

Circulating microRNAs: Potential Markers of Cardiotoxicity in Children and Young Adults Treated With Anthracycline Chemotherapy

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Background—Biomarkers for early detection of anthracycline (AC)-induced cardiotoxicity may allow cardioprotective intervention before irreversible damage. Circulating microRNAs (miRNAs) are promising biomarkers of cardiovascular disease, however, have not been studied in the setting of AC-induced cardiotoxicity. This study aimed to identify AC-induced alterations in plasma miRNA expression in children and correlate expression with markers of cardiac injury.

Methods and Results—Candidate plasma profiling of 24 miRNAs was performed in 33 children before and after a cycle of AC (n=24) or noncardiotoxic chemotherapy (n=9). Relative miRNA changes between the pre- and postcycle time points (6, 12, and 24 hours) were determined within each treatment group and compared across groups. Plasma miRNA expression patterns were further explored with respect to AC dose and high-sensitivity troponin T. Greater chemotherapy-induced dysregulation was observed in this panel of candidate, cardiac-related plasma miRNAs in patients receiving anthracyclines compared with those receiving noncardiotoxic chemotherapy (24-hour MANOVA; $P=0.024$). Specifically, plasma miRs-29b and -499 were upregulated 6 to 24 hours post-AC, and their postchemotherapy expression significantly correlated with AC dose. Patients with acute cardiomyocyte injury (high-sensitivity troponin T increase ≥ 5 ng/L from baseline) demonstrated higher expression of miR-29b and miR-499 post-AC compared with those without.

Conclusions—In this pilot study, cardiac-related plasma miRNAs are dysregulated following ACs. Plasma miR-29b and -499 are acutely elevated post-AC, with dose response relationships observed with anthracycline dose and markers of cardiac injury. Further evaluation of miRNAs may provide mechanistic insight into AC-induced cardiotoxicity and yield biomarkers to facilitate earlier intervention to mitigate cardiotoxicity. (*J Am Heart Assoc.* 2017;6:e004653. DOI: 10.1161/JAHA.116.004653.)

Key Words: anthracycline • cancer • cardiotoxicity • microRNA • pediatric

Anthracycline (AC)-related cardiotoxicity is among the most significant long-term threats to cancer survivors, with cardiac events comprising the most common nonmalignant cause of death in this population.^{1,2} The estimated

prevalence of cardiomyopathy in 50-year-old survivors of childhood cancer exposed to cardiotoxic chemotherapy or radiation is 21%.³ Once AC-induced cardiomyopathy develops, it is commonly progressive, with no definitive treatment other than implantation of a left ventricular assist device and/or heart transplantation.^{4–6} Currently, methods to detect subclinical cardiac injury early in the course of progressive cardiotoxicity are lacking. Early identification of patients at high risk for cardiac morbidity could allow implementation of cardioprotective measures during a stage when these therapies are likely to impact cardiac outcome.^{7,8} Pediatric studies exploring anthracycline (AC)-induced trends in cardiac troponin demonstrate a correlation between early troponin elevations and late echocardiographic abnormalities.^{9,10} However, studies have yet to reveal quantitative trends in troponin levels that specifically predict late cardiac outcomes, and thus troponins are insufficient to guide cardioprotective intervention.

MicroRNAs (miRNAs) are short, noncoding RNAs that regulate fundamental cellular processes and play a critical role in cardiovascular development and disease.^{11,12}

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Accompanying Data S1, Tables S1 through S3, and Figure S1 are available at <http://jaha.ahajournals.org/content/6/4/e004653/DC1/embed/inline-supplementary-material-1.pdf>

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Circulating miRNAs are promising biomarkers of cardiovascular disease attributed to their surprisingly high stability in plasma, tissue- or cell-specific distribution, and ease of quantitative measurement.^{13,14} The demonstrated sensitivity and specificity of miRNAs in various cardiovascular disease states indicate a potential role for miRNAs in early detection of cardiotoxicity.¹⁵

Although the role of miRNAs in modulating anthracycline-induced cardiac injury has been demonstrated in animal models,^{16–18} little is known about AC-induced alterations in plasma miRNAs. The aim of this study was to explore acute AC-induced alterations in plasma miRNA expression in children and correlate miRNA expression with known markers or predictors of cardiac injury. We hypothesized that children receiving AC chemotherapy would demonstrate a unique plasma miRNA profile compared with controls receiving

noncardiotoxic chemotherapy, and that this plasma miRNA signature could identify children with AC-induced cardiac injury.

Methods

Study Design

This was a prospective cohort study of patients aged <18 years receiving AC or noncardiotoxic chemotherapy (controls) at Children's Medical Center Dallas (Figure 1), approved by the University of Texas Southwestern Institutional Review Board (Dallas, TX). Patients with a history of congenital heart disease, Down syndrome, or those who received radiation to the heart were ineligible. Patients receiving AC, high-dose cyclophosphamide (>2000 mg/m² per cycle or

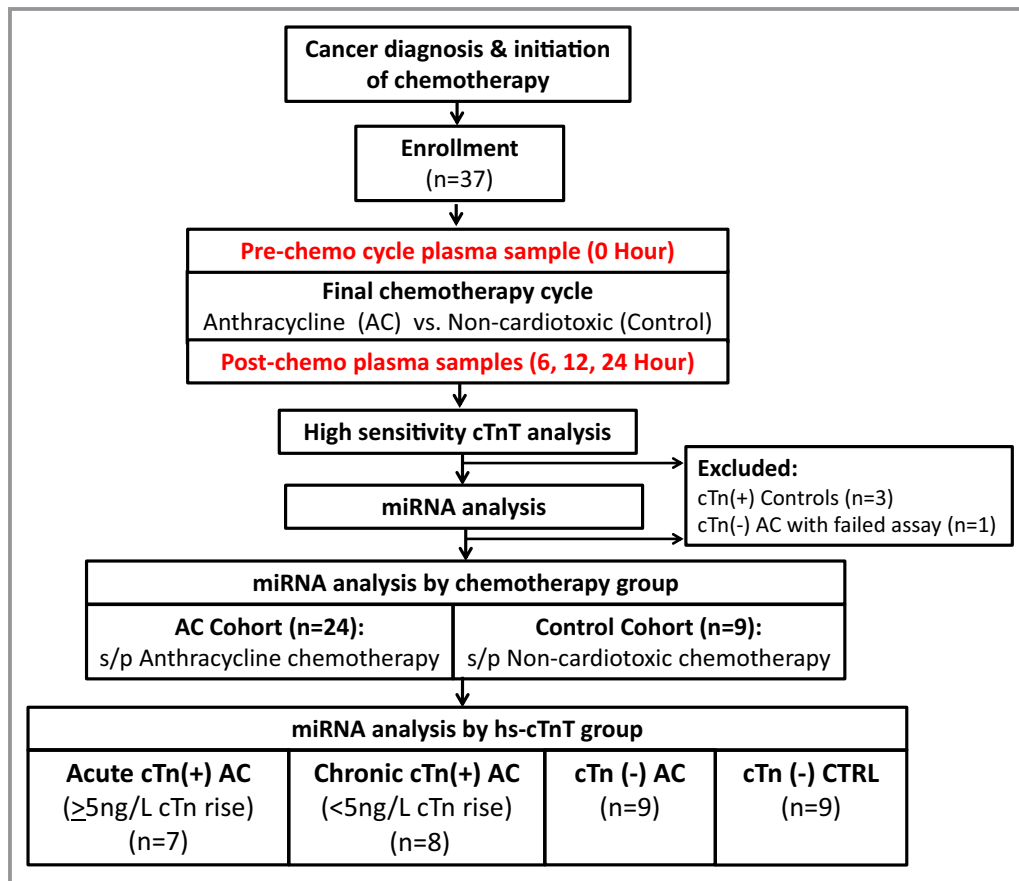


Figure 1. Study schematic and patient classification. Children being treated with chemotherapy for cancer were enrolled before a cycle of anthracycline (AC) or noncardiotoxic (Control) chemotherapy. Plasma was collected before and at several points following the chemotherapy cycle. Plasma miRNA expression was evaluated according to chemotherapy group (AC vs noncardiotoxic) and troponin elevation. AC indicates anthracycline; Acute cTn(+), patients with hs-cTnT ≥ 14 ng/L whose post-AC cTnT was ≥ 5 ng/L above the precycle-AC baseline; Chronic cTn(+), patients with hs-cTnT ≥ 14 ng/L whose pre- and postcycle-AC troponin levels remained stable (< 5 ng/L rise from baseline); cTn(-), patients with plasma cTnT level < 14 ng/L at all time points; cTn(+), patients with plasma hs-cTnT level ≥ 14 ng/L at any time point (0, 6, 12, or 24 hours); CTRL, control group; hs-cTnT, high-sensitivity cardiac troponin T assay.

>1200 mg/m² per dose), or tyrosine kinase inhibitors or those with any evidence of cardiac injury (high-sensitivity cardiac troponin T [hs-cTnT] >14 ng/L) were not eligible for the control group. Potential subjects were identified and referred for recruitment by their primary oncology provider. Those meeting eligibility criteria were approached for enrollment and informed consent was obtained. In all cases, AC chemotherapy was given by bolus infusion over 15 to 30 minutes. In most (n=21 of 25) cases, the AC group underwent sample collection during their final cycle of AC; controls underwent collection during a late treatment cycle of a noncardiotoxic chemotherapy regimen. In the AC group, a “pre-AC” cycle baseline sample was drawn before the first cycle dose of AC, with post-AC sample collection 6, 12, and 24 hours following the final AC dose. The 12-hour sample was only drawn from patients who were hospitalized (collected in 24 of 33 patients). Because this study was designed to assess miRNA alteration following the maximal cumulative AC exposure, all but 1 patient in the AC group had anthracycline exposure before to their “day 0” precycle baseline. Controls underwent the same schedule of plasma collection timed around a dose of noncardiotoxic chemotherapy.

Sample Processing, RNA Isolation, and MicroRNA Profiling

Plasma samples were collected either by peripheral venipuncture or from a central line (see Data S1 for details). Briefly, RNA was isolated using a miRNeasy serum/plasma microRNA isolation kit (Qiagen, Hilden, Germany) and reverse transcribed using the Taqman miRNA reverse transcription kit. Custom TaqMan Array MicroRNA Cards (Product# 4346799, Format 32; Applied Biosystems, Foster City, CA) were designed to assay 24 candidate miRNAs and 7 miRNAs for quality assessment and normalization. Candidate miRNAs were selected based on their demonstrated roles as biomarkers or mediators of cardiovascular disease (Table S1). The final endogenous miRNA controls used for miRNA normalization were chosen based on stability score (Table S2).

High-Sensitivity Troponin Assay

hs-cTnT was measured using an automated immunoassay (Troponin T hs STAT, Elecsys-2010; Roche Diagnostics, Indianapolis, IN).¹⁹ Five nanograms per liter represents the limit of detection for this assay. An absolute threshold of ≥ 14 ng/L is considered abnormal for this assay.^{20,21} Assessment of change in troponin over time is recommended to discriminate patients with and without acute cardiomyocyte injury.^{22,23} Therefore, we considered patients to have a significant chemotherapy-induced troponin rise if hs-cTnT 6 to 24 hours after chemotherapy was at least 5 ng/L greater

than the precycle baseline. Patients were categorized into 3 hs-cTnT categories, those with: acute hs-cTnT elevations (hs-cTnT ≥ 14 ng/L at any time point, plus ≥ 5 ng/L rise from baseline); chronic elevation (hs-cTnT >14 ng/L at any time point, but <5 ng/L rise from baseline); or normal (<14 ng/L at all time points).

Statistical Analysis

Comparisons across groups were made using Fisher's exact tests for nominal characteristics and rank-sum tests for continuous characteristics. Individual patient troponin values across 0-, 6-, 12-, and 24-hour time points were plotted by type of chemotherapy received (Figure S1). Differences in miRNA regulation from baseline were compared between AC and control groups at each time point (6, 12, and 24 hours) using linear mixed-effects regression models for each of the 24 target miRNAs. The models included fixed effects of group (anthracycline versus noncardiotoxic chemotherapy or cTnT rise ≥ 5 ng/L versus <5 ng/L), time (0, 6, 12, and 24 hours), condition (target miRNA versus control miRs-140 and -484), and all 2- and 3-factor cross-terms. The models included random intercept, time, condition, and time \times condition by patient effects to account for correlated cycle threshold (Ct) levels, changes over time, differences between conditions, and differences between changes over time by condition within patients, respectively. Additionally, MANOVA was applied to miRNA levels averaged by patient over the three Ct replicates at the 6- and 24-hour time points and the 2 control conditions to test the hypothesis of no overall group effect on the entire panel of candidate miRNAs. The number of samples collected at 12 hours was insufficient to perform this test.

Twenty-four miRNAs were measured in the cohort and 3 were selected for further analyses based on their consistent differential regulation between treatment groups with between group $P < 0.05$ (miRs-1 and -29b) or between group $P < 0.1$ with miRNA with fold-change >2 at all time points in the AC group with no change in the treatment group (miR-499). These miRNAs were subsequently compared across troponin groups (hs-cTnT rise ≥ 5 ng/L versus <5 ng/L) using similar regression modeling to that used to analyze treatment groups. Two miRNAs with differential expression between troponin groups were further analyzed to explore their relationship with relevant covariates including interim and cumulative AC dose, wherein normalized miRNA cycle threshold values at each time point were regressed linearly and separately on each covariate using data averaged over the 3 Ct replicates by patient and time. Finally, logistic regression models were fit in the subgroup of patients receiving ACs to explore the sensitivity and specificity of normalized miR-29b and -499 expression for predicting patients with a significant rise in hs-cTnT post-AC (≥ 5 ng/L above baseline). These models

Table. Characteristics of the Study Population

	Anthracycline (AC), n=24				Controls (CTRL), n=9	P for AC vs CTRL
	Acute cTnT Elevation*	Chronic cTnT Elevation [†]	No cTnT Elevation [‡]	P Across AC Groups		
No. of patients	7	8	9	...	9	...
Median age [range in y]	4 [0.5–16]	10.5 [3–16]	13 [7–16]	P=0.09	14 [1.8–18]	P=1.0
No. female (%)	4 (57)	1 (12)	2 (22)	P=0.20	2 (23%)	P=1.0
Diagnoses (n)	AML (6), NBL	Sarcoma (4), HL (2), ALL, APL	Sarcoma (3), NHL, HL (3), AML (2)	...	GCT (4), HBL, LCH, MED, NPC, RMS	...
Cumulative AC dose: [§] median [range in mg/m ²]	442 [75–442]	375 [75–600]	300 [75–450]	P=0.25
Interim AC dose: median [range in mg/m ²]	192 [75–192]	67.5 [25–75]	60 [25–192]	P<0.01
No. receiving dexrazoxane (%)	3 (43)	2 (25)	3 (33)	P=0.87
No. with SF reduction ≥20% from AC naïve baseline (%) [¶]	2 (28)	2 (25)	1 (11)	P=0.69
% SF reduction: [¶] median [range]	8 [3–35]	11 [0–26]	0 [0–24]	P=0.30
Peak hs-cTnT: median [range in ng/L]	32 [20–149]	30 [14–57]	9 [0–12]	P<0.01	6 [0–10]	P<0.01
Max hs-cTnT increase from baseline: median fold-change [range]	2.7 [1.6–6.4]	1.1 [0.8–1.4]	1.1 [0.7–1.5]	P<0.01	1 [0.8–1.4]	P=0.49

ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; GCT, germ cell tumor; HBL, hepatoblastoma; HL, Hodgkin's lymphoma; hs-cTnT, high-sensitivity cardiac troponin T; LCH, Langerhans histiocytosis; MED, medulloblastoma; NBL, neuroblastoma; NHL, non-Hodgkin's lymphoma; Max, maximum; NPC, nasopharyngeal carcinoma; RMS, rhabdomyosarcoma.

*Acute cTnT elevation: hs-cTnT ≥14 ng/L at any time point, plus ≥5 ng/L rise in hs-cTnT between the precycle baseline (0 hour) and postcycle hs-cTnT (6, 12, or 24 hours).

[†]Chronic cTnT elevation: hs-cTnT ≥14 ng/L at any time point, but <5 ng/L rise in hs-cTnT between the precycle baseline (0 hour) and postcycle hs-cTnT (6, 12, or 24 hours).

[‡]No cTnT elevation: hs-cTnT <14 ng/L at all time points.

[§]Cumulative anthracycline dose=total anthracycline dose given before 6, 12, or 24 hours postanthracycline sample collection.

^{||}Interim anthracycline dose=dose around which the pre- (0 hour) and postplasma (6, 12, and 24 hours) samples were collected. Anthracycline doses were converted to the equivalent doxorubicin dose: daunorubicin×0.83, epirubicin×0.67, idarubicin×5, and mitoxantrone×4.

[¶]SF: shortening fraction; echocardiograms were obtained in patients receiving anthracyclines, before any anthracycline exposure and 1 to 3 months following the final anthracycline dose.

used average Ct data by patient, time, and miRNA to construct receiver operator characteristic curves.

A priori, we targeted recruitment of 34 patients (24 AC-exposed; 10 controls), which would provide 80% power to detect a 2-fold mean group change in miRNA expression from baseline between groups, with an alpha of 0.05. Reported *P* values were not adjusted for multiple comparisons. All analyses were programmed in SAS/STAT software (version 9.4; SAS Institute Inc., Cary, NC). Additional details on both mRNA and troponin assays as well as the statistical modeling can be found in Data S1.

Results

Clinical Data and High-Sensitivity Troponin T Assay

A total of 37 patients were enrolled (n=25 AC and n=12 control), 33 of whom were included in the miRNA analysis

(n=24 AC and n=9 control). Three patients receiving intermediate-dose cyclophosphamide (1200 mg/m²) were excluded from the control group because of low-level elevations in hs-cTnT, in order to maintain a control group free of any evidence of cardiac injury. Additionally, 1 patient receiving doxorubicin had uniformly low Ct values (global mean <2 SD below that of the entire group) in 3 of the 4 sample time points, thus was excluded because of technical problems with the sample/assay. The AC group contained patients with various diagnoses (Table) who received doxorubicin (n=15), mitoxantrone (n=7), daunorubicin (n=1), or idarubicin (n=1) fractionated over 1 to 5 days. The mean cumulative anthracycline dose before baseline plasma collection and the individual cycle dose around which the plasma samples were collected were 321±30 and 102±13 mg/m² in doxorubicin equivalents, respectively. Eight patients received dexrazoxane in addition to their cycle dose of anthracycline, either per institutional protocol (n=7) or for left ventricular dysfunction following a previous anthracycline

cycle (n=1). The control group contained patients with a variety of tumor types who received non-AC containing chemotherapeutic regimens, including cisplatin, carboplatin, cyclophosphamide, ifosfamide, etoposide, bleomycin, clofarabine, 5-fluorouracil, vincristine, or dactinomycin. The AC and control groups did not differ by age ($P=0.83$) or sex ($P=0.81$) at study entry.

Of the 24 AC patients analyzed, 15 had hs-cTnT concentrations ≥ 14 ng/L following the cycle dose(s) of AC (cTn(+) AC). Of these, 8 patients had troponin ≥ 14 ng/L at baseline, preceding this cycle of AC (mean cTnT: $23 \pm \text{SD } 4.1$ ng/L; Figure 1). Seven of the 15 hs-cTnT-positive patients had an acute hs-cTnT rise of ≥ 5 ng/L from baseline. Nine patients had no hs-cTnT elevations at any time point. Within the AC group, the patients with acute elevations were similar in age, sex, and cumulative AC dose compared to those with chronically elevated or normal hs-cTnT (Table). However, the patients with acute elevations received a higher interim dose of AC than those with normal or chronically elevated hs-cTnT ($P=0.004$).

Evaluation of Plasma MicroRNA Dysregulation Over Time by Chemotherapy Group

Greater overall chemotherapy-induced dysregulation was observed in the 24 candidate plasma miRNAs in patients receiving AC chemotherapy compared with those receiving noncardiotoxic chemotherapy (MANOVA at 24 hours, $P=0.024$). Change in plasma expression from baseline of 2 specific miRNAs differed significantly between AC and controls (P value for anthracycline versus control at 6 hours, <0.05 ; Table S3). Plasma miR-1 was significantly upregulated 6, 12, and 24 hours post-AC, compared with controls in whom it was unchanged at all time points (Figure 2; Table S3). Plasma miR-29b demonstrated 2.7-fold upregulation 6 hours post-AC, while unchanged in controls (Figure 2). Additionally, miR-499 was significantly upregulated by >2 -fold 6, 12, and 24 hours post-AC compared with controls in whom it was unchanged and showed a trend toward differential regulation between treatment groups at 24 hours ($P=0.09$; Figure 2; Table S3).

Relationship Between Plasma miR-29b and -499 Expression and Anthracycline Dose

When comparing miRNA expression and AC dose, a significant relationship was identified between 6-hour miR-29b, 6-hour miR-499, and 24-hour miR-499 expression and interim AC dose, that is, higher miRNA expression with increasing AC dose for the cycle around which the plasma was collected (parameter estimates for miRNA Δ Ct: 6-hour miR-29b = -0.017 ; $P=0.020$; 6-hour miR-499 = -0.018 ; $P=0.004$; 24-hour miR-499 = -0.028 ; $P<0.001$). Additionally, there was a significant relationship

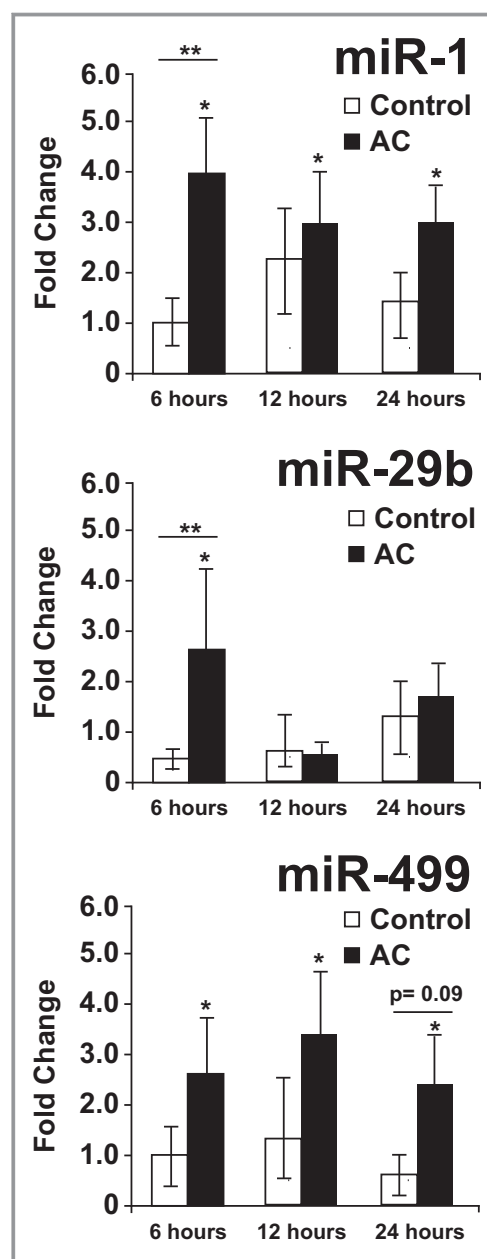


Figure 2. MicroRNA (miRNA) upregulation from baseline postanthracycline versus noncardiotoxic chemotherapy. Normalized miR-1, -29b, and -499 were significantly upregulated at multiple time points following the cycle anthracycline (AC; n=24), while unchanged in controls (n=9) receiving noncardiotoxic chemotherapy. * $P<0.05$ within-group change in miRNA postchemo (from baseline). ** $P<0.05$ between group (AC vs control) difference in miRNA regulation postchemo.

between 6- and 24-hour miR-499 and cumulative AC dose (estimate 6-hour = -0.0065 ; $P=0.006$; 24-hour = -0.0084 ; $P=0.010$). Finally, when controlling for concomitant cardioprotective therapy with dexrazoxane, a significant relationship between 6-hour miR-29b and cumulative anthracycline dose

was observed (parameter estimate = -0.0387 ; $P=0.033$), whereas the relationship between 24-hour miR-499 and cumulative dose was no longer significant.

Evaluation of Plasma MicroRNA Expression by Troponin Concentration

Further analysis of chemotherapy-induced plasma miR-1, -29b, and -499 regulation revealed significant differences between patients with and without evidence of acute cardiac injury following chemotherapy (hs-cTnT increase ≥ 5 ng/L from baseline). Patients with acute AC-induced hs-cTnT elevations had significant upregulation in miR-29b at 6 hours (6.6-fold; $P<0.01$) and miR-499 at 24 hours (5.2-fold; $P=0.03$) compared with AC or control patients with chronically elevated or normal hs-cTnT (Figure 3; between group $P=0.01$ for miR-29b and $P=0.07$ for miR-499). Plasma miR-1 was similarly upregulated over time regardless of hs-cTnT concentrations.

Similar results were observed when normalized plasma miR-29b and miR-499 expression (Δ Ct) was compared across groups at each pre- and postchemotherapy time point. Patients with acute cardiac injury demonstrated significantly higher plasma expression of miR-29b at 6 hours and miR-499 at 6 and 24 hours compared with those with chronically elevated or normal hs-cTnT (Figure 4).

Six-hour miR-29b, 6-hour miR-499, and 24-hour miR-499 individually moderately discriminated patients with evidence of acute cardiac injury post-AC (hs-cTnT rise, ≥ 5 ng/L; area under the curve [AUC], 0.75, 0.82, and 0.80, respectively). Discrimination for acute cardiac injury improved when using an aggregate of 6-hour miR-29b and miR-499 (AUC, 0.90). Individual or aggregate 12-hour miR-29b or miR-499 levels did not accurately discriminate patients with evidence of acute cardiac injury (AUC, 0.61, 0.68, 0.63 for miR-29b, miR-499, and miR-29b/-499, respectively).

Discussion

This study is the first to analyze circulating miRNAs in the setting of AC-induced cardiotoxicity. Although these findings are preliminary given the exploratory nature and small sample size, there were several key findings that support further exploration of miRNAs as clinically relevant markers of AC-induced cardiotoxicity. First, overall dysregulation of a panel of candidate plasma miRNAs with cardiac relevance was greater following AC compared with noncardiotoxic chemotherapy. Second, plasma miR-29b and miR-499 were upregulated in children with acute AC-induced cardiac injury, as evidenced by rising troponin concentrations post-AC. Third, post-AC miR-29b and miR-499 expression significantly correlated with cumulative AC dose, an established predictor of

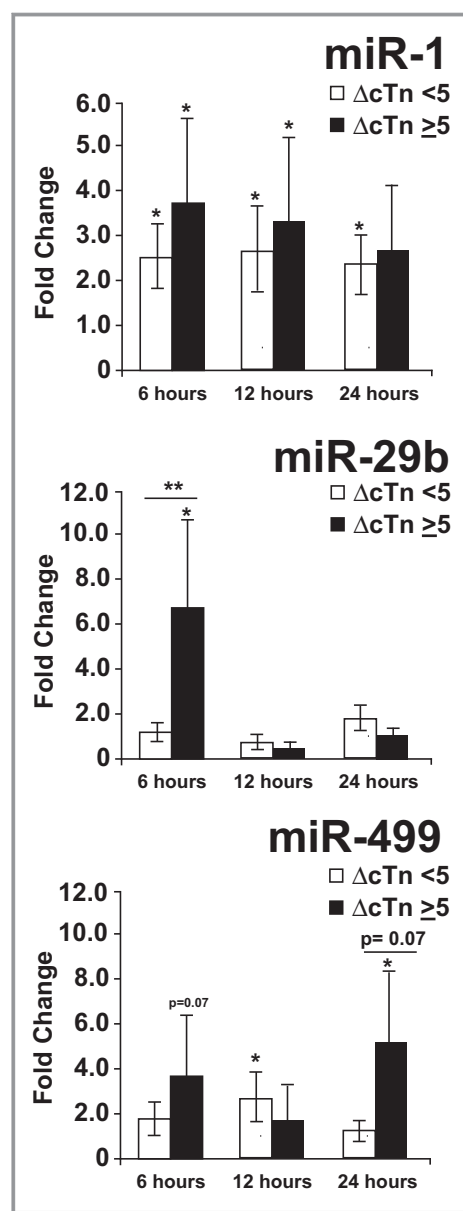


Figure 3. miRNA upregulation from baseline postchemotherapy by troponin group. Patients with ≥ 5 -ng/L postanthracycline rise in troponin from precycle baseline (Δ cTn ≥ 5 ; $n=7$) demonstrated upregulation of miR-29b 6 hours following anthracycline chemotherapy when compared with patients with stable or negative troponin following anthracycline or noncardiotoxic chemotherapy (Δ cTn < 5 ; $n=26$; between group, $P=0.013$). Whereas miR-499 demonstrated a trend toward differential regulation between troponin groups, this difference was nonsignificant ($P=0.07$). Plasma miR-1 was similarly upregulated across troponin groups. * $P<0.05$ within-group change in miRNA postchemo (from precycle baseline). ** $P<0.05$ between-group (Δ cTn ≥ 5 vs cTn < 5) difference in miRNA regulation postchemo. AC indicates anthracycline; cTn, cardiac troponin; miRNA, microRNA.

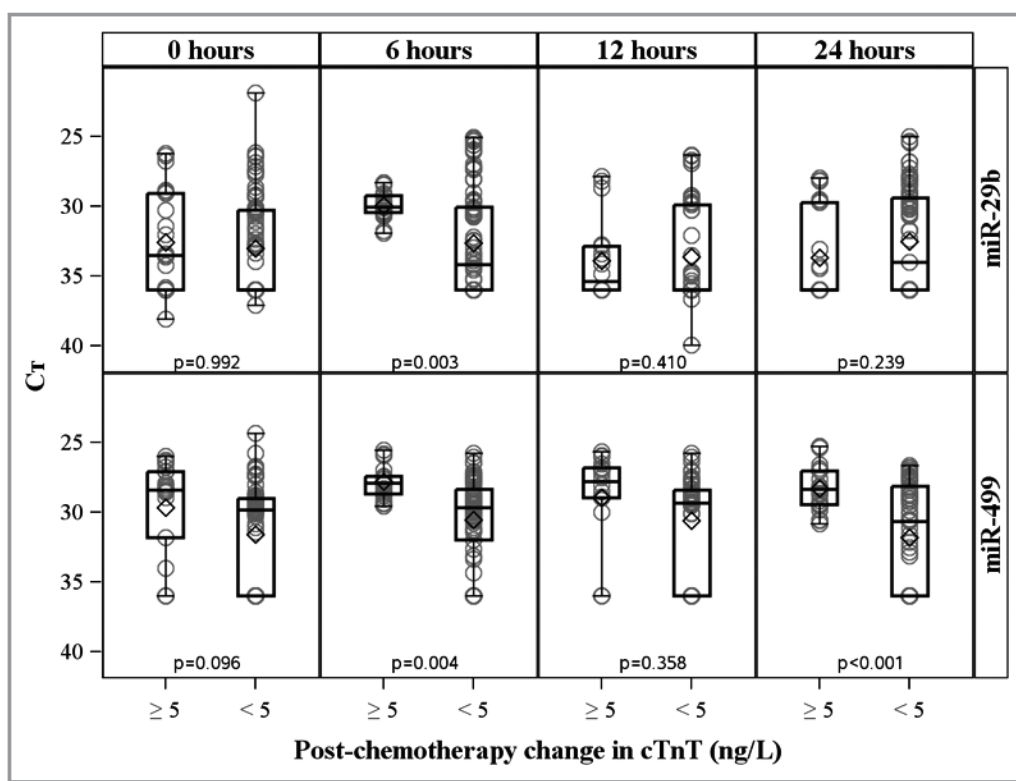


Figure 4. Normalized miRNA expression at each time point by troponin group. Normalized plasma miR-29b (top row) and miR-499 (bottom row) expression (Δ Ct [cycle threshold]) of patients with acute troponin elevations (rise in cTnT of ≥ 5 ng/L; $n=7$) compared with that of patients with stable or negative troponin postchemotherapy ($n=26$). Patients with troponin evidence of acute cardiac injury demonstrated higher plasma expression of miR-29b at 6 hours and miR-499 at 6 and 24 hours post-AC compared with those with chronically elevated or normal hs-cTnT. Mean Ct difference expressed on the y -axis is the cycle threshold of each candidate miRNA normalized to the aggregate Ct of endogenous controls (miR-484 and miR-140). Each box represents the interquartile range of miRNA expression (25th–75th percentile), with the horizontal line representing the median and the diamond representing the mean. hs-cTnT, high-sensitivity cardiac troponin T; miRNA, microRNA.

cardiotoxicity risk. Finally, plasma miR-29b and miR-499 expression levels 6 to 24 hours following AC provided moderate discrimination of patients with and without acute, cardiac injury. Together, these findings indicate that miRNAs may have a role in detecting subclinical cardiotoxicity.

miRNAs are critical modulators of cardiovascular development and disease, wherein miRNA-guided expression of downstream targets governs the process of regeneration, repair, or pathological remodeling.¹¹ Specific profiles of miRNA expression have been identified in numerous cardiovascular disease states, wherein circulating expression profiles may provide insight into underlying injury or remodeling occurring in the cardiac tissue.^{24,25} These circulating miRNA profiles are not only windows into the molecular landscape of heart disease, but offer the potential for novel therapeutics aimed at the modulation of miRNA and downstream target expression.¹⁵ Additionally, accumulating evidence supports the value of miRNAs as robust biomarkers of cardiovascular disease.^{13,15} Relevant to our findings, miR-499 is a muscle-

specific, cardiomyocyte-enriched miRNA that has been shown to have high sensitivity and specificity for detecting myocardial ischemia in previous reports.^{15,26,27} Plasma miR-499 upregulation postinfarction has been observed even earlier than hs-cTnT and thus may provide superior early sensitivity than troponins.²⁸ Additionally, higher plasma miR-499 expression following acute myocardial infarction has been correlated with increased risk for heart failure or mortality, indicating that miRNA profiles in the setting of acute injury may have important prognostic implications.²⁷ The protective role of miR-499 against oxidative-stress-induced apoptosis following acute myocardial infarction²⁹ has important relevance to AC-induced cardiotoxicity given that the most accepted mechanism of AC-induced cardiotoxicity is through the generation of reactive oxygen species and mitochondrial dysfunction within the cardiomyocyte.³⁰ Interestingly, in our small cohort, the protective role of dexrazoxane against AC-induced oxidative stress abrogated the relationship between cumulative anthracycline dose and miR-499

elevations, perhaps indicating that dexrazoxane was protective against oxidative-stress-related miR-499 elevations, even at high anthracycline doses.

The miR-29 family members are potent inhibitors of cardiac fibrosis and play a key role in cardiac remodeling following cardiomyocyte injury.³¹ Plasma miR-29a upregulation has been reported following myocardial injury, where the degree of miR-29a upregulation was associated with the extent of late remodeling post-acute myocardial infarction.²⁴ miR-29b, reported herein, targets many genes involved in the extracellular matrix (ECM), such as fibronectin, collagen, and matrix metalloproteinases. Given the critical role of early and late ECM remodeling in response to AC-induced cardiotoxicity,^{32,33} the upregulation of plasma miR-29b in this study has intriguing mechanistic relevance and may reflect early remodeling in response to AC-induced cardiac injury. Furthermore, modulation of miR-29 in various settings has successfully reduced maladaptive remodeling postinjury and allowed a more-efficient ECM remodeling response.^{31,34} With ongoing progress toward miRNA-directed therapy, the elucidation of the role of miR-29b in AC-induced cardiotoxicity could reveal an important therapeutic target that might allow prevention of AC-induced remodeling leading to preserved cardiac function.

This study has several notable limitations. First, the miRNA expression patterns identified in this study are based on a small, heterogeneous group of patients who received varying anthracycline regimens for a variety of malignancies. Additionally, the disproportionate sample sizes between the control group and AC group poses a significant statistical limitation. However, despite this heterogeneity and small sample size, miRNA expression correlated with multiple indicators/predictors of cardiotoxicity, including AC dose and hs-cTnT. Although troponin elevation has not consistently been shown to correlate with late cardiac outcomes in AC-exposed childhood cancer survivors, it is clearly a reflection of cardiomyocyte injury.³⁵ Thus, use of high-sensitivity troponin changes as a cardiotoxicity correlate for this preliminary miRNA analysis is reasonable. Unfortunately, changes in echocardiogram parameters are typically a late finding in pediatric cardiotoxicity occurring many years from AC exposure. Thus, more-definitive imaging correlates on which to compare miRNA expression were not feasible. The candidate approach to miRNA assessment in the setting of AC-induced cardiotoxicity may have limited our ability to identify miRNAs with important diagnostic or mechanistic implications. However, the 2 miRNAs found to have the most significant plasma dysregulation in patients with AC-induced cardiac injury are promising, particularly given their intriguing mechanistic relevance to that implicated in cardiotoxicity.

A final limitation of this and other circulating miRNA profiling studies is that the etiology and mechanistic significance of plasma miRNA dysregulation is unknown. Although

it is clear that many miRNAs (eg, miRs-499, -208, -133, and -1) are released into the plasma following cellular injury (as demonstrated after myocardial infarction), miRNAs are also exported from intact cells by being bound to stabilizing proteins or packaged within exosomes or microvesicles. Given evidence of troponin elevations following AC exposure, plasma miRNA expression described herein may, in part, reflect miRNA release following myocardial injury. Given the small sample size of this study, it cannot be definitively concluded that the other candidate miRNAs previously associated with cardiomyocyte injury (eg, in the setting of acute myocardial infarction, such as miR-133a)¹⁵ are not associated with AC-induced cardiomyocyte injury. However, the lack of relationship observed in this study between these cardiac miRNAs and AC might reflect the unique molecular functions of each individual miRNA, which might not be significantly involved in the heart's response to AC. Further work is warranted to determine the source and mechanistic implications of miR-29b and 499 elevations in the plasma.

In conclusion, we report that plasma miR-29b and -499 elevations occur in the acute setting following AC exposure and may be useful in detecting cardiomyocyte injury. Further work is warranted to evaluate the mechanistic role of these miRNAs in AC-induced cardiac injury and determine whether these miRNAs are useful for identifying patients at high risk of developing AC-induced cardiomyopathy.

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SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS:

Sample processing and RNA isolation

Plasma samples were collected following patient enrollment into an Institutional Review Board approved research protocol (University of Texas Southwestern IRB). Study investigators obtained informed consent from each participant or each participant's guardian. Plasma samples were collected either via peripheral venipuncture or from the patient's central line after sufficient waste was withdrawn to avoid heparin contamination. Within one hour of collection, samples were processed via refrigerated centrifuge at 2500 x G for 15 minutes. Plasma used for RNA isolation underwent a second high speed spin at 14,000 x G for 10 minutes to decrease platelet and particle contamination. RNA was isolated using Qiagen's miRNeasy serum/ plasma microRNA isolation kit (Qiagen, cat no. 217184) according to the manufacturers protocol with the following modifications: (1) 750uL Qiazol Lysis reagent added to 200uL plasma (vs. the 5:1 ratio recommended in the protocol) (2) 1.25µl 0.8 µg/µl bacteriophage MS2 RNA added upon addition of Qiazol solution in order to improve RNA yield (3) Wash with RPE repeated x 3 (in place of ETOH step). (3) RNA eluted in 20uL of nuclease free water.

RNA Quantification & Processing of miRNA Expression Data

Custom TaqMan® Array MicroRNA Cards (Applied Biosystems; Product# 4346799, Format 32) were designed to assay 24 candidate miRNAs and 7 miRNAs for quality assessment and normalization. Candidate miRNAs were selected based on their demonstrated roles as biomarkers or mediators of cardiovascular disease (Table S1) and include let-7g, miRs-1, 133a, 133b, 145, 146a, 195, 208a, 208b, 21, 210, 214, 215, 216b, 29a, 29b, 29c, 30a-5p, 34c, 361, 367, 423-3p, 423-5p, and 499-5p. A group

of candidate endogenous control miRNAs, including miRs-103, 140, 191-5p, 484, U6, and synthetic cel-miR-39, were also selected based on reports of successful normalization achieved using these miRNAs.[1-4] Additionally miR-451 and miR-23a were assayed as a quality control measure to assess sample hemolysis.[5]

Reverse transcription utilizing the Taqman custom RT primer pool was performed using the Taqman miRNA reverse transcription kit. Reaction volumes were modified based on the Taqman technical note entitled “Optimized protocols for human or rodent microRNA profiling with precious samples” (Product bulletin 4478705), including the following volumes: 3uL undiluted RNA, 4uL custom primer pool, 0.2uL dNTP, 2uL reverse transcriptase, 1uL 10X buffer, and 0.13uL RNase inhibitor for a total volume of 10.33uL. Pre-amplification was performed utilizing the Taqman PreAmp Mastermix and custom primer pool, with volumes and number of amplification cycles modified based on the Taqman technical note (Product bulletin 4478705). The reaction included 5uL cDNA, 3.75uL water, and 3.75uL custom PreAmp primer pool and underwent 14 amplification cycles. Finally, 2.25uL of the undiluted preamp product was combined with 112.5uL Taq PCR mastermix and 110.25uL nuclease free water (total volume of 225uL for 2 fill ports) and pipetted onto the custom miRNA TLDA cards. Real time analysis was performed per the manufacturer’s protocol using an Applied Biosystems 7900HT Real time PCR System.

Cycle threshold data for all samples were imported into OmicsOffice Statminer software program’s real time qPCR analysis feature. Undetermined cycle threshold values were set to 36, the detection limit. Candidate endogenous control miRNAs were evaluated using the normfinder algorithm aimed at identifying the miRNA(s) with the lowest variation within groups (AC vs. control). The aggregate of miRs-140 and -484 was selected as the endogenous control for this study based on its lowest stability value (Table S2).

Plasma microRNA Normalization

Currently there is a lack of consensus regarding circulating miRNA normalization. Although many groups are normalizing serum and plasma miRNA expression to a spiked in synthetic miRNA, this does not account for the effects of plasma processing and other pre-analytic variables on circulating miRNA measurement.[6] Given the high content of many intracellular miRNAs relative to that of plasma, hemolysis and platelet contamination of plasma can introduce significant miRNA variability from one sample to the next.[6, 7] We employed several strategies to overcome these technical challenges, including an additional high-speed centrifugation step to minimize platelet contamination and assessing the degree of RBC-derived miRNA contamination due to hemolysis.[7, 8] Fortunately, tissue specific miRNAs with little or no expression in circulating blood cells (including miR-499 from this study) are relatively unaffected by these processing variables. However, miR-29b and other miRNAs examined herein are highly expressed in blood cells, thus may be sensitive to processing conditions and inadequately normalized by the synthetic spike-in strategy.[9] For this reason, endogenous miRNA controls, chosen based on stability score (Table S2), were used for candidate miRNA normalization in this study.

High-sensitivity Troponin Assay

cTnT was measured in sample aliquots previously stored at -80°C using a highly sensitive automated immunoassay (Troponin T hs STAT, Elecsys-2010, Roche Diagnostics, Indianapolis, Indiana), with a limit of detection of 5 ng/l and a limit of blank of 3 ng/l. The lowest hs-cTnT concentration that can be measured with a coefficient of variation $\leq 10\%$ with this assay is 13 ng/l.[10]

Statistical Analysis

Plasma miRNA dysregulation over time by chemotherapy group. For each patient and candidate miRNA, 3 cycle threshold measurements were made at each time point. Linear mixed effects regression models for each target miRNA were used to estimate normalized mean cycle threshold values over time by chemotherapy group and allow comparison across groups. The models included fixed effects of group (anthracycline versus non-cardiotoxic chemotherapy), time (0, 6, 12, and 24 hours), condition (target miRNA vs. endogenous control miRs-140 and -484), and all 2- and 3- factor cross-terms. The models included random intercept, time, condition, and time x condition by patient effects to account for correlated cycle threshold levels, changes over time, differences between conditions, and differences between changes over time by condition within patients, respectively. The models allowed the residual variation in repeated measurements across time to differ by condition within patients. Normalized cycle threshold by group and time was estimated as the difference between the estimated mean target cycle threshold by group and time and the arithmetic average of the corresponding mean cycle thresholds of the two endogenous controls.

Plasma miRNA dysregulation over time by troponin group. Similar mixed effects regression models were constructed to assess miRNA expression over time by troponin group. These models were identical to that used for chemotherapy group, except that troponin group (≥ 5 versus < 5 ng/L post-chemotherapy change in troponin) was used in place of chemotherapy group. While population studies of normal adults support a threshold of 14 ng/L for cTnT concentrations above the 99th percentile, more recent data supports the use of change in troponin over time to discriminate patients with and without acute cardiomyocyte injury.[11-14] The threshold for acute cardiomyocyte injury of 5 ng/L rise 6-24

hours post chemotherapy is consistent with the clinically meaningful rise over a similar time period in the setting of myocardial infarction.[13, 14]

Sample size

A priori, we targeted recruitment of 34 patients (24 anthracycline-exposed; 10 controls), which would provide 80% power to detect a 2 fold-mean group change in miRNA expression from baseline between groups, with an alpha of 0.05. Variability in miRNA expression was calculated based on pilot data in the Mammen Laboratory revealing cycle threshold variability of 1.5-2.5 standard deviations, and we made a conservative estimate of sample size (n=30) based on SD of 2.5. Reported p-values were not adjusted for multiple comparisons. All analyses were programmed in SAS/STAT® version 9.4 (SAS Institute, Cary, NC, USA).

Table S1. Selection of candidate (A) and control (B) miRNAs based on published literature

(A) Candidate miRNAs	
Biomarker of cardiomyocyte (CM) injury post-myocardial infarction and/or mechanistically involved in cardiomyocyte proliferation & survival	miR-1 [15, 16] miR-133a/b [16, 17] miR-195 [18] miR-208a/b [19, 20] miR-21 [21] miR-30a-5p [22] miR-499-5p [19, 23]
Biomarker of heart failure	miR-423-5p [24]
Mediator/ Biomarker of Cardiac Fibrosis	miR-29a/b/c [25, 26]
Mediator of ROS signaling within the cardiomyocyte	miR-145 [27] miR-210 [28] miR-214 [29] miR-361 [30]
Mediators of (or dysregulated in) doxorubicin-induced cardiac injury models	miR-21 [31] miR-34c [32] miR-146a [33] miR-215 [32] miR-216b [32] miR-367 [32] miR-423-3p* let-7g [34]
(B) Endogenous Control miRNAs	
Role in normalizing plasma miRNAs	miR-103 [3] miR-140 [†] miR-191-5p [1] miR-484 [1] cel-miR-39 [2]
miRNAs used to assess degree of hemolysis	miR-23a [5] miR-451 [5]

[] Denotes manuscript reference

*Selected based on differential regulation observed in pilot data involving plasma miRNA array profiling (performed by Exiqon) of patients with chronic anthracycline cardiomyopathy compared to controls.

[†]Selected based on a low stability score in pilot data involving plasma miRNA array profiling (performed by Exiqon) on samples drawn and processed in the same manner by the same institution

Table S2. Stability score of potential endogenous controls, as determined by normfinder algorithm[35]

<u>Endogenous Control(s)</u>	<u>Stability Score</u>
hsa-miR-140 + miR-484	0.057
hsa-miR-484	0.058
miR-103 + miR-484	0.092
miR-140	0.097
miR-191 + miR-484	0.098
miR-103+ miR-140	0.101
miR-140+ miR-191	0.106
miR-103 + miR-191	0.128
cel-miR-39 + miR-484	0.166
cel-miR-39 + miR-140	0.171
miR-103	0.174
cel-miR-39 + miR-103	0.185
miR-191	0.186
cel-miR-39 + miR-191	0.189
cel-miR-39	0.326

Table S3. Dysregulation of candidate miRNAs post-anthracycline or non-cardiotoxic chemotherapy

		Anthracycline Group				Non-Cardiotoxic Chemotherapy Group				Between Group Difference
		Fold Change	Lower 95% CL	Upper 95% CL	Pr > t	Fold Change	Lower 95% CL	Upper 95% CL	Pr > t	Pr > t
miRNA	Time (hr)									
let-7g	6	1.3	0.9	1.7	0.108	1.0	0.6	1.6	0.841	0.312
	12	1.3	0.9	1.8	0.158	1.1	0.7	1.8	0.722	0.609
	24	1.1	0.8	1.4	0.675	0.7	0.4	1.1	0.088	0.095
miR-1	6	3.9	2.2	7.0	<0.001	1.0	0.4	2.5	0.973	0.015
	12	3.0	1.6	5.9	0.001	2.2	0.8	5.8	0.113	0.598
	24	3.0	1.7	5.3	<0.001	1.4	0.5	3.6	0.486	0.178
miR-133a	6	1.1	0.8	1.5	0.441	0.9	0.5	1.5	0.725	0.482
	12	1.0	0.7	1.4	0.851	1.1	0.6	1.8	0.800	0.752
	24	1.2	0.9	1.7	0.208	1.0	0.6	1.7	0.944	0.544
miR-133b	6	1.4	0.8	2.4	0.210	2.1	0.9	5.1	0.108	0.472
	12	1.3	0.7	2.4	0.487	0.5	0.2	1.4	0.198	0.146
	24	0.9	0.5	1.6	0.797	0.9	0.4	2.1	0.776	0.916
miR-145	6	1.2	0.9	1.6	0.288	1.5	0.9	2.6	0.096	0.384
	12	1.1	0.8	1.6	0.616	1.3	0.8	2.2	0.307	0.578
	24	1.0	0.8	1.4	0.852	1.1	0.7	1.8	0.678	0.799
miR-146a	6	1.0	0.7	1.3	0.758	1.2	0.8	1.8	0.499	0.461
	12	0.9	0.6	1.2	0.404	1.3	0.8	2.0	0.299	0.184
	24	1.1	0.8	1.4	0.719	1.5	1.0	2.4	0.055	0.148
miR-195	6	1.4	1.1	1.7	< 0.001	1.2	0.9	1.6	0.252	0.430
	12	1.6	1.3	2.0	< 0.001	1.5	1.1	2.1	0.011	0.742
	24	1.7	1.4	2.1	< 0.001	1.3	0.9	1.7	0.160	0.099
miR-21	6	1.6	1.3	2.0	< 0.001	1.9	1.3	2.7	< 0.001	0.390
	12	1.6	1.2	2.1	< 0.001	2.0	1.4	2.8	< 0.001	0.365
	24	1.6	1.3	2.0	< 0.001	1.7	1.2	2.4	0.002	0.703

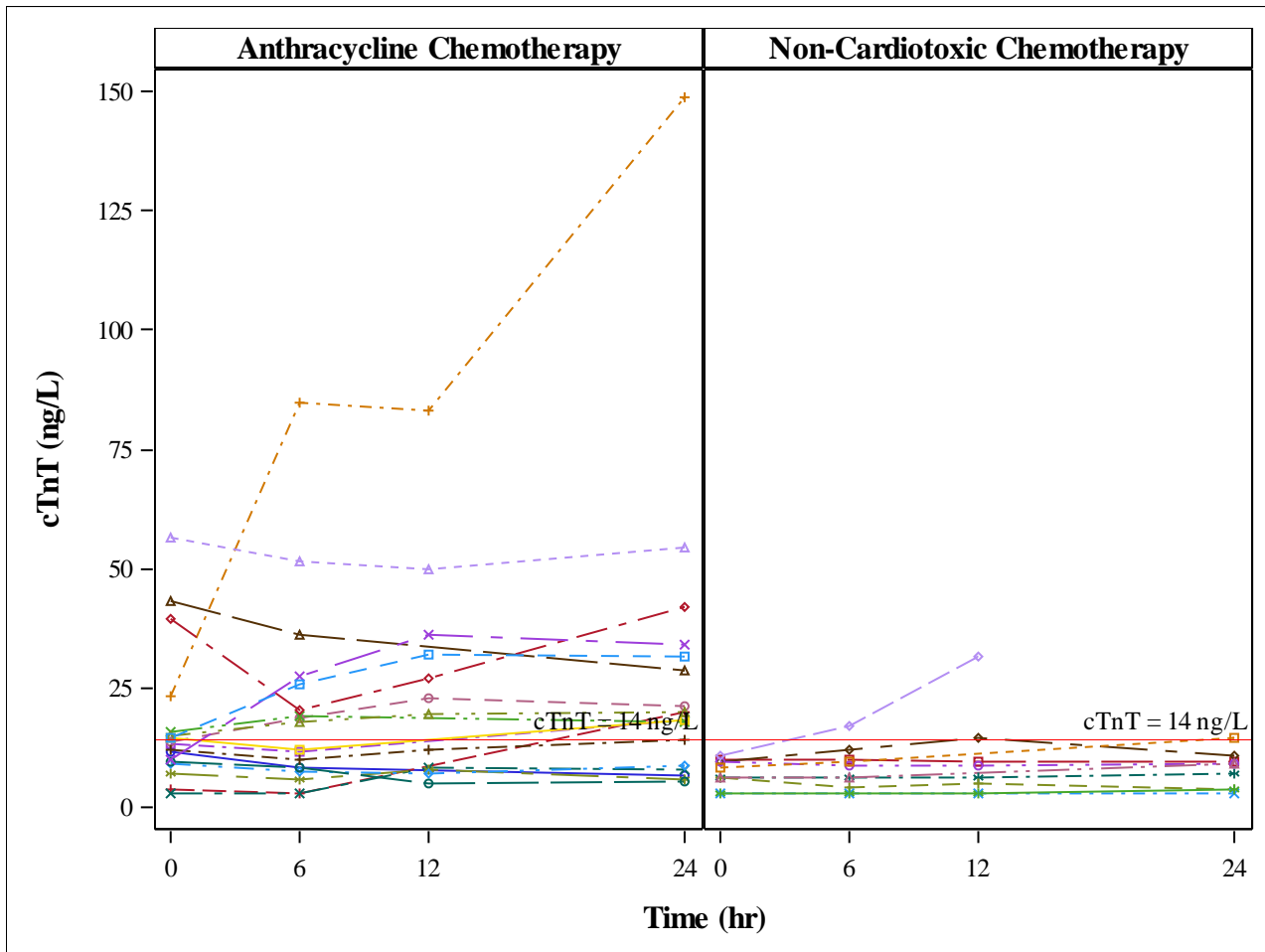
miR-210	6	1.3	1.1	1.5	< 0.001	1.3	1.0	1.6	0.050	0.870
	12	1.3	1.1	1.6	0.003	1.3	1.0	1.7	0.024	0.891
	24	1.4	1.2	1.6	< 0.001	1.4	1.1	1.8	0.004	0.806
miR-214	6	1.1	0.8	1.5	0.612	1.0	0.6	1.5	0.837	0.660
	12	1.5	1.1	2.2	0.019	1.2	0.7	1.9	0.551	0.391
	24	1.5	1.1	2.1	0.007	0.9	0.6	1.5	0.794	0.096
miR-215	6	1.1	0.8	1.5	0.676	1.0	0.6	1.7	0.952	0.868
	12	1.2	0.8	1.7	0.393	1.6	0.9	2.7	0.095	0.374
	24	1.6	1.2	2.3	0.003	0.9	0.5	1.5	0.694	0.059
miR-216b	6	0.6	0.3	1.2	0.157	0.4	0.1	1.3	0.126	0.571
	12	1.6	0.7	3.7	0.269	0.6	0.2	1.9	0.347	0.161
	24	0.6	0.3	1.3	0.227	0.5	0.2	1.7	0.264	0.755
miR-23a	6	1.3	1.0	1.7	0.041	1.6	1.0	2.4	0.048	0.538
	12	1.4	1.0	1.9	0.045	1.5	0.9	2.3	0.096	0.823
	24	1.5	1.1	2.0	0.004	1.7	1.1	2.7	0.019	0.627
miR-29a	6	1.3	1.0	1.6	0.025	1.3	0.9	1.8	0.120	0.878
	12	1.2	0.9	1.5	0.233	1.4	1.0	2.0	0.068	0.407
	24	1.1	0.9	1.4	0.343	1.2	0.8	1.7	0.320	0.731
miR-29b	6	2.7	1.4	5.4	0.004	0.4	0.1	1.1	0.084	0.003
	12	0.6	0.3	1.4	0.263	0.8	0.3	2.5	0.710	0.742
	24	1.8	0.9	3.6	0.092	1.3	0.4	3.8	0.666	0.599
miR-29c	6	1.4	1.1	1.7	0.002	1.4	1.0	2.0	0.034	0.882
	12	1.4	1.1	1.7	0.009	1.5	1.0	2.0	0.031	0.776
	24	1.4	1.1	1.7	0.002	1.3	0.9	1.8	0.122	0.752
miR-30a	6	1.2	1.0	1.5	0.026	1.3	1.0	1.8	0.045	0.585
	12	1.3	1.1	1.6	0.016	1.2	0.9	1.6	0.230	0.693
	24	1.4	1.2	1.7	< 0.001	1.4	1.0	1.8	0.037	0.810
miR-34c	6	1.3	0.8	2.0	0.268	1.1	0.6	2.3	0.726	0.779
	12	2.6	1.6	4.3	< 0.001	1.5	0.7	3.0	0.315	0.209
	24	1.9	1.3	3.0	0.003	1.0	0.5	2.1	0.920	0.139
miR-361	6	1.1	0.9	1.5	0.435	1.6	1.0	2.4	0.044	0.187

	12	1.1	0.8	1.5	0.447	1.4	0.9	2.2	0.153	0.456
	24	1.4	1.1	1.9	0.013	1.4	0.9	2.2	0.104	0.949
miR-367	6	1.1	0.8	1.6	0.521	2.1	1.1	3.9	0.018	0.091
	12	1.0	0.7	1.6	0.899	1.9	1.0	3.7	0.044	0.111
	24	1.3	0.9	1.9	0.181	1.1	0.6	2.0	0.824	0.604
miR-423_3P	6	1.0	0.8	1.2	0.907	1.4	0.9	2.0	0.104	0.148
	12	0.7	0.6	0.9	0.017	1.0	0.7	1.4	0.968	0.180
	24	0.9	0.7	1.1	0.212	1.3	0.9	1.9	0.155	0.063
miR-423_5p	6	1.1	0.9	1.3	0.407	1.5	1.1	2.1	0.008	0.065
	12	1.0	0.8	1.3	0.699	1.5	1.0	2.0	0.027	0.109
	24	1.1	0.9	1.3	0.421	1.3	1.0	1.9	0.064	0.248
miR-499	6	2.7	1.2	5.9	0.014	1.0	0.3	3.5	0.960	0.183
	12	3.3	1.3	8.1	0.011	1.5	0.4	5.6	0.542	0.345
	24	2.4	1.1	5.2	0.035	0.6	0.2	2.3	0.495	0.091

miR-208a and b not included because undetectable in essentially all patients

Bolded red miRNAs are those with between group (AC vs. C) change in cycle threshold $p < 0.05$

Figure S1. Troponin trajectory over time by treatment group



Each color and symbol represents an individual patient's plasma troponin prior to (0 hour) and following (6, 12, and 24 hours) a cycle of anthracycline or non-cardiotoxic chemotherapy. The dotted red line represents the standard threshold for a "positive troponin" at 14ng/L.

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