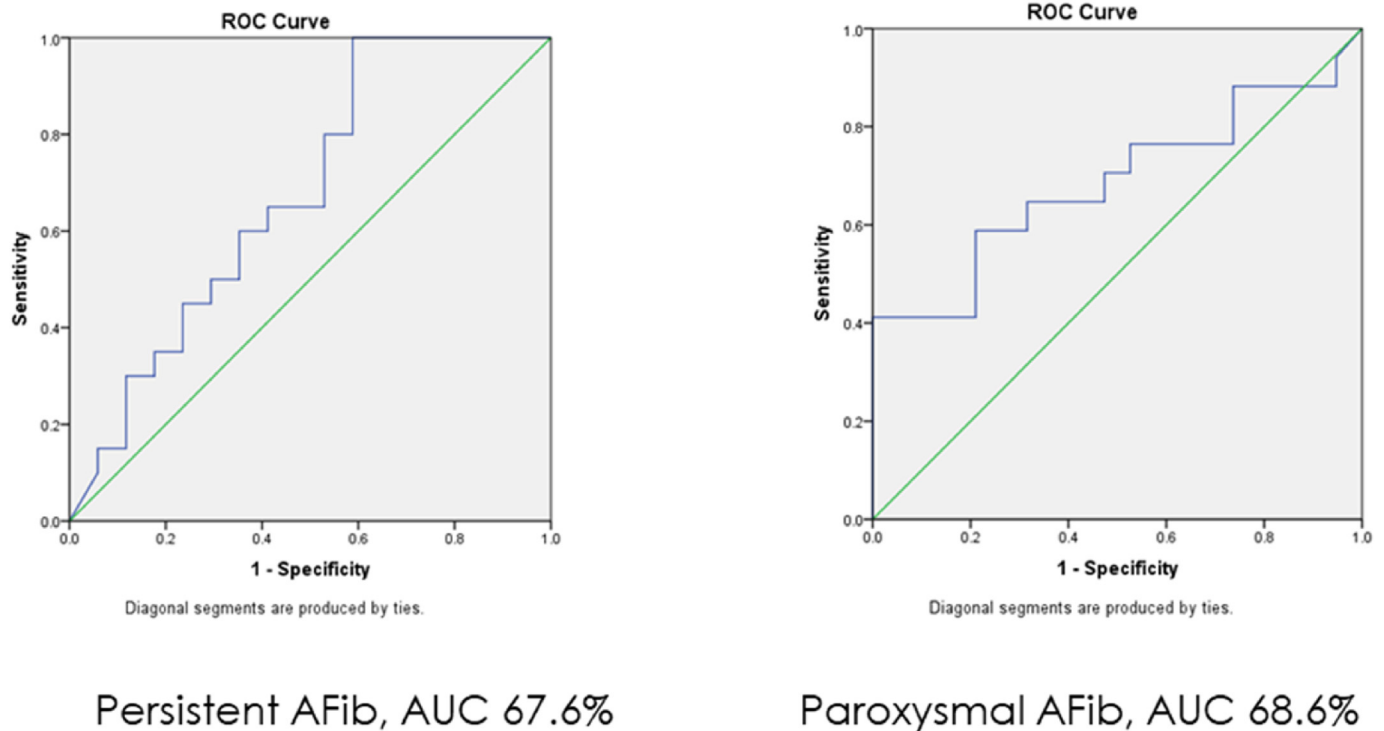


Fig. 2. ROC curve for Persistent and Paroxysmal AFib. AUC was significant for both groups. AUC was not significant for cryptogenic CVA group (not shown).

this an absolute fold-change of the miRNA208b could also not be established. Further studies with greater sample size are required to firmly establish pathological levels of selected miRNA. Furthermore, this was a single centre study, which included samples from a single population subgroup. Hence, population variation in miRNA levels, which have not been evaluated, could alter the outcomes in larger studies.

6. Conclusion

There was a statistically significant elevation of serum miRNA 208-b levels in paroxysmal and persistent AFib. In our study with miRNA 17 as a baseline, a ΔCt of 4.18 was found to be significant for paroxysmal AFib, while it did not reach statistical significance in Persistent AFib.

The most significant clinical application of this study is the potential of miR-208 b as a marker to aid in detecting an otherwise undiagnosed AFib with a subsequent change in therapeutic strategy. A blood marker that reflects the ionic and structural changes in AFib would theoretically help in rapid and complete diagnosis. Tissue inhibitors of miRNA could play a role in targeted anti-atrial remodelling therapy. There are currently no serum biomarkers that accurately detect paroxysmal AFib, which can go undetected by current conventional rhythm monitoring devices. This pilot study, done in a relatively small number of patients from a single centre, shows the potential of this marker in diagnosis.

Acknowledgments

The authors would like to thank the senior and junior faculty and laboratory staff of Departments of Cardiology, Neurology and Molecular Biology of Amrita Institute of Medical Sciences, Kochi for the conduct of research. The study was supported by internal seed grant by Amrita Vishwa Vidyapeetham.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ihj.2021.06.018>.

References

- Schnabel RB, Yin X, Gona P, et al. 50 year trends in atrial fibrillation prevalence, incidence, risk factors, and mortality in the Framingham Heart Study: a cohort study. *Jul 11 Lancet*. 2015;386(9989):154–162. [https://doi.org/10.1016/S0140-6736\(14\)61774-8](https://doi.org/10.1016/S0140-6736(14)61774-8). Epub 2015 May 7. PMID: 25960110; PMCID: PMC4553037.
- Chugh SS, Havmoeller R, Narayanan K, et al. Worldwide epidemiology of atrial fibrillation: a global burden of disease 2010 study. *Feb 25 Circulation*. 2014;129(8):837–847. <https://doi.org/10.1161/CIRCULATIONAHA.113.005119>. Epub 2013 Dec 17. PMID: 24345399; PMCID: PMC4151302.
- Vora A, Kapoor A, Nair M, et al. Clinical presentation, management, and outcomes in the Indian heart rhythm society-atrial fibrillation (IHRs-AF) registry. *Indian Heart J*. 2017;69(1):43–47.
- Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Oct 1 Circulation*. 1995;92(7):1954–1968.
- Yeh Y-H, Wakili R, Qi X-Y, et al. Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure. *Jun 1 Circ Arrhythm Electrophysiol*. 2008;1(2):93–102.
- A signature pattern of Stress-Responsive microRNAs that can evoke cardiac hypertrophy and heart failure [internet] [cited 2019 Nov 22]. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1838739/>.
- Luo X, Yang B, Nattel S. MicroRNAs and atrial fibrillation: mechanisms and translational potential. *Feb Nat Rev Cardiol*. 2015;12(2):80–90.
- Atrial natriuretic peptide is negatively regulated by microRNA-425. - PubMed - NCBI [internet] [cited 2019 Nov 22]. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/23867623>.
- Gallagher Mark, Camm John. Classification of atrial fibrillation. *Pacing Clin Electrophysiol*. 1997;20. <https://doi.org/10.1111/j.1540-8159.1997.tb03527.x>.
- Gladstone DJ, Spring M, Dorian P, et al. Atrial fibrillation in patients with cryptogenic stroke. *Jun 26 N Engl J Med*. 2014;370(26):2467–2477.
- Sanna T, Diener H-C, Passman RS, et al. Cryptogenic stroke and underlying atrial fibrillation. *Jun 26 N Engl J Med*. 2014;370(26):2478–2486.
- Luo X, Zhang H, Xiao J, Wang Z. Regulation of human cardiac ion channel genes by microRNAs: theoretical perspective and pathophysiological implications. *Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol*. 2010;25(6):571–586.

13. Chistiakov DA, Orekhov AN, Bobryshev YV. Cardiac-specific miRNA in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). May 1 *J Mol Cell Cardiol.* 2016;94:107–121.
14. Nishi H, Sakaguchi T, Miyagawa S, et al. Impact of microRNA expression in human atrial tissue in patients with atrial fibrillation undergoing cardiac surgery. Sep 12 *PLoS One.* 2013;8(9), e73397.
15. Cañón S, Caballero R, Herraiz-Martínez A, et al. miR-208b upregulation interferes with calcium handling in HL-1 atrial myocytes: implications in human chronic atrial fibrillation. Oct *J Mol Cell Cardiol.* 2016;99:162–173.
16. Ma R, Wang J, Wu X, et al. MiR-499 is a diagnostic biomarker of paroxysmal atrial fibrillation involved in the development of atrial fibrillation. Jan 1 *Int J Clin Exp Pathol.* 2017;10:4221–4231.
17. Steinberg Daniel H, Staubach Stephan, Franke Jennifer, Sievert Horst. Defining structural heart disease in the adult patient: current scope, inherent challenges and future directions, 1 September *Eur Heart J Suppl.* 2010;12(suppl_E):E2–E9. <https://doi.org/10.1093/eurheartj/suq012>.
18. <https://www.qiagen.com/us/resources/resourcedetail?id=f02862d7-29df-4466-a457-ebde78beb891&lang=en>.
19. <https://www.qiagen.com/ca/resources/resourcedetail?id=20e0ddd1-ad97-43ef-9edf-eef6a6d02a1d&lang=en>.
20. Gidlöf Olof, Smith J Gustav, Miyazu Kazuma, Gilje Patrik, Spencer Anna, Blomquist Sten, et al. Circulating cardio-enriched microRNAs are associated with long-term prognosis following myocardial infarction. *BMC Cardiovasc Disord.* 2013;13(1):1–9.
21. D'Alessandra Y, Devanna Paolo, Limana Federica, Straino Stefania, Di Carlo Anna, Brambilla Paola G, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Nov Eur Heart J.* 2010;31(22):2765–2773. <https://doi.org/10.1093/eurheartj/ehq167>. Epub 2010 Jun 9. PMID: 20534597; PMCID: PMC2980809.
22. Harling L, Lambert J, Ashrafian H, Darzi A, Gooderham NJ, Athanasiou T. Elevated serum microRNA 483-5p levels may predict patients at risk of postoperative atrial fibrillation. *Jan Eur J Cardio-Thorac Surg Off J Eur Assoc Cardio-Thorac Surg.* 2017;51(1):73–78.
23. Remote ischemic preconditioning and incidence of postoperative atrial fibrillation. Cochrane Library [Internet]. [cited 2019 Dec 2]. Available from: https://www.cochranelibrary.com/content?templateType=related&urTitle=%2Fcentral%2Fdoi%2F10.1002%2Fcentral%2FCN-01255323&doi=10.1002%2Fcentral%2FCN01255323&p_p_id=scolariscontentdisplay_WAR_scolariscontentdisplay&_scolaris_contentdisplay_WAR_scolariscontentdisplay_action=relatedcontent&p_p_lifecycle=0&mode=view&type=central&contentLanguage=.
24. van den Berg NWE, Kawasaki M, Berger WR, et al. MicroRNAs in atrial fibrillation: from expression signatures to functional implications. *Jun Cardiovasc Drugs Ther.* 2017;31(3):345–365.
25. Chistiakov DA, Orekhov AN, Bobryshev YV. Cardiac-specific miRNA in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). May 1 *J Mol Cell Cardiol.* 2016;94:107–121.