Great Expectations:

MicroRNA-30d and Cardiac Resynchronization Therapy

Running title: Dorn; miR-30d as a biomarker for resynchronization success

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“The broken heart. You think you will die, but you just keep living, day after day after terrible day.” - Charles Dickens’ Great Expectations

There seem to be as many approaches to managing heart failure as there are causal factors. Heart failure management is evolving from a one-size-fits-all approach centered around therapy with neurohormonal antagonists toward strategies tailored to provide the optimal clinical benefit based upon individual patient profile. Thus, a major collective enterprise of the translational research community has been to identify subsets of patients that will derive greater benefit from one or another management scheme. The initial observation that, like cancer and other pathologies, microRNAs are regulated in heart disease was coupled with observations that microRNAs are found circulating in stable form in the blood to raise expectations that microRNAs would prove useful as disease biomarkers, providing insights into aspects of heart disease not revealed through traditional clinical testing. In the current issue of Circulation, Melman et al propose miR-30d as a biomarker for heart failure responsiveness to cardiac resynchronization therapy. This manuscript aptly illustrates the promise, problems, and pitfalls with the current state of evaluating microRNAs as biomarkers of cardiovascular disease. To quote Pip’s sister in Great Expectations: “Answer him one question, and he’ll ask you a dozen directly”.

Melman et al initially identified miR-30d as one of several microRNAs exhibiting statistically significant differences or a strong trend toward being differentially abundant in the plasma of patients who had undergone cardiac resynchronization therapy. The pilot microarray study of 12 male non-ischemic cardiomyopathy patients reflected the two extremes of response; six non-responder subjects had no change or a decrease in left ventricular ejection fraction.
(LVEF) and 6 responder subjects exhibited marked functional improvement (increase in LVEF averaging 24%). In an expanded cohort of 40 subjects, three of the microRNAs were confirmed as having significantly different circulating levels between categorical responders (here defined as an increase in LVEF of at least 10%) and categorical nonresponders (all others). The correlation data between log2 relative [miR-30d] and change in LVEF after synchronized biventricular pacing as depicted in Figure 2b of the manuscript, and are redrawn here and modified for purposes of illustration (Figure 1). The correlation coefficient is reported as 0.39, p=0.01. These data reveal a 10 log2 range in relative miR-30d concentration. After censoring two outliers in which miR-30d levels are 2 and 4 logs greater than the highest values reported for the other 40 subjects (Figure 1) the authors indicate that the correlation between miR-30d plasma concentration and change in %LVEF after resynchronization no longer achieved significance. However, binary probabilistic modeling of categorical discrimination between responder and non-responder using circulating miR-30d levels as the variable (Figure 2c of Melman, et al6) remained significant after removing the outliers.

What is currently known about miR-30d in the heart? Interrogation of extensive human and mouse heart miR-Seq data deposited in public databases reveals that miR-30d is one member of a family of identical or nearly-identical microRNAs (miR-30a, b, c, d, and e) that are abundant and present at remarkably similar levels in normal hearts of these two species7,8 (Table 1, Supplemental Table 1). In both human and mouse hearts, four of the five miR-30 family members are among the top 20 most abundant cardiac microRNAs: miR-30a is the most abundant miR-30 family member, and the 9th most abundant cardiac microRNA in both species. miR-30d is the 11th most abundant miR in normal human hearts and the 12th most abundant miR in mouse hearts. Although the sequencing read data are reported differently in another small
RNA sequencing study\textsuperscript{9}, miR-30e, -30d, -30a, -30c, and -30b were ranked the 14\textsuperscript{th}, 15\textsuperscript{th}, 17\textsuperscript{th}, 26\textsuperscript{th}, and 35\textsuperscript{th} most abundant myocardial microRNAs. In failing human hearts, cardiac levels of miR-30 family members are either similar to\textsuperscript{8} or modestly lower than\textsuperscript{9} non-failing human myocardium (Table 1, Supplemental Table 1). The latter study also assayed plasma samples and found that the most abundant miR-30 family member in plasma (assayed by small RNA sequencing) was miR-30d, which ranked as 12\textsuperscript{th} most abundant and was present at approximately 10\% of the level of the most abundant circulating microRNA, miR-21\textsuperscript{9}. Neither cardiac nor circulating blood microRNA sequencing data are available for experimental murine heart failure, but the entire miR-30 family is downregulated \textasciitilde 20\% in the myocardium of mice early after experimental pressure overload induced by surgical transverse aortic coarctation\textsuperscript{10} (Table 1, Supplemental Table 1). As confirmed by Melman and colleagues\textsuperscript{6}, microRNA sequencing has shown that miR-30d and the other miR-30 family members are more abundant in cardiac myocytes than in non-myocytes\textsuperscript{7}.

Previous reports have suggested that plasma miR-30d levels are a biomarker for type 2 diabetes mellitus\textsuperscript{11}, whereas whole blood levels of miR-30c are part of a panel of regulated circulating microRNAs associated with myocardial infarction\textsuperscript{12}. miR-30d was recently observed to mediate programmed cardiomyocyte death in diabetic cardiomyopathy\textsuperscript{13}, which contrasts with the anti-apoptotic actions reported by Melman, et al\textsuperscript{6}.

It is not clear how increased circulating plasma miR-30d levels might identify heart failure patients more likely to respond to ventricular resynchronization. A recent microarray microRNA profiling study detected a decrease in circulating miR-30d in human heart failure\textsuperscript{14}, which contrasts with the observation by Melman, et al that circulating plasma miR-30d levels are markedly higher in heart failure subjects than in non-failing volunteers\textsuperscript{6}; small RNA sequencing
of plasma from advanced human heart failure showed no significant change in circulating miR-30d plasma small RNA sequence data from advanced human heart failure. Thus, as has been recognized by virtually all investigators who are contributing to this nascent field, different microRNA assay methodologies and technical differences in sample handling or preparation complicate direct comparative analyses of these types of data.

Because all of the miR-30 family members are highly abundant in hearts, miR-30d may be passively released by injured or dying cardiomyocytes. Importantly however, Melman et al detected an increase in vesicular miR-30d released from cultured neonatal rat cardiac myocytes subjected to repetitive mechanical deformation, suggesting that miR-30d can be actively secreted. Nevertheless, stretching cultured neonatal cardiomyocytes is an imperfect model of ventricular dys-synchrony, and it is important to consider that increases in steady state plasma levels of a given microRNA may reflect greater miR secretion, enhanced miR biosynthesis, increased miR stability, or a combination of these mechanisms.

The promise of a new biomarker is that it will improve clinical discrimination for the condition that it marks, compared to the existing gold standard. The current gold standard for predicting a favorable response to ventricular resynchronization in heart failure is electrocardiographic QRS duration. As a categorical variable, QRS duration was part of the inclusion criteria for the study by Melman, et al. However, likely because of limited cohort sizes, QRS duration did not predict resynchronization response. Thus, it is not possible to compare the new biomarker to the existing gold standard here because the gold standard failed. It will be interesting to see if larger studies might in the future discover that the lack of exact predictive concordance between circulating miR-30d and QRS duration is advantageous, reflecting different aspects of heart failure that considered in combination comprise a superior
response biomarker to either factor alone.

As noted by the authors, additional work is necessary before miR-30d can be accepted in either its proposed clinical role as a biomarker for predicting success of ventricular resynchronization therapy for heart failure, or mechanistically as a factor that is dynamically regulated in heart failure and favors cell growth over cell death. These uncertainties are certainly not unique to miR-30d, and seemingly reflect the current state of the field. Here, I will follow Mr. Jaggers’ advice to Pip in Great Expectations “Take nothing on its looks; take everything on evidence”¹ and suggest ways in which research in this rapidly evolving area might evolve in order to fully meet our expectations:

1. When possible, initial biomarker discovery studies of circulating microRNAs should employ next generation sequencing. Hypothesis-generating research too often has to compromise breadth of data coverage to assure accuracy of read-out, or vice versa. Fortunately, next generation sequencing is both the most comprehensive and most accurate method to profile RNA species of all sizes. Coverage is essentially complete; if a microRNA is present in even miniscule amounts it will be detected provided that sequencing depth is sufficient. miR-Seq provides better accuracy for microRNAs that undergo alternative processing (known as isomiRs) and that may not be readily detected by standard arrays or RT-qPCR¹⁸. Furthermore, RNA sequencing is inherently quantitative, whereas “spike-in” protocols such as the one used by Melman, et al ⁶ to normalize RT-qPCR results, have limitations that include differing stabilities of the endogenous and exogenous microRNAs. miR-Seq results are not adversely impacted by annotation errors in miR sequence that can impair the efficacy of array probes and PCR primers designed around erroneous sequences¹⁸. A recent comparison of quantitative microRNA expression platforms concluded that the standard analytical filters used for comprehensive
sequencing may reduce sensitivity of the technique for extremely rare RNAs, but when compared to dedicated qPCR sequencing was unparalleled for capturing expression differences\(^{19}\).

2. **Case-control associations should be validated in independent populations.** The essence of experimental validation is independent replication. In the current era of comprehensive genomics it is standard practice for an initial report of a given genotype-phenotype association to demonstrate independent replication of the index finding in one or more separate populations, as our group did some years ago with a common heart failure-associated polymorphism at 1p36.13\(^{20,21}\). Of course, true validation requires that separate groups confirm the findings in even more independent cohorts, as was accomplished with 1p36.13\(^{22,23}\). Adding additional subjects to an index cohort\(^{6}\), while helping to increase statistical confidence, is not sufficient for independent validation.

3. **The predictive value of a biomarker, either for at-risk populations or individuals, must ultimately be demonstrated prospectively.** Even when a statistical association is demonstrated in multiple independent case-control populations, correlative data are not sufficient to demonstrate efficacy for a given metric to stratify individual risk or response. Indeed, there are many valid associations between levels of a marker and prevalence of a condition that, when applied to individuals, are poor clinical stratifiers or predictors because the correlation is not sufficiently tight, the variance is too great, or the marker is confounded by extraneous environmental or co-morbid factors. It can be argued that including actual predictive data for individuals is too high a bar to set for initial biomarker discovery and characterization, in favor of binary probabilistic modeling\(^{6}\). Fortunately, it is not necessary to actually perform a prospective trial to assess the predictive value of a novel biomarker. Existing outcomes data for the condition of interest
can be analyzed blindly to ascertain the predictive efficacy of the prospective biomarker according to models developed from the discovery data. Such analyses performed for individuals within the discovery cohort can help gauge real-world clinical utility of the biomarker and are useful to optimize design of larger studies with longer follow-up, as are proposed by Melman and colleagues.

Drs. Melman, Das and colleagues are to be congratulated for attempting such a challenging project. A biomarker that improves the selection of heart failure cases for ventricular resynchronization has the potential to improve patient care, lower medical costs, and prevent ineffective device implantation in non-responders. Circulating microRNAs seem to be the “next big thing” in biomarkers, and for good reason. The current study adds to accumulating data supporting the concept that circulating microRNAs will become useful as biomarkers of heart disease, and extends this notion to a biomarker predicting the specific therapeutic response to a form of targeted disease management. Circulating microRNAs would be readily obtainable from patients via simple venipuncture, and a limited panel of dozens or hundreds of validated microRNA biomarkers could be assayed in whole blood, serum, or plasma using standardized arrays; such data would be comparable across different centers. Because release or secretion of microRNAs is likely modulated by features of cardiovascular diseases that differ from those that promote the release of circulating protein biomarkers assayed by standard clinical tests, one of the expectations is that circulating microRNA biomarkers will add to our understanding of disease pathogenesis as well as refine clinical decision making. For this purpose, whether a circulating microRNA is actively or passively released and the extent to which it induces a biological effect (as via cell-to-cell communication) does not impact its utility as a biomarker. Both issues are interesting and important, but are capable of being addressed independently.
Conflict of Interest Disclosure: None.

References:


**Table 1.** Cardiac microRNA sequence data for miR-30 family members in human (top) and mouse (bottom) hearts.

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<th>isch CM</th>
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<table>
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Sequence data are expressed as reads per million aligned microRNA reads. CM is cardiomyopathy, isch is ischemic, TAC is traverse aortic coarctation. GSE numbers are NCBI Gene Expression Omnibus sub-series files for the original sequence data from references 7, 8, and 10.
Figure Legend:

**Figure 1.** Relationship between circulating plasma miR-30d levels and change in left ventricular ejection fraction after cardiac resynchronization therapy, modified from Figure 2b of Melman, et al. Rose highlighting indicates miR-30d outliers, green are categorical responders, blue are categorical non-responders.
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