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Exosomal MicroRNA for Detection of Cardiac Sarcoidosis

To the Editor:

Sarcoidosis is a multisystem, granulomatous disease of unknown cause that most commonly affects adults 25–50 years of age, causing significant morbidity and mortality. Studies indicate that sarcoidosis-related mortality is on the rise, perhaps relating to improved disease detection (1). Cardiac sarcoidosis (CS) is the second leading cause of sarcoidosis-related death, and young adults are particularly at risk (2). A contributing factor is failure to detect CS during routine clinical screening, including patient history, physical examination, and electrocardiography (1). As a result, many cases are detected for the first time after a cardiovascular complication, ranging from frequent premature ventricular contractions and atrial arrhythmias to sudden cardiac death (1). Whereas high-resolution cardiac imaging techniques, such as cardiac magnetic resonance or fludeoxyglucose F 18–positron emission tomography/computed tomography ([^{18}F]FDG-PET/CT), greatly improve CS detection (1), these modalities are expensive and present certain patient risks (e.g., radiation exposure with [^{18}F]FDG-PET/CT [3]), making them impractical for routine screening for CS. To our knowledge, no circulating biomarker has been shown to reliably detect CS.

Noncoding RNAs are readily detected in human blood and are reported to serve as biomarkers of diseases including acute myocardial damage (4). A fraction of the microRNAs (miRNAs) in blood are encapsulated within lipid bilayer vesicles, referred to as *exosomes*, where they are shielded from enzymatic degradation (5). Exosomes originate from multivesicle bodies within cells containing proteins, nucleic acids, and lipids, and as such, exosomal molecular content reflects its cellular origin (6). Thus, we hypothesized that exosome-derived miRNA could serve as an informative source of biomarkers for cardiac sarcoidosis.

With institutional review board approval, we conducted a retrospective study of 21 subjects with histologically proven sarcoidosis who had clinical and radiographic evidence of CS, based on established criteria (7, 8), compared with 21 subjects with sarcoidosis with no evidence of CS (non-CS), and 11 healthy human volunteers. We randomly divided the plasma samples obtained into two groups: discovery (10 CS, 10 non-CS, and 5 control) and validation (11 CS, 11 non-CS, and 6 control) cohorts. The demographic and clinical characteristics are presented in Table 1, with no significant differences found between the groups in any of the demographic characteristics. The plasma samples were collected within the scope of the National Institutes

Table 1. Patient Demographics and Clinical Manifestations

Group	Age (*yr*) (*Mean ± SE*)	Race (*W/B/O*)	Sex (*M/F*)	Smoking Status (*N/C/F*)	MRI*	PET†	Heart Block‡	V-tach§	HF‖
First cohort (NGS analysis)									
Control (n = 5)	50.0 ± 3.4	4/1/0	2/3	3/0/2					
Cardiac sarcoidosis (n = 10)	51.8 ± 3.1	9/0/1	5/5	8/0/2	50%	30%	60%	50%	40%
Noncardiac sarcoidosis (n = 10)	52.9 ± 3.8	8/1/1	2/8	5/0/5					
Second cohort (qRT-PCR analysis)									
Control (n = 6)	44.0 ± 5.2	5/1/0	3/3	3/0/3					
Cardiac sarcoidosis (n = 11)	54.1 ± 4.1	4/7/0	4/7	10/0/1	27%	55%	64%	27%	36%
Noncardiac sarcoidosis (n = 11)	51.2 ± 3.7	8/3/0	5/6	8/0/3					

Definition of abbreviations: HF = heart failure; M/F = male/female; MRI = magnetic resonance imaging; N/C/F = never/current/former; NGS = next-generation sequencing; PET = positron emission tomography; qRT-PCR = real-time quantitative reverse transcription–polymerase chain reaction; V-tach = ventricular tachycardia; W/B/O = white/black/other.
*Late gadolinium enhancement on cardiac MRI.
†Patchy left ventricular and/or septal uptake by PET with fludeoxyglucose F 18.
‡Second- or third-degree heart block or bifascicular block.
§Nonsustained or sustained spontaneous or inducible V-tach.
‖Evidence of systolic (left ventricular ejection fraction < 50%) or diastolic left ventricular dysfunction by echocardiogram or MRI.

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of Health–funded GRADS (Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis) research study and were obtained after patient diagnosis and from those who would benefit from subsequent treatment (i.e., active cardiac sarcoidosis as opposed to a more chronic form not requiring intervention). Exosomes were isolated from the plasma, using a total exosome isolation kit (Life Technologies, Carlsbad, CA), and total RNA was extracted (total exosome RNA and protein isolation kit; Life Technologies). RNA integrity and concentration were measured with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). To identify potential miRNA biomarkers, we assessed the miRNA spectrum in the discovery samples by next-generation sequencing (NGS). Small-RNA sequencing libraries were constructed with an NEBNext small-RNA library prep set (New England BioLabs,

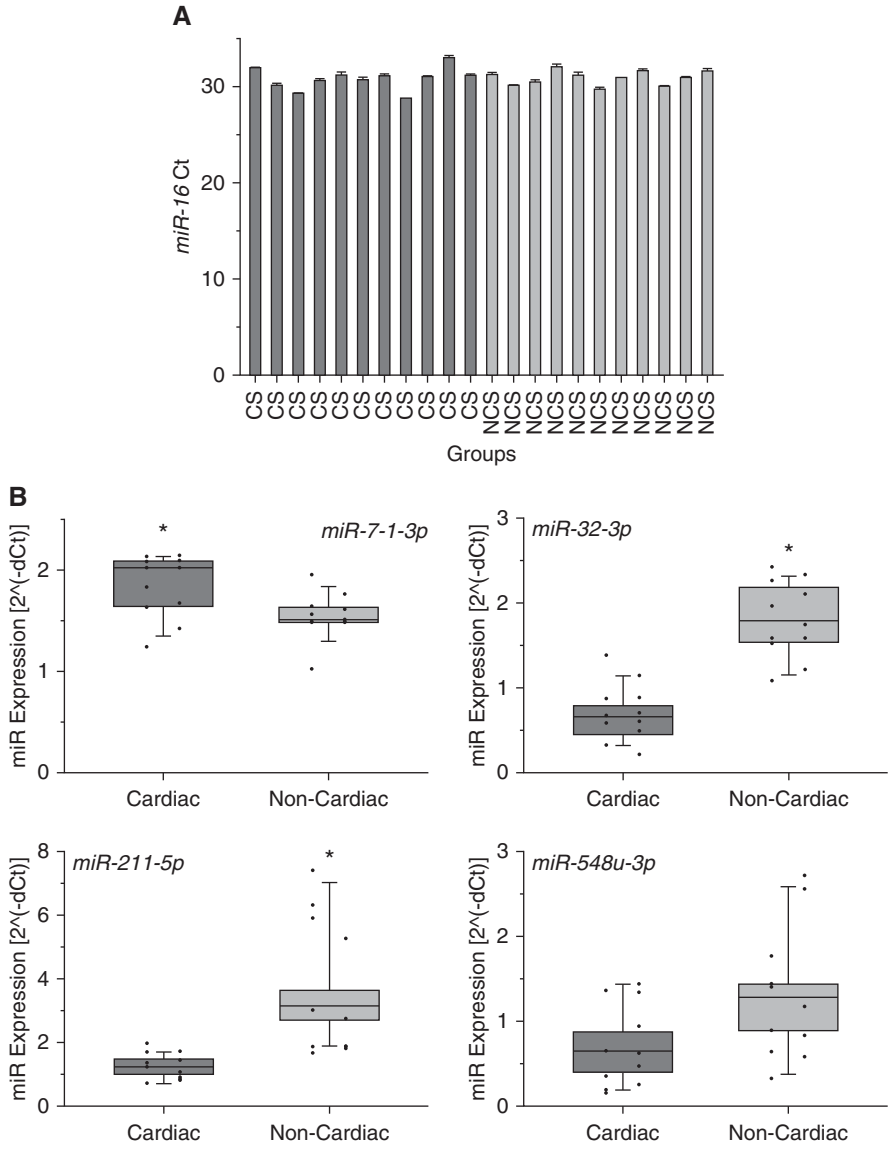

Figure 1. Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) validation of selected differentially expressed (DE) transcripts in plasma exosomal fractions from patients with cardiac sarcoidosis (CS) and patients with sarcoidosis with no evidence of CS (NCS). Of the 13 DE microRNAs identified by next-generation sequencing (NGS) analysis between CS and NCS plasma exosomal fractions, a select subgroup of the most DE transcripts was validated by qRT-PCR. (*A*) Mean + SE qRT-PCR cycle threshold (C_t) values for *miR-16* (to be used for normalization as in NGS) for all the CS and NCS plasma exosomal samples in the validation cohort. As anticipated, the values were relatively consistent across all samples. The mean C_t value for *miR-16* as determined in the control samples was 30.3 ± 0.9. (*B*) Relative qRT-PCR expression of select transcripts (*miR-7-1-3p*, *miR-32-3p*, *miR-211-5p*, and *miR-548u-3p*) normalized to *miR-16* between the CS and NCS plasma exosomal fractions. Three of these four transcripts identified by NGS were also significantly DE by qRT-PCR analysis (*$P < 0.02$, Mann-Whitney [rank sum] *U* test). In the box-and-whisker plots, the *middle line* represents the median; the *top* and *bottom* of the *box* represent the third and first quartiles, respectively; and the *upper* and *lower whiskers* represent the highest and lowest data points within 1.5 × the interquartile range from the *top* and *bottom* of the *box*, respectively. miR = microRNA.

Ipswich, MA) and then run on NextSeq 500 high-output kits (Illumina, San Diego, CA) with single-end 50-nucleotide read length. Data processing and mapping were performed as described previously (9), with subsequent miRNA expression internally normalized to *miR-16* levels because of its low concentration variation among samples used in the study (three independent real-time quantitative reverse transcription–polymerase chain reaction [qRT-PCR] results from validation samples are shown in Figure 1A). Statistical comparisons of differentially expressed miRNAs (identified when comparing the CS and non-CS groups) were corrected for multiple hypothesis testing, based on the Benjamini-Hochberg procedure to reduce false discovery (10).

The concentrations of 13 miRNAs were specifically different in exosomes, and not in plasma or exosome-depleted plasma, using a twofold concentration change threshold when comparing CS with non-CS ($P < 0.05$). These miRNAs were as follows: *miR-7-1-3p*, *miR-32-3p*, *miR-211-5p*, *miR-409-5p*, *miR-517a-3p*, *miR-548u-3p*, *miR-660-3p*, *miR-942-3p*, *miR-1262-5p*, *miR-3179-1-3p*, *miR-3940-3p*, *miR-6855-5p*, and *miR-7854-5p*. Among these, we selected several of the most affected miRNAs (*miR-7-1-3p*, *miR-32-3p*, *miR-211-5p*, and *miR-548u-3p*) for evaluation by qRT-PCR (normalized to *miR-16*) in the validation cohort. Notably, *miR-7-1-3p* and *miR-211-5p* are reported biomarkers of ischemic heart disease (11–13). The concentrations of these miRNAs were significantly different in all patients with sarcoidosis, taken collectively, relative to control subjects. Two (*miR-32-3p* and *miR-211-5p*) were found to have higher concentrations in the non-CS group compared with the CS group, and *miR-7-1-3p* was shown by qRT-PCR to be significantly elevated in patients with CS (Figure 1B). The concentration of *miR-548u-3p* was not significantly different between the CS and non-CS groups within the validation cohort.

Exosomal contents offer a rich source of potential biomarkers and may reflect the underlying molecular mechanisms of disease (5). *miR-7-1-3p* and *miR-211-5p* have been shown to be markers of, and in the case of *miR-7-1-3p*, incriminated in the pathogenesis of ischemic cardiac disease (11–13). However, the role of *miR-7* in the pathogenesis of CS, if any, remains to be determined.

Despite the small sample size, statistically significant concentration differences for specific exosomal miRNAs were apparent. Given the known limitations of NGS technologies (14), it was recommended to validate the results using techniques such as qRT-PCR. Limited by the small quantities of RNA obtained from exosomes, we were able to validate only a few selected miRNAs. Although the exosome isolation kit has been claimed to be highly selective (15), there is a significant amount of protein copurified with exosomes. It is possible that some of the miRNA/protein complexes were coprecipitated in the exosomal fraction. Nevertheless, our NGS data showed that the concentration changes for each of the 13 differentially expressed miRNAs were observed only in the exosomal fraction of the plasma and not in the exosome-depleted plasma samples. Even though ultracentrifugation offers a more pure exosomal isolation, it is low throughput, time-consuming, and prohibitory for use in most clinical applications with limited sample size. Given the risks and low sensitivity of myocardial biopsy, the diagnosis of CS is typically established on the basis of clinical criteria. In this regard, advanced imaging modalities, including magnetic resonance imaging and [^{18}F]FDG-PET/CT scan, are considered to be highly sensitive for the detection of CS (1) and can differentiate active inflammation from more chronic disease manifestations (e.g., fibrosis) (16). We suspect that the performance of an exosomal miRNA–based CS biomarker would be even better if we restricted the analysis to samples from those having evidence of active myocardial inflammation.

Our study suggests that specific exosomal miRNAs could serve as biomarkers of myocardial involvement in patients with sarcoidosis. Further investigation is needed to determine whether exosomal miRNA can reflect changes in cardiac activity (e.g., in response to immune suppression) or whether miRNA markers from exosomes can detect and differentiate other sarcoidosis phenotypes (e.g., neurosarcoidosis). ■

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The Timing of Early Antibiotics and Hospital Mortality in Sepsis: Playing Devil's Advocate

To the Editor:

In this issue of the *Journal*, Liu and colleagues have reported that hourly delays in antibiotic administration were associated with increased odds of hospital mortality even among patients receiving antibiotics within 6 hours (1) [pp. 856–863]. The overarching theme and implication of these findings is that clinicians should strive to deliver antibiotics to patients presenting to the emergency department (ED) with presumed sepsis as expeditiously as possible to improve survival (1). We applaud the authors' intentions of providing additional evidence that prompt administration of appropriate antimicrobial therapy in sepsis is lifesaving, but making this conclusion without following the outcomes of those patients without sepsis who received prompt, but unnecessary, antimicrobial therapy, leads to potentially skewed and biased assumptions (1–5). In fact, the authors make mention of this in their introduction when they list the potential harms of timely antibiotic administration (i.e., receipt of antibiotics unnecessarily culminating in adverse patient and community consequences, decreased attention to other diseases and patient-specific needs), but overlook this important fact when discussing their results and conclusions (1). The intent of this letter is to highlight the ramifications that neglecting to include those nonseptic patients who needlessly received antibiotics conceivably had on the researchers' results, while urging the investigators to reevaluate their findings in light of this potential bias.

The authors discuss their approach, which led to the 35,000 patients with sepsis who were included in their retrospective analysis, which included incorporating patients admitted with sepsis-specific *International Classification of Diseases* codes who received antibiotics within 6 hours of ED registration time (1). However, the authors neglect to include, and fail to mention, the exclusion of those patients who received prompt antibiotics who were later found not to be septic (i.e., presumably those with systemic inflammatory response syndrome resulting from noninfectious causes or viral infections) (1). It is these patients who received antibiotics unnecessarily, and their direct and indirect downstream health consequences of receiving unneeded antibiotics, that have significant potential to bias the authors' conclusion that prompt antibiotic administration improves survival in patients with sepsis. A more accurate conclusion given the study's methodology might be: for those patients presenting to the ED who received antibiotics within 6 hours and were admitted with a sepsis-specific diagnosis, rapid administration of antibiotics was associated with less odds of mortality.

It is safe to assume that a significant fraction of those nonseptic patients who received antibiotics unnecessarily had poorer outcomes and possibly higher mortality than if they never received antibiotics in the first place (6, 7). To list the potential ways inappropriate and unnecessary antibiotic administration can cause harm is beyond the scope of this letter, but suffice it to say there are many (6, 7).

Overall, we commend the authors for aspiring to demonstrate that antibiotics administered as quickly as possible in patients presenting to the ED with a systemic inflammatory response may improve sepsis survival, but making this conclusion without incorporating the potential harms of delivering unneeded antibiotics to nonseptic patients can lead to potentially inaccurate interpretations. Thus, despite these most recent findings, it remains imperative that clinicians weigh the benefits of prompt antibiotic administration with antibiotic stewardship. ■

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