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Long-term impact of anthracycline in early-stage breast cancer, bridging of MiRNAs profiler for early cardiotoxicity

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Abstract

Background Anthracyclines are essential in early breast cancer chemotherapy but pose long-term cardiotoxicity risks.

Objectives This study aims to investigate the long-term incidence of cancer therapy-related cardiac dysfunction (CTRCD), bridging with the miRNAs profiler representing acute cardiac injury.

Methods We conducted a prospective cohort including stage I-III breast cancer patients who received anthracycline between 2007 and 2012. Echocardiography was performed before and 12 weeks after anthracycline administration. The miRNAs profiler was conducted by NanoString and RT-PCR. Long-term cardiac magnetic resonance imaging (CMR) was evaluated in 24.2% of asymptomatic participants.

Results At a median follow-up of 11 [IQR 6–12] years, 194 patients who completed follow-up echocardiography after anthracycline were included in the analysis. The median age at diagnosis was 50 [26–72] years. An early LVEF decline of ≥ 10% was found in 32.9% of participants. The cumulative equivalent dose of doxorubicin was 223.2 ± 21.6 mg/m2. At the time of censoring, sixty-four participants (32.9%) died, 70% from breast cancer. Nine participants (4.6%) reported cardiovascular events compatible with the CTRCD definition. Forty-seven participants (24.2%) underwent long-term cardiac evaluation. The miRNAs profiler and RT-PCR at different time points, 3 weeks and 6 weeks, respectively, revealed significantly diverse expressions of miR-1-3p and miR-16-5p in participants with and without an early LVEF decline of ≥ 10%. Despite cardiac injury demonstrated by dynamic miR-1-3p and miR-16-5p, CMR parameters revealed no significant differences.

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Conclusions Our study demonstrates a very low incidence of long-term symptomatic CTRCD. The diverse expression patterns of miR-16-5p and miR-1-3p at different time points also provide valuable biological insights. Within-normal results of an exact and comprehensive CMR, in asymptomatic and any LVEF change participants, indicate the long-term safety of limited-dose anthracycline-containing use.

Keywords Cancer therapy-related cardiac dysfunction, Long-term cardiotoxicity, Cardiac MRI, MicroRNAs

Introduction

Doxorubicin chemotherapy, approved by the US FDA since 1974, is essential for treating various cancers. However, there is a significant concern about long-term cardiac problems, particularly in survivors of early-stage breast cancer, who are at risk of anthracycline-induced cardiotoxicity (AIC). Previously, AIC was diagnosed when participants developed symptomatic cardiac dysfunction or significant decline in left ventricular ejection fraction (LVEF) using echocardiogram evaluation, and the definition has varied over time. Currently, the term "cancer therapy-related cardiac dysfunction" (CTRCD), as defined by the 2022 International Cardio-Oncology Society (IC-OS), is used for evaluating cardiotoxicity, and cardiac magnetic resonance imaging (CMR) has been established as a preferred method for assessing cardiac function [1, 2]. Despite dosage restrictions, early AIC in breast cancer patients has been reported in 3–5% of cases during short-term follow-up [3, 4]. While most cases are asymptomatic cardiomyopathy, information on long-term cardiac issues according to an updated definition of cardiotoxicity and long-term CMR findings has been limited.

Given the previous clinical benefits and limitations of anthracycline, exploring predictive biomarkers for early detection of AIC is crucial to optimize patient outcomes. Previously, noninvasive circulating biomarkers such as cardiac-specific isoenzymes troponins (cTn) and N-terminal brain natriuretic peptides (NT-proBNP) were reported to be associated with cardiac dysfunction during chemotherapy in breast cancer participants [5]. However, some data gave conflicting results, confounding factors from physiological changes, and a relatively short half-life in some markers have yet to be determined [6–8]. Recent data suggest that innovative circulating biomarker microRNAs (miRNAs) have a potential role as a predictive biomarker for AIC [9–11]. The miRNAs are involved in multiple gene regulation and were released into the circulation even in an early phase of cellular injury. Integrating miRNAs profiler will confirm the early cellular phase of cardiac injury after anthracycline treatment. This study aims to assess long-term cardiac complications (CTRCD) in breast cancer survivors treated with anthracyclines, with or without early AIC, and to bridge the results with a comprehensive miRNAs analysis. Part of this study was presented at the 2023 European Society for Medical Oncology (ESMO) congress (Abstract 259P).

Materials and methods

Study participants

The prospective cohort enrolled 227 of stage I-III breast cancer participants who received neoadjuvant or adjuvant anthracycline combination treatment regimen between January 2007 and December 2012 at the Division of Medical Oncology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand (Fig. 1). All participants had pathology proof of breast cancer and received at least one cycle of anthracycline. Demographic characteristics, co-morbid disease, and all breast cancer treatments provided were obtained from the electronic medical database. The participant's death date was validated by The Bureau of Registration Administration, Ministry of Interior, Bangkok, Thailand. The baseline cardiovascular toxicity risk was evaluated using the Heart Failure Association of the European Society of Cardiology Cardio-Oncology Study Group in collaboration with the International Cardio-Oncology Society (HFA-ICOS) Cardio-Oncology cardiovascular risk assessment tool [12]. The long-term follow-up was conducted as our institute's standard practice. Participants who exhibited any signs or symptoms of cardiac problems were referred to the cardiologist for further evaluation. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB. 998/64). Written informed consent was obtained from all participants (See Fig. 2).

Cardiac functional assessment

Echocardiography

Complete paired echocardiography was performed at baseline before anthracycline combination regimen treatment and 12 weeks after in 194 participants. Two-dimensional (2D) echocardiography was performed by a cardiologist who was blinded to biomarker assessment using Aloka proSound SSD–5500 SV (Hitachi Aloka, Tokyo, Japan). The left ventricular end-diastolic dimension (LVEDD) and the left ventricular end-systolic dimension (LVESD) were measured in the parasternal long-axis view by the manual edge detection method in 2D-guided M-mode. LVEF was measured using Teicholtz's method. Fractional shortening (FS) was calculated as (LVEDD-LVESD)/LVEDD×100. Participants with a post-anthracycline (12 weeks after) LVEF decline of ≥ 10% from baseline were defined as an early AIC case based on pre-2012 research and expert consensus of the

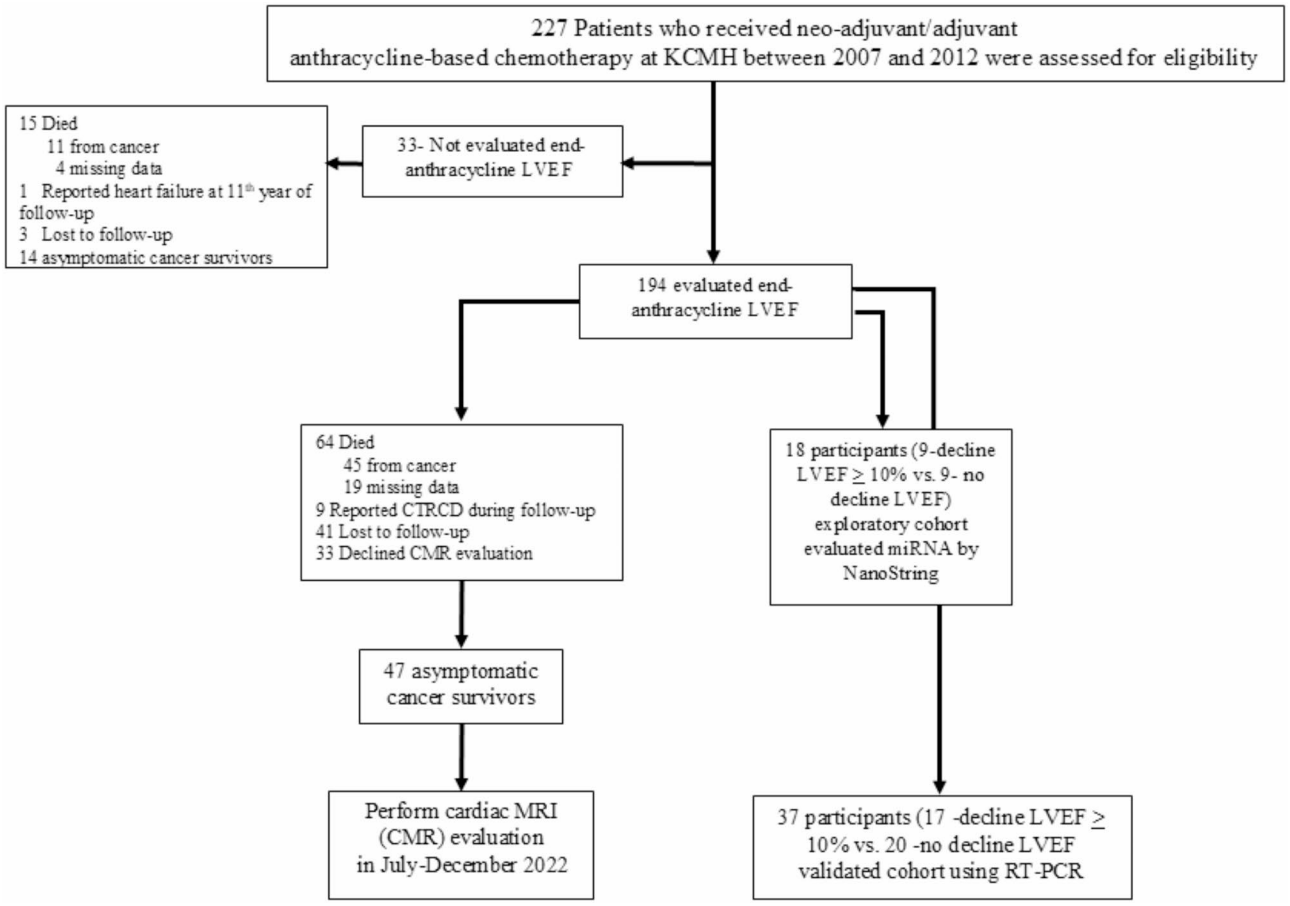

Fig. 1 Consort diagram for prospective cohort and long-term evaluation

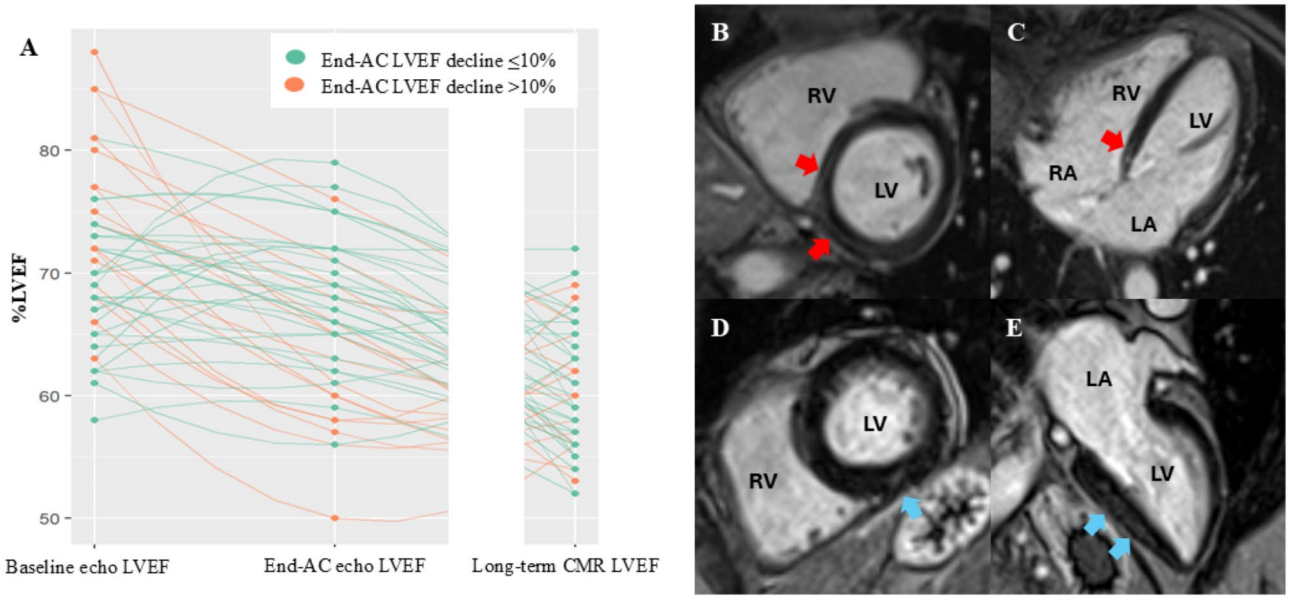

Fig. 2 Individual correlation between baseline, end-anthracycline, and long-term CMR LVEF between 16 cases and 31 controls (**A**). Cardiac magnetic resonance imaging with delayed gadolinium enhancement technique demonstrated two scar patterns. The basal short-axis (**B**) and 4-chamber view (**C**) showed mid-wall scar (red arrows) at basal inferoseptal and basal inferior segments of the LV. The middle short-axis (**D**) and 2-chamber view (**E**) revealed patchy scars (blue arrows) at mid-to-apical inferior segments of the LV. (LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle)

2014 American Society of Echocardiography and the European Association of Cardiovascular Imaging [13, 14]. Those without LVEF decline beyond the threshold were defined as controls.

Cardiac magnetic resonance (CMR) imaging

Between January and June 2022, 47 participants (25%) with a pair baseline and 12 weeks of echocardiography since the start of the anthracycline treatment and who did not document long-term symptomatic CTRCD were invited to be evaluated for subclinical and access severity of late cardiac dysfunction based on 2022 ESC classification by CMR. CMR was performed on a 1.5 Tesla system (Siemens Magnetom Sola) with phased array coil systems and analyzed using image analysis software (Circle Cardiovascular Imaging Inc., Calgary, AB, Canada). All CMR measurements were evaluated by a cardiac radiologist and a cardiologist. Details on the CMR imaging protocol & analysis were provided in the supplementary materials.

Plasma MiRNAs NanoString and validation RT-PCR study

Whole blood samples were collected in an EDTA container from all participants at baseline, 3 weeks, 6 weeks, and 9 weeks after the 1st cycle of anthracycline combination treatment. Blood samples were centrifuged at 1600 g for 10 min to collect plasma and then stored at − 80 °C until further used. In the exploratory cohort, miRNAs expression was performed in pretreatment and 3 weeks after the 1st cycle of anthracycline combination treatment in early AIC cases ($n = 9$) and control ($n = 9$), using nCounter Human v2 miRNA expression assay (NanoString Technologies, Inc., Seattle, WA, USA). The miRNAs extraction and NanoString profiler protocol were provided in supplementary materials. Significant miRNAs were selected and validated by RT-PCR in the validation cohort ($n = 37$) composed of early AIC cases ($n = 17$) and controls ($n = 20$) using TaqMan Advanced miRNA Assays. The details are provided in supplementary materials. All plasma samples were obtained from the Biobank facility of the Faculty of Medicine, Chulalongkorn University.

Raw data of the NanoString miRNAs was exported from nSolver™ analysis software version 3.0 to perform downstream analysis pipeline. Quality control using technical QC flags in R, which included imaging quality flag, blinding density flag, limit of detection flag, and linearity of positive controls flag. Data normalization was performed using R packages "DESeq2" and "RUVSeq". The RUVSeq package demonstrated robust outperform compared to nSolver in removing differences across technical sources of variation [15]. Differential housekeeping gene expression was assessed, which was not correlated to the biological phenotype of interest. Upper quantile and housekeeping gene normalization were conducted. Relative log expression gene expression before and after normalization is shown in Figure S2. Differential gene expression analysis was conducted according to each phenotype. Differential gene expression using p-value < 0.01, FDR p-value < 0.1 was considered significant (Table S2).

Statistical analysis

The frequency of CTRCD was assessed by calculating the proportion of individuals who met the criteria outlined in the 2022 European Society of Cardiology (ESC) guideline. An exploratory analysis was carried out to investigate differences in the characteristics of participants who underwent CMR altogether with diverse CMR parameters, and the differential miRNA expression was conducted based on the early change of LVEF before and 12 weeks after anthracycline combination treatment. Categorical variables were compared using $\chi 2$ or Fisher's exact test, while continuous variables were compared using t-tests or variance analysis. A significance threshold of p-value < 0.05 was used to determine statistical significance.

Results

Cardiovascular outcome and cardiac assessment

As of October 31, 2023, a total of 227 participants with early-stage breast cancer were enrolled in this cohort with a median follow-up time of 11 [IQR 6–12] years. One hundred ninety-four participants who completed follow-up echocardiography after anthracycline treatment were included in the final analysis. The median age of participants at the time of breast cancer diagnosis was 50 [IQR 26–72] years, and the mean cumulative equivalent dose of doxorubicin was 223.2 ± 21.6 mg/m^2. Sixty-four participants (32.9%) died, 70% from breast cancer, while the remaining died from unknown causes. Nine participants (4.6%) reported cardiovascular events such as CTRCD during the follow-up period (Fig. 1**).** Sixty-four participants (32.9%) experienced a decline in LVEF ≥ 10%, while 130 participants (67.1%) did not experience a decline in LVEF beyond this threshold. The baseline characteristics of participants are shown in Table 1. No significant differences were observed in the median age at breast cancer diagnosis, baseline cardiovascular risk according to HFA-ICOS criteria, BMI, cumulative doxorubicin-equivalent dose, or receipt of left chest wall radiation. Subsequent anti-HER2 therapy was administered to 15.9% of participants. Those who experienced a decline in LVEF ≥ 10% (case group) were more likely to receive anti-HER2 therapy (25%) compared to those who did not experience a decline in LVEF (control group) (13.8%) (Table 1). However, this difference was borderline statistically significant (p-value 0.07). Participants in the case group had a higher baseline LVEF than those in the control group. However, after the end of anthracycline therapy, participants in the case group exhibited a statistically significant decrease in

Table 1 Baseline characteristics of 194 stage I-III breast cancer participants treated with anthracyclines between 2007 and 2012. All participants with pair baseline and 12 weeks since start anthracycline LVEF evaluation by echocardiogram were divided into case (early LVEF decline > 10%) and control (no LVEF decline)

Parameters	All participants *n* = 194	Participants with pair baseline and 12 weeks since start anthracycline LVEF evaluation by echocardiogram (*n* = 194)		
		LVEF decline ≥ 10% (*n* = 64)	No LVEF decline (*n* = 130)	*p*-value
Median age at breast cancer diagnosis, [range] (years)	50 [26–72]	51 [26–70]	50 [30–72]	0.76
BMI (kg/m2) at diagnosis	24.6 ± 4.6	24.5 ± 4.3	24.6 ± 4.7	0.84
Hypertension, diabetes mellitus or dyslipidemia, n (%)	74 (38.1)	23 (35.9)	43 (33.1)	0.75
Baseline cardiovascular risk (HFA-ICOS), n (%)				
-Low risk	60 (30.9)	17 (26.6)	43 (33.1)	0.64
-Moderate risk	59 (30.4)	21 (32.8)	38 (29.2)	
-High risk	75 (38.7)	26 (40.6)	49 (37.7)	
History of smoking, n (%)	5 (2.6)	1 (1.6)	2 (1.5)	1.00
Menopause, n (%)	65 (33.5)	20 (31.3)	45 (34.6)	0.63
Cumulative doxorubicin-equivalent dose (mg/m^2)	223.2 ± 21.6	223.7 ± 18	222.9 ± 23.2	0.81
Baseline LVEF (%) by echocardiogram	70.2 ± 7.1	74.3 ± 6.6	68.2 ± 6.5	< 0.01*
End-anthracycline LVEF by echocardiogram (%)	66.6 ± 6.8	61.9 ± 6.1	68.9 ± 5.9	< 0.01*
Change of baseline and end-anthracycline LVEF (%)	-4.5 ± 11.9	-16.5 ± 5.7	1.5 ± 9.4	< 0.01*
Subsequent anti-HER2 therapy, n (%)	34 (17.5)	16 (25.0)	18 (13.8)	0.07
Left chest wall radiation, n (%)	68 (35.1)	25 (39.1)	43 (33.1)	0.52
Documented symptomatic CTRCD, n (%)	9 (4.6)	1 (1.6)	8 (6.2)	1.00

LVEF: left ventricular ejection fraction, CMR: cardiac magnetic resonance imaging, CTRCD: cancer therapy-related cardiac dysfunction, HER2; human epidermal growth factor receptor2, HFA-ICOS: The Heart Failure Association of the European Society of Cardiology Cardio-Oncology Study Group in collaboration with the International Cardio-Oncology Society, BMI: body mass index

LVEF from baseline (-16.5 ± 5.7%) compared to the control group (+ 1.5 ± 9.4%).

Short-term report of symptomatic CTRCD (within 1 year) after the last dose of anthracycline

Out of 194 participants with complete pair echocardiography, there was a rare incidence of symptomatic CTRCD who met the 2022 IC-OS CTRCD ESC definition in an early phase of treatment. Only one in the case group developed symptomatic heart failure after the third cycle of anthracycline combination treatment. This patient had a pre-existing condition of morbid obesity (BMI 40 kg/m^2) and a baseline LVEF of 64%. After stopping anthracycline and receiving appropriate treatment for heart failure, her condition improved to NYHA class I-II. Adjuvant chemotherapy was continued with a taxane regimen. Follow-up echocardiography revealed an LVEF of 54% without any regional wall motion abnormality. No further cardiac events were reported.

Long-term report of symptomatic CTRCD after anthracycline therapy

We censored the date of follow-up participants on October 31, 2023, representing a median follow-up time of 11 [IQR 6–12] years. Long-term CTRCD events were identified through the electronic medical database at The King Chulalongkorn Hospital. Symptomatic participants, as well as other LV dysfunctions, were investigated and managed, following our institute's standard practice. Those included referral to a cardiologist to evaluate the cause of cardiomyopathy and starting cardioprotective agents to prevent further cardiac complications. Nine cases (4.6%) of CTRCD were observed. These included participants with a post-anthracycline LVEF decline of ≥ 10% ($n = 1$) and participants without post-anthracycline LVEF decline ($n = 8$). Among those without post-anthracycline LVEF decline, two participants experienced heart failure, and six had non-fatal arrhythmias. None of the symptomatic CTRCD die from cardiac events. Most events were reported more than eight years after completing anthracycline therapy. The details of all 9 CTRCD participants are provided in the supplementary material.

Long-term cardiac assessment in asymptomatic participants without prior CTRCD

The remaining asymptomatic participants, despite decline in LVEF ≥ 10%, were followed up as usual practice, the same as those without a decline in the LVEF. According to this, we could not define the severity of long-term CTRCD in all asymptomatic participants upon the 2022 ESC classification. However, forty-seven asymptomatic CTRCD participants were invited for cardiac evaluation with physical examination, EKG, and CMR. The median time from baseline echocardiogram to CMR was 12 years (IQR 11–12). All participants had normal findings of physical examinations and no cardiac arrhythmias by

Table 2 Patient characteristics in long-term CMR evaluation cohort (*n* = 47)

Characteristics	All participants (*n* = 47)	Participants with pair baseline and 12 weeks since start anthracycline LVEF evaluation by echocardiogram (*n* = 47[#])		
		LVEF decline ≥ 10% (*n* = 16)	No LVEF decline (*n* = 31)	*p*-value
Median age at breast cancer diagnosis, (range) (y)	46 (26–64)	44 (31–53)	49 (26–64)	0.84
Baseline HFA-ICOS cardiovascular risk				
- Low (%)	42 (89.4)	14	28	0.35
- Moderate (%)	5 (10.6)	2	3	
- High (%)	0	0	0	
BMI (kg/m2) at diagnosis	24.0 ± 4.4	23.7 ± 4.1	24.2 ± 4.6	0.69
Cumulative doxorubicin-equivalent dose (mg/m2)	225.5 ± 11.3	226 ± 11.5	224 ± 11.3	0.55
Baseline LVEF by echocardiogram (%)	70.7 ± 6.7	75.2 ± 7.2	68.4 ± 5.2	< 0.01*
End-anthracycline LVEF by echocardiogram (%)	66.3 ± 6.2	62.7 ± 6.5	68.1 ± 5.3	< 0.01*
Subsequent anti-HER2 therapy, n (%)	9 (19.1)	5 (31.2)	4 (12.9)	0.25
Left chest wall radiation, n (%)	19 (40.4)	7 (43.8)	12 (38.7)	0.36
Duration from baseline echocardiogram to CMR, (range) (y)	12 (10–15)	12 (11–14)	12 (11–15)	0.12
Age at time of CMR, (range) (y)	59 (37–74)	56 (44–66)	61 (37–74)	0.29

LVEF: left ventricular ejection fraction, CMR: cardiac magnetic resonance imaging, CTRCD: cancer therapy-related cardiac dysfunction, HER2; human epidermal growth factor receptor2, HFA-ICOS: The Heart Failure Association of the European Society of Cardiology Cardio-Oncology Study Group in collaboration with the International Cardio-Oncology Society, BMI: body mass index

Table 3 CMR parameters and characteristics in long-term safety cohort

Long-term CMR parameters	Normal limited of value [47, 48]	Total (*n* = 47)	End-anthracycline LVEF decline ≥ 10% (*n* = 16)	End-anthracycline no LVEF decline (*n* = 31)	*p*-value
LVEF by CMR (%)	64 ± 5.8	60.4 ± 5.2	60.3 ± 4.9	60.4 ± 5.3	0.87
Cardiac scar, n (%)	N/A	6 (12.8)	1 (6.3)	5 (16.1)	0.13
Pattern			RV insertion	RV insertion = 3, mid wall = 2	
Native T1 (ms)					
-Basal segment	885–1059	905.9 ± 53.5	914.5 ± 56.2	901.4 ± 52.4	0.43
-Mid segment		832.6 ± 69.2	837.2 ± 67.6	830.2 ± 71	0.75
-Apical segment		777.1 ± 95.6	800.1 ± 105.4	764.8 ± 89.5	0.23
LV mass index (g/m^2)	57.4 ± 10	41.3 ± 5.6	42.0 ± 4.8	40.9 ± 6.0	0.52
LVEDV (mL)	115 ± 28.5	112.7 ± 16.2	113.1 ± 17.2	112.5 ± 15.9	0.91
LVEDVI	68 ± 11.7	69.3 ± 9.0	70.1 ± 9.7	68.8 ± 8.8	0.65
LVESV (mL)	41.9 ± 14.3	45.1 ± 9.7	45.0 ± 9.4	45.2 ± 10.0	0.96
LVESVI	24.3 ± 6.5	27.8 ± 6.0	27.9 ± 5.4	27.7 ± 6.4	0.91
Global longitudinal strain (GLS)	− 20.4 ± 3.5	-17.6 ± 2.5	-17.3 ± 3.2	-17.7 ± 2.0	0.67
RVEF by CMR (%)	57.5 ± 5.9	59.9 ± 5.0	59.4 ± 5.3	60.1 ± 4.9	0.75

CMR: cardiac magnetic resonance imaging, LVEF: left ventricular ejection fraction, LVEDV: left ventricular end-diastolic volume, LVEDVI: left ventricular end-diastolic volume index, LVESV: left ventricular end-diastolic volume, LVESVI: left ventricular end-diastolic volume index RVEF: right ventricular ejection fraction

EKG. The characteristics of the participants for the long-term CMR evaluation cohort are shown in Table 2. 34% of CMR participants had an end-anthracycline LVEF decline of ≥ 10%. Despite the dynamic change of end-anthracycline LVEF, there were no significant differences in age at breast cancer diagnosis, baseline cardiovascular risk, BMI, cumulative doxorubicin-equivalent dose, left chest wall radiation, time from baseline echocardiogram to CMR, and age at CMR. However, those who experienced a decline in LVEF ≥ 10% were more likely to receive anti-HER2 therapy (31.2%) compared to those who did not experience a decline in LVEF (12.9%) (Table 2), although this difference was not statistically significant (*p*-value 0.25).

Regarding CMR findings (Table 3), none of the participants who experienced decreased LVEF in CMR met the 2022 IC-OS CTRCD criteria. There were no differences in CMR parameters, including LVEF, LVEDVI, LVESVI, LV mass index, LVEDV, LVESV, RVEF, T1 mapping, and strain between participants with end-anthracycline LVEF declined ≥ 10%, and those without LVEF declined. One participant (6.3%) with end-anthracycline LVEF decline ≥ 10% had an RV insertion scar, while five participants (16.1%) without LVEF decline had cardiac scars: three RV insertion and two mid-wall scars.

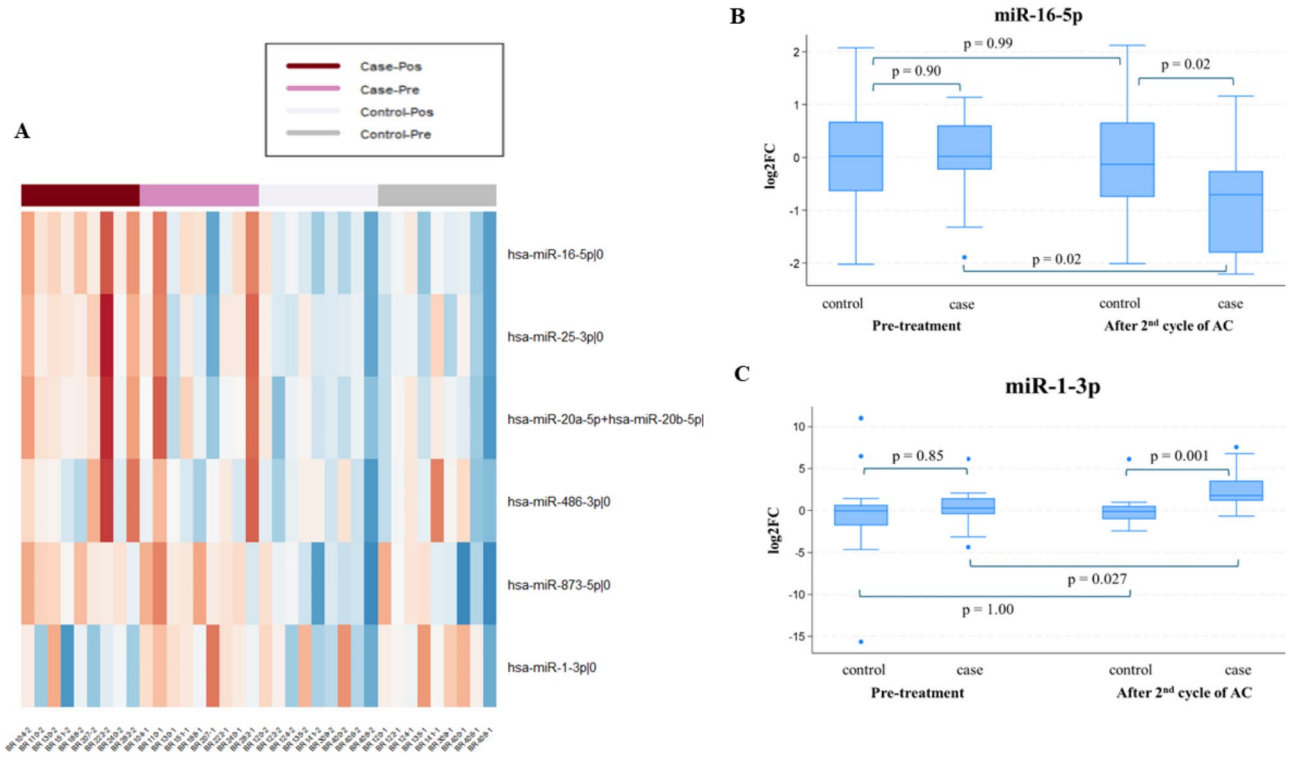

Fig. 3 Heatmap of significant five miRNAs expression and miR-1-3p by NanoString profiler in the discovery cohort ($n = 18$) at baseline and 3 weeks after anthracycline combination therapy (**A**). The validation cohort of selected miRNAs was conducted in 37 participants at baseline and 6 weeks after anthracycline combination therapy. The miR-16-5p (**B**) revealed significantly down-regulated with a mean log2 fold change of -0.77 (p-value 0.02) in the case group compared to the control group. While miR-1-3p was significantly up-regulated in the case compared to the control group with a mean log2 fold change of 2.48 (p-value 0.001) (**C**)

Plasma biomarker for the early detection of cardiotoxicity

MiRNA expression using targeted multiplex analysis panel

Thirty-six plasma samples from 18 participants (9 early AIC cases and 9 controls) at two timepoints (pre-treatment and 3 weeks post 1st cycle of anthracycline) were explored miRNAs using NanoString technology to identify & confirm potential biomarkers for early cardiotoxicity detection compared to previously published data. Differential miRNAs expression and principal component analysis (PCA) based on anthracycline-induced cardiotoxicity according to the timepoint of specimen collection using DESeq2 [16] and adjusting for multiple testing with the Benjamini-Hochberg [17]. PCA was performed on the top 50 gene expression variances according to group and timepoint (Figure S1). In each comparison, the PCA score (PC1 and PC2) did not show much difference, ranging from 20 to 37%. The heatmap of the highest 35 gene expressions from the NanoString cohort and the dendrogram of an unsupervised approach hierarchical clustering are shown in Figure S2. However, the most significant differential miRNA expressions when comparing cases to controls at 3 weeks after the 1st cycle of anthracycline were miR-16-5p, miR-20-5p, miR-25-3p, miR-486-3p, and miR-873-5p consistent with a previous report [18]. There are up-regulated in the range of 1.76–2.98, referred from log2 fold changes ranging from 0.82 to 1.58 (p-value < 0.01, FDR p-value < 0.1) (Table S2, Fig. 3A). We speculated miR-1-3p, which had previously been reported to correlate with anthracycline-induced cardiomyopathy [10, 19]. However, in our study, miR-1-3p was not significantly upregulated in the case group compared to the control group at 3 weeks after the first cycle of anthracycline combination therapy. The normalized and raw data in the discovery cohort are shown in Table S3 and Table S4.

Plasma MiRNAs expression using RT-PCR

To validate biomarkers for early detection of cardiotoxicity, the significant and biologic meaningful miR-16-5p was selected to perform validation in the validation cohort using RT-PCR. miR-16-5p was previously identified as a biomarker associated with cardiomyopathy [20] and anthracycline-related cardiotoxicity [11]. We also included miR-1-3p, which is not a significant gene expression of miRNAs profiler in the validation cohort. The optimal time point for exploring miRNA to detect anthracycline-induced cardiotoxicity varies from study to study. Based on findings from previous studies [11, 21], we examined the validation cohort at different time points to the discovery cohort to enhance differential

expressions. RT-PCR was performed using plasma at 6 weeks after initiating the anthracycline combination treatment compared to baseline. The validation cohort was composed of 17 cases and 20 controls. miR-16-5p was significantly down-regulated with a mean log2 fold change of -0.77 (p-value 0.02) in the case group (6th week) compared to the control group (6th week). The diverse down-regulated miR-16-5p in the case group at the 6th week compared to the baseline was found, with a mean log2 fold change of -0.77 (p-value 0.02). While non-significant miR-1-3p expression in an exploratory cohort was significantly up-regulated in the case group at the 6th week compared to the baseline (mean log2 fold change 2.48, p-value 0.02, respectively) (Fig. 3B and C). Despite the possibility of technical issues, diverse patterns of 2 miRNAs (miR-1-3p and miR-16-5p) expression in a time-dependent manner might represent dynamic changes of miRNAs correlated with biological meaning.

Discussion

This study investigates long-term cardiac complications, specifically cancer therapy-related cardiac dysfunction (CTRCD), in breast cancer survivors who received anthracycline combination treatment. Although earlier reports from before the 2000s indicated cardiotoxicity rates from anthracycline use ranged from 10 to 60%, current meta-analyses and long-term follow-up studies showed a lower incidence of cardiotoxicity with limited doses of anthracycline chemotherapy protocols [22–25]. Most participants (90.7%) in our study received four cycles of anthracycline combined with cyclophosphamide, with an average anthracycline dose of 223.2 ± 21.6 mg/m². The prevalence of symptomatic CTRCD was observed in less than 5% of this cohort, confirming findings from prior studies indicating a low incidence of symptomatic CTRCD during long-term follow-up [24, 26]. Among participants with symptomatic CTRCD, heart failure was the most common complication. Other issues included cardiac arrhythmias such as atrial fibrillation, atrial tachycardia, AVNRT, and PVC. Some reports suggest an increased risk of cardiac arrhythmias associated with anthracycline treatment [27, 28]. Most patients (90%) who developed symptomatic CTRCD in our study were classified as moderate to high risk for baseline cardiovascular toxicity, as evaluated using the HFA-ICOS criteria.

Anthracycline is a topoisomerase II blocker. Its inhibition leads to the activation of cell death pathways and suppression of mitochondrial biogenesis in cardiac myocytes. This is now considered the fundamental mechanism of anthracycline-induced cardiotoxicity (AIC), leading to permanent structural changes and long-term effects on cardiac function [29, 30]. The identification of early myocardial injury in cancer patients treated with anthracyclines often relies on cardiac imaging. Monitoring LVEF by echocardiography is widely recognized as the consensus for identifying cardiotoxicity. However, its sensitivity is limited in detecting subtle myocardial dysfunction. Cardiac magnetic resonance imaging (CMR) is regarded as the reference standard for measuring ventricular volumes and function, making it an optimal tool for cardiotoxicity assessment. CMR offers highly detailed, precise, and comprehensive imaging, often surpassing the capabilities of other cardiac imaging modalities. Furthermore, CMR exhibits lower inter-reader variability than echocardiography when evaluating left ventricular function and volumes, enhancing its reliability in clinical and research settings. Although echocardiography is the method of early AIC assessment in our cohort, we chose CMR to evaluate long-term safety due to the superiority of CMR in late consequences of anthracycline-related cardiomyopathy [1, 31].

Numerous studies define cardiotoxicity by serial decline in LVEF using different thresholds. In our study, early AIC was defined as LVEF decline of ≥ 10% from baseline, based on the studies before 2012 [13, 32, 33]. Although one-third of participants experienced acute AIC, no significant differences in clinical parameters were observed, for example, the median age at breast cancer diagnosis, baseline cardiovascular risk according to HFA-ICOS criteria, BMI, cumulative doxorubicin-equivalent dose, or receipt of left chest wall radiation. The early AIC exhibited a decrease in LVEF after anthracycline therapy (p-value < 0.01) and more subsequent anti-HER2 treatment (p-value 0.07) compared to the control group (Table 1). However, this decline did not translate into a significant increase in symptomatic CTRCD. We evaluated all CMR parameters, including myocardial strain, scarring, increased native T1 mapping, and decreased LV mass index [2, 34]. The decline in global longitudinal strain (GLS) has been associated with the development of cardiotoxicity in many studies [35]. None of the participants in either group exhibited a decrease in LVEF on CMR that met the 2022 IC-OS CTRCD criteria. No significant differences were observed in CMR parameters, including LVEDV, LVESV, LV mass index, RVEF, T1 mapping, and GLS. The average long-term LVEF measured by CMR was comparable between the two groups, with an average LVEF of 60%, aligning with findings from recent prospective studies on healthy populations in the same age range [36]. Cardiac scarring was identified in one participant (6.3%) from the case group and five (16.1%) from the control group. However, cardiac scars at RV insertion and mid-wall scarring were reviewed and found to be potentially non-specific [37]. Our findings suggest that decreased in LVEF during anthracycline therapy does not predict significant cardiac events or long-term cardiac consequences. The usual CMR findings in asymptomatic

participants might support the long-term cardiac safety of anthracycline usage.

Cardiac biomarkers, such as troponins (cTn) and NT-proBNP, have been previously reported to identify patients at higher risk of developing myocardial dysfunction and may aid in detecting subclinical anthracycline-related cardiotoxicity [5, 38, 39]. However, the utility of these biomarkers is limited [6–8]. Several studies have also focused on the role of microRNAs (miRNAs) in anthracycline-induced toxicity. The miRNAs were stored in exosomes, associated with RNA-binding proteins, and have been reported to have long-term stability. We bridge comprehensive miRNAs profiler through the NanoString platform to long-term CMR results. Our results revealed that the dynamic miRNAs profiler was consistent with previous reports [10, 18]. However, the diverse differential gene expression signal of selected miRNAs miR-1-3p and miR-16-5p from miRNAs profiler of the early period (the 3rd week since the start of anthracycline) and confirmatory RT-PCR of the later period (the 6th week since the start of anthracycline) was revealed. The controversial change in the level of miR-1-3p was previously reported [10]. The upregulation of miR-1 was reported in one study [11], while the downregulation of miR-1 was reported in another study [40]. Consistent with our study, significant upregulated miR-1-3p expression was found at the 6th week but not for the 3rd week since the start of anthracycline. The dynamic significant downregulation of miR-16-5p and upregulation of miR-1-3p in the subsequent cycle might have biological implications consistent with previous studies [41, 42]. This discrepancy in miRNA expression might be due to biological roles and regulatory mechanisms. miR-1-3p had biological functions involved in cardiac development, remodeling, and promoting differentiation of embryonic stem cells [43–45]. Meanwhile, miR-16-5p had a biologic function that promoted endoplasmic reticulum stress-induced apoptosis and oxidative stress in cardiomyocytes [41, 42, 46]. This is the first study to utilize comprehensive miRNA profiling to investigate miRNAs associated with anthracycline-induced cardiac injury and the long-term cardiac safety of anthracycline treatment. Dynamic microRNAs, miR-1-3p, and miR-16-5p gene expression detection at the 6th week since the start of the anthracycline might be very early detection of anthracycline-induced cardiotoxicity in subtle clinical impact by usual echocardiography. Without a long-term effect of cardiac function by CMR, dynamic change of microRNA detection might not impact standard management. The findings of this study may enhance physician confidence in managing breast cancer treatment while addressing concerns regarding potential cardiac risks.

Lastly, we acknowledge several limitations in our study. First, our long-term prospective study was conducted in a single tertiary academic center since 2007. Since then, various definitions of CTRCD were periodically changed. At that time, mild CTRCD was defined as a decline of LVEF ≥ 10%. Among these cases, none of them had baseline LVEF ≤ 60%. Furthermore, we employed the Teicholz formula to measure left ventricular ejection fraction (LVEF), which was widely used as a standard method in our institute at the time of study conduct. The Teicholz formula exhibited more significant variability than advanced 2D and 3D volumetric techniques. Second, the long-term follow-up led to a loss of participants over time. Although we obtained the death dates through The Bureau of Registration Administration, Ministry of Interior, information on the causes of death was partly unavailable. Third, we selected participants from asymptomatic breast cancer survivors who are still attending follow-up in the clinic to perform CMR. Thus, we could not define the severity of CTRCD in all asymptomatic participants upon the 2022 ESC classification. Moreover, all CMR participants had low to moderate baseline HFA-ICOS cardiovascular risk. This selection may have introduced bias and led to an underestimation of the incidence of CTRCD. Fourth, CMR was recently integrated into clinical practice. The lack of baseline CMR data made it impossible to directly compare changes in long-term CMR parameters, including LVEF, GLS, and T1 mapping. Finally, we observed the dynamic expression of interested miRNAs, miR-1-3p and miR-16-5p, from RT-PCR at the 6th week and miRNAs profiler at the 3rd week since the start of treatment. Non-confirmatory results of miRNA profilers might be the technical issues or downstream analysis. However, in our definition of case vs. control participants, significantly altered expression of miR-1-3p and miR-16-5p by RT-PCR at the 6th week exhibits a diverse AIC effect. Thus, we preferred the explanation of the biological relevance of the time-dependent dynamics of miRNAs, which is consistent with previous reports.

In conclusion, our study demonstrates a very low incidence of long-term symptomatic CTRCD, despite acute anthracycline-induced cardiac injury, confirmed by dynamic changes of miRNAs. The diverse expression patterns of miR-16-5p and miR-1-3p at different time points also provide valuable biological insights. Within-normal results of an exact and comprehensive CMR, in asymptomatic and any LVEF change participants, indicate the long-term safety of limited-dose anthracycline-containing use.

Abbreviations

HFA-ICOS	The Heart Failure Association of the European Society of Cardiology Cardio-Oncology Study Group in collaboration with the International Cardio-Oncology Society
IC-OS	International Cardio-Oncology Society
CTRCD	Cancer therapy-related cardiac dysfunction
AIC	Anthracycline-induced cardiotoxicity
LVEF	Left ventricular ejection fraction

LVEDV	Left ventricular end-diastolic volume
LVESV	Left ventricular end-diastolic volume
RVEF	Right ventricular ejection fraction
cTn	cardiac-specific isoenzymes troponins
NT-proBNP	N-terminal brain natriuretic peptides
BMI	Body mass index

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40959-025-00337-2.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Biospecimen collection was supported by Biobank, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The authors would like to thank the nurse staff of the medical oncology unit for specimen collection.

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Funding
This research was supported by the Ethinic Research Initiatives, GlaxoSmithKline Oncology International to VS.

Data availability
Data availability statement: The miRNAs profiler data set supporting this study's findings is in a supplementary file (Table S3 and Table S4).

Declarations

Ethical approval
The authors are responsible for all aspects of the work and ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All participants provided written informed consent. This study was approved by the Institutional Review Board of the Faculty of Medicine at Chulalongkorn University (No. 998/64) and was performed in accordance with the Health Insurance Portability and Accountability Act and the Declaration of Helsinki (as revised in 2013).

Competing interests
The authors declare no competing interests.

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Received: 17 January 2025 / Accepted: 10 April 2025
Published online: 23 April 2025

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