Juvenile exposure to doxorubicin alters the cardiovascular response to adult-onset psychosocial stress in mice

Marianne K. O. Grant**, Maria Razzoli**, Ibrahim Y. Abdelgawad†, Rachel Mansk‡, Davis Seelig§, Alessandro Bartolomuccili‡ and Beshay N. Zordoky‡†

**Department of Experimental and Clinical Pharmacology, University of Minnesota College of Pharmacy, Minneapolis, MN, USA; ‡Department of Integrative Biology and Physiology, University of Minnesota Medical School, Minneapolis, MN, USA; †Department of Veterinary Clinical Sciences, University of Minnesota College of Veterinary Medicine, St. Paul, MN, USA

ABSTRACT
Childhood cancer survivors have a high risk for premature cardiovascular diseases, mainly due to cardiotoxic cancer treatments such as doxorubicin (DOX). Psychosocial stress is a significant cardiovascular risk factor and an enormous burden in childhood cancer survivors. Although observational studies suggest that psychosocial stress is associated with cardiovascular complications in cancer survivors, there is no translationally relevant animal model to study this interaction. We established a “two-hit” model in which juvenile mice were administered DOX (4 mg/kg/week for 3 weeks), paired to a validated model of chronic subordination stress (CSS) 5 weeks later upon reaching adulthood. Blood pressure, heart rate, and activity were monitored by radio-telemetry. At the end of CSS experiment, cardiac function was assessed by echocardiography. Cardiac fibrosis and inflammation were assessed by histopathologic analysis. Gene expressions of inflammatory and fibrotic markers were determined by PCR. Juvenile exposure to DOX followed by adult-onset CSS caused cardiac fibrosis and inflammation as evident by histopathologic findings and upregulated gene expression of multiple inflammatory and fibrotic markers. Intriguingly, juvenile exposure to DOX blunted CSS-induced hypertension but not CSS-induced tachycardia. There were no significant differences in cardiac function parameters among all groups, but juvenile exposure to DOX abrogated the hypertrophic response to CSS. In conclusion, we established a translationally relevant mouse model of juvenile DOX-induced cardiotoxicity that predisposes to adult-onset stress-induced adverse cardiac remodeling. Psychosocial stress should be taken into consideration in cardiovascular risk stratification of DOX-treated childhood cancer survivors.

Introduction
The 5-year survival rate of children diagnosed with cancer has increased from less than 60% in the 1970s to more than 80% in 2010 (Lipshultz, Diamond, et al., 2014; Lipshultz, Sambatakos, et al., 2014). Unfortunately, young childhood cancer survivors suffer from several long-term adverse health complications (Ariffin et al., 2017; Ness et al., 2015; Blauuwbroek et al., 2007). In particular, childhood cancer survivors have a considerably increased risk for premature cardiovascular diseases (Faber et al., 2018), with an estimated 15 times higher risk of heart failure than their siblings who did not have cancer (Oeffinger et al., 2006). Nearly 50% of pediatric cancer patients receive anthracyclines such as doxorubicin (DOX), despite its known cardiotoxic effects (van Dalen et al., 2011). Although it has been widely accepted that the risk of anthracycline-induced cardiotoxicity increases with a higher anthracycline cumulative dose (Nysom et al., 1998), recent studies have demonstrated that latent cardiotoxicity occurs in children who receive low doses of anthracyclines (Leger et al., 2015; Vandecruys et al., 2012; Yeh & Vejpongsa, 2015). This latent cardiotoxicity predisposes anthracycline-treated survivors to other cardiovascular risk factors later in life in a “two-hit” manner (Armstrong et al., 2013). Psychosocial stress, i.e. stress derived from interrelation of social interactions and individual thought and behavior, is a significant cardiovascular risk factor (Rozanski et al., 1999; Ford et al., 1998; Kivimaki et al., 2006; Kamphuis et al., 2006) and an enormous burden in up to 63% of childhood cancer survivors (Brinkman et al., 2016; Prasad et al., 2015; Johannsdottir et al., 2017; Tremolada et al., 2016; Kuba et al., 2019). Although observational studies demonstrate that psychosocial stress is associated with higher rates of cardiovascular complications in cancer survivors (Ehrhardt et al., 2018; Schoormans et al., 2016; Vuotto et al., 2017; Schoormans et al., 2017), there is a
pauclity in translationally relevant animal models that study
the intriguing interaction between juvenile exposure to
anthracyclines and adult-onset psychosocial stress.

We have recently demonstrated that exposure to low
doses of DOX (4 mg/kg/week for 3 weeks) produced no overt
cardiac dysfunction in young mice, yet was sufficient to
cause modest cardiac atrophy and to induce molecular
markers of cardiotoxicity such as atrial natriuretic peptide
(ANP) (Matsumura et al., 2018; Maayah et al., 2021;
Abdelgawad et al., 2021). Intriguingly, exposure of young
mice to these low doses of DOX aggravated the detrimental
cardiovascular effects of adult-onset angiotensin II (ANGII)-
induced hypertension resulting in overt cardiac dysfunction
and severe cardiac fibrosis (Matsumura et al., 2018; Maayah
et al., 2021; Agostinucci et al., 2022). We have also developed
and validated a chronic subordination stress (CSS) model in
which experimental male mice are housed with aggressive
males and exposed to daily defeat episodes and chronic sen-
sory housing, allowing a continuous threat of aggression
while limiting physical injuries (Bartolomucci et al., 2001).
Subordinate mice manifest several hallmarks of chronic stress
(Razzoli et al., 2014, 2015; Bartolomucci et al., 2004), signifying
the validity of the CSS model to recapitulate human psy-
chosocial stress. Intriguingly, we and others have shown that
CSS alone caused modest structural and functional cardiovas-
cular effects in experimental animals (Costoli et al., 2004;
Simas et al., 2018; Agrimi et al., 2019) and exacerbated the
cardiovascular complications when combined with other car-
diovascular risk factors such as obesity (Simas et al., 2018;
Agrimi et al., 2019). Therefore, the objective of the current
work is to determine how juvenile exposure to DOX may
aggravate the cardiovascular response to adult-onset psycho-
social stress in a clinically relevant mouse model.

Materials and methods

Animals

Male C57BL/6N mice were purchased from Charles River
Laboratories at 4 weeks of age and housed in groups of 4
with free access to food and water during the study period,
under 12:12 h light: dark cycle at 22 ± 2°C. After a one-week
acclimation period, mice were randomized to receive either
4 mg/kg/week DOX or equivalent volume of sterile saline by
intraperitoneal injection for 3 weeks. CD1 male mice that
sed in the CSS model as aggressive residents were pur-
chased from Charles River Laboratories and individually
housed in static cages (257 mm × 483 mm × 152 mm) to be
used for the CSS protocol and provided with food, water,
and bedding under the same general environmental condi-
tions as described above. At the end of the experiment, ani-
mals were humanely euthanized by decapitation under
isoflurane anesthesia. Hearts were harvested, rinsed in phos-
phate-buffered saline, flash frozen in liquid nitrogen, and
stored at −80°C. All experimental procedures were approved
by the Institutional Animal Care and Use Committee (IACUC)
of the University of Minnesota (Protocol ID: 1807-36187 A).
The number of experimental subjects used for each experi-
ment is indicated in the figure legends.

Chronic subordination stress (CSS)

Experimental animals were exposed to CSS 5 weeks after the
last dose of DOX or saline had been administered. The proto-
col used is a modified version of our standard CSS procedure
(Razzoli et al., 2014; Bartolomucci et al., 2004; Razzoli et al.,
2020; Bartolomucci et al., 2010). Saline- and DOX-exposed
mice entered a one-week baseline period before being exposed
to daily (between 8 and 10 AM) social defeat expos-
ure by a CD1 mouse for a maximum of 10 min and continu-
ous sensory contact (allowing auditory, olfactory, and visual
stimuli but preventing physical interaction) for 14 d (Figure
1A). According to our standard protocol, each experimental
mouse was only paired with one CD1 for the entire duration
of the study, thus enabling the establishment of a true and
stable social hierarchy between the two subjects. During the
social interaction, offensive behaviors of the animals were
manually recorded and social status was determined by dis-
play of upright postures, flight behavior, and squeaking
vocalization as previously described (Bartolomucci et al.,
2010; Dadomo et al., 2011). To prevent injuries, mice were
continuously monitored and were separated if fights esca-
lated. Mice in the CSS group were housed in large colony
boxes (257 mm × 483 mm × 152 mmH; 1241 cm² of floor
space) divided lengthwise in two equally sized compartments
(~620 cm² each) by a divider consisting of a white plexiglass
frame and wire mesh netting; controls were individually
housed in standard housing cages (186 mm × 298 mm
× 128 mmH; 554 cm² of floor space) and received daily gentle
handling at the same time as the social defeat procedure.

Radio-telemetry for hemodynamic monitoring

A cohort of experimental mice underwent surgery for the
implantation of a pressure catheter in the femoral artery
attached to a telemetry device (PA-C10; Data Sciences
International, Saint Paul, MN) 1 week prior to CSS exposure.
Briefly, mice were prepared for surgery under aseptic condi-
tions, and anesthetized with 2–3% isoflurane. The body of
the transmitter was implanted in the flank through a small
incision. Another incision was made in the groin to expose
the femoral artery. The transmitter was inserted in a small
incision of the femoral artery. A suture was placed around the femoral artery
and the catheter to hold it in place. Subsequently, the body
of the transmitter was tunneled to the flank and the incisions
were closed with 5–0 silk sutures. Following the surgery,
mice were individually housed during a 7-d recovery period.
Blood pressure, heart rate, and activity were then continu-
ously monitored following the recovery period for the dur-
ation of the experiment.

Metabolic study and body composition analysis

Food intake and body weight were measured regularly
throughout the experiment. Energy efficiency was calculated
from the ratio between the body weight gain and the food
consumed. A separate cohort of mice was used for the ana-
lysis of body composition. Body composition parameters
Figure 1. Effect of juvenile exposure to DOX, adult-onset CSS, and their combination on body composition. (A) Experimental design of the two-hit model of latent DOX-induced cardiotoxicity using CSS as a second hit. Male 5-week-old C57BL/6N mice were administered DOX (4 mg/kg/week) or saline for 3 weeks and allowed to recover for 5 weeks prior to exposure to 14 d of CSS. Effect of juvenile exposure to DOX one day prior to CSS on (B) body weight (n=27–29 per group), (C) Fat mass (n=12 per group), and (D) fat free mass (n=12 per group). (E) Average number of attacks received during the daily social defeat interactions in CSS (n=14).

Comparisons between the two groups in (B–E) were performed using unpaired student t-test. (F) Daily body weight gain during CSS where a significant stress × time interaction is found (F(4,208)=5.915, p<.001) and asterisks denote significant binary differences within each time point among groups following Tukey’s HSD post hoc test. (G) Total amount of food consumed in the course of CSS (n=13–15 per group). (H) Delta fat free mass (n=6 per group), and (I) Delta fat mass (n=6 per group) following CSS. Values are represented as means ± SEM. Statistical analysis was performed by two-way ANOVA with Tukey’s HSD post-hoc tests as appropriate (*p<.05, **p<.01, ***p<.001, ****p<.0001). Detailed statistical analysis is provided in Supplementary Table 2.
including fat and fat free mass were measured with EchoMRI 3-in-1 (EchoMRI LLC, Houston, TX) one day before (baseline) and on day 12 of CSS exposure in a subset of mice.

**Echocardiography**

Cardiac function was assessed by trans-thoracic echocardiography 1 day following the end of the CSS experiment in a subset of mice. Echocardiography was performed using the Vevo 2100 system (VisualSonics, Inc., Toronto, Ontario, Canada) equipped with an MS400 transducer. Anesthesia was induced with 3% isoflurane in oxygen and maintained at 1–2% during the procedure. Mice were secured in a supine position on a heated physiologic monitoring stage. Parasternal short axis images of the left ventricle (LV) were obtained in M-Mode at the level of the papillary muscles. Endocardial and epicardial borders were manually traced over 3–4 cardiac cycles and parameters of cardiac function and morphometry were calculated using VisualSonics cardiac measurement package of the Vevo 2100. Pulsed Doppler images of mitral valve flow were acquired in the apical 4-chamber view and peak velocities of the early (E) and atrial (A) peaks were measured to calculate E/A ratio.

**Histopathology**

LV heart sections were collected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (HE) or Masson’s trichrome stain. Histopathologic evaluation was performed by a board-certified veterinary pathologist who was blinded to the experimental group. The severity of fibrosis on the Masson’s trichrome stained section was assessed for fibrosis as follows: 0, absent; 1, minimal inflammation or fibrosis; 2, mild inflammation or fibrosis; 3, moderate inflammation or fibrosis; and 4, marked inflammation or fibrosis.

**Quantitative real-time PCR**

Total RNA was extracted from 20 mg frozen ventricular tissue using 300 µL Trizol reagent (Life Technologies, Carlsbad, CA) according to manufacturer’s instructions. RNA concentrations were calculated by measuring absorbance at 260 nm using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). First-strand cDNA was synthesized from 1.5 µg total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Specific mRNA expression was quantified by SYBR Green (Applied Biosystems) based real-time PCR performed on an ABI 7900HT instrument (Applied Biosystems) using 384-well optical reaction plates. Thermal cycling parameters were as follows: 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Gene expression was determined using previously published primers for cyclooxygenase-2 (Cox-2), interleukin-6 (IL-6), collagen 1α1 (Col1α1), transforming growth factor-beta1 (TGF-beta1), and galectin-3. Primer sequences are listed in Supplementary Table 1. The mRNA expression levels were normalized to β-actin and are expressed relative to the saline-treated controls. Relative gene expression was determined by the ΔΔCT method, and primer specificity and purity of the final PCR product were verified by melting curve analysis.

**Statistical analysis**

Statistical significance was determined by one- or two-way ANOVA, one- or two-way ANOVA for repeated measures with Tukey’s HSD post-hoc test, or two-way mixed effects ANOVA with Sidak’s post hoc test (Wilcox, 1987). Unpaired student t-test was used to compare between two groups when appropriate. Data are presented as mean ± SEM. Statistical analyses for histopathologic grading were performed using the non-parametric Kruskal–Wallis followed by Mann–Whitney U tests. A p value of <.05 was taken to indicate statistical significance. Statistica 13.5 (TIBCO Software Inc., Palo Alto, CA), R (Version 1.3.1093 © 2009-2020 RStudio, PBC), and GraphPad Prism (La Jolla, CA) were used for analyses. The specific statistical test used for each experiment is indicated in the figure legends.

**Results**

**Effect of CSS on body weight, food intake, and body composition in saline- and DOX-exposed mice**

Juvenile exposure to DOX caused a significant decline in body weight gain during the treatment weeks (Supplementary Figure 1(A)), resulting in a significantly lower body weight than that of saline-injected mice prior to the start of CSS (Figure 1(B)). The reduction in body weight in DOX-treated mice is attributed to a reduction in both fat mass (Figure 1(C)) and fat free mass (Figure 1(D)). Five weeks after the last DOX or saline injection, mice were randomized to undergo the 14 d CSS protocol or to serve as controls as described in the Methods section. Juvenile exposure to DOX did not alter the number of attacks received by the CD1 during the aggressive interactions (Figure 1(E)). In the course of the 14 d of CSS, the body weight gain measured was significantly changed as a function of both time and stress, revealing that stress groups independently from DOX exposure, gained more weight than control mice groups (Figure 1(F) and Supplementary Figure 1(B)). CSS had a significant impact on the amount of food ingested by stressed mice, where, independently from DOX exposure, they exhibited a clear hyperphagic response (Figure 1(G)) becoming more pronounced as the CSS progressed and is strengthened by the normalization of amount of food ingested by body weight (Supplementary Figure 1(C)). CSS exposure modestly but significantly increased body weight gain (Figure 1(F)), an effect that was due to an increase in fat free mass (Figure 1(H)) in the presence of decreased fat mass (Figure 1(I)) and % fat mass (Supplementary Figure 1(D)). These metabolic effects were significant in CSS-saline group only (Supplementary Table 2). Due to differences in baseline body weight caused by DOX treatment, % change in body weight was also analyzed. The overall effect mirrors the result with weight gain, although
post hoc comparisons are not significant (Supplementary Figure 1(B)). In general, this result is consistent with previous reports on the same model and other strains of mice when mice were fed a standard diet (Sanghez et al., 2016; Razzoli et al., 2016; Sanghez et al., 2013).

### Juvenile exposure to DOX blunted CSS-induced hypertension but not CSS-induced tachycardia

Juvenile exposure to DOX did not result in any significant difference in blood pressure measurements compared to saline-treated mice at baseline level (Figure 2). However, exposure to CSS caused profound cardiovascular changes. In particular, mean arterial pressure (MAP) was found to be significantly elevated in saline-treated but not in DOX-treated mice exposed to CSS (Figure 2A,B; Supplementary Table 3). This result was reflected in changes in both systolic and diastolic pressure which were found significantly elevated in saline-treated but not in DOX-treated CSS-exposed mice (Figure 2C–F; Supplementary Table 3). Remarkably, unlike what was observed in MAP, CSS exposure caused significant tachycardia in both saline-treated and DOX-treated mice (Figure 2).

**Figure 2.** Juvenile exposure to DOX prevented CSS-induced hypertension. Male 5-week-old C57BL/6N mice were administered DOX (4 mg/kg/week) or saline for 3 weeks and allowed to recover for 5 weeks prior to exposure to CSS. Average daily values and average values at baseline and at the end of CSS of (A and B) Mean arterial blood pressure (MAP), (C and D) systolic, and (E and F) diastolic blood pressure recorded by radio telemetry prior to and over the course of CSS (n = 4–8 per group); shaded area on the graphs represents time during CSS protocol. Values are represented as means ± SEM. Statistical analysis was conducted by repeated measures ANOVA followed by Tukey’s HSD post-hoc tests (**p < .01, ***p < .001). Detailed statistical analysis is provided in Supplementary Table 3.
The same outcome was true for activity level, where CSS induced a similar level of hyperactivity in both saline- and DOX-treated mice compared to controls (Figure 3(C,D); Supplementary Table 3). Finally, we analyzed MAP and heart rate during rest (defined as intervals with <2 activity counts per minute). Results demonstrate that MAP remained significantly elevated at rest in CSS-Saline treated, but not in CSS-DOX treated mice (Supplementary Figure 2(A)). Conversely, heart rate at rest is not increased by CSS (Supplementary Figure 2(B)).

### Juvenile exposure to DOX abrogated the hypertrophic response to CSS

Trans-thoracic echocardiography was utilized to determine the effects of juvenile exposure to DOX, adult-onset CSS, and the combination thereof on cardiac function and morphometry. Representative echocardiographic images obtained in M-Mode from each group are displayed in Figure 4(A). There were no statistically significant differences in cardiac function parameters among all these groups, as evidenced by no change in ejection fraction, fractional shortening, E/A ratio or cardiac output (Figure 4(B-E); Supplementary Table 4). Nevertheless, 2 weeks of CSS caused cardiac hypertrophy in C57BL/6N mice as evidenced by a significant increase in heart weight to tibia length (HW/TL) (Figure 4(F); Supplementary Table 4). Echocardiography-derived morphometric measurements showed a statistically significant effect of DOX exposure to reduce LV mass and a statistically significant effect of CSS to increase LV mass (Table 1). CSS-induced increase in LV mass was associated with a significant effect to increase LV posterior wall thickness (Table 1). Intriguingly, juvenile exposure to DOX significantly reduced HW/TL and LV mass, thus abrogating CSS-induced cardiac hypertrophy (Figure 4(F); Table 1).

### Juvenile exposure to DOX exacerbated CSS-induced myocardial inflammation and fibrosis

Histopathology analysis using HE and Masson’s trichrome stains revealed inflammation and cardiac fibrosis that were more severe and significantly increased in the DOX/CSS group as compared to the saline control (Figure 5(A,B,C)). Although a few mice in the DOX/control and saline/CSS groups also showed signs of cardiac fibrosis, neither DOX nor CSS alone was sufficient to cause a statistically significant effect on cardiac fibrosis (Figure 5(C)). Consistent with the histopathologic findings, gene expressions of multiple key inflammatory markers and fibrotic markers such as Cox-2 (Figure 6(A)) and IL-6 (Figure 6(B)) were significantly induced.
The IL-6/Cox-2 signaling pathway has been shown to be a key mediator of cardiac inflammation and fibrosis via different mechanisms (Chou et al., 2018; Liao et al., 2020). Similarly, Col1a1 (Figure 6(C)), TGF-β1 (Figure 6(D)), and galectin-3 (Figure 6(E)) were significantly upregulated in DOX-treated mice following CSS (Supplementary Table 5). Col1a1, TGF-β1, and galectin-3 are markers as well as mediators of myocardial fibrosis as reported by several studies (Shen et al., 2018). Intriguingly, neither juvenile exposure to DOX nor adult-onset CSS alone resulted in significant upregulation of any of these markers (Figure 6).

Figure 4. Juvenile exposure to DOX abrogated CSS-induced hypertrophy without changes in cardiac function. Male 5-week-old C57BL/6N mice were administered DOX (4 mg/kg/week) or saline for 3 weeks and allowed to recover for 5 weeks prior to exposure to CSS. Cardiac function was assessed by trans-thoracic echocardiography (n = 7–9 per group) at the end of CSS. (A) Representative M-Mode images from parasternal short axis view of the heart, (B) ejection fraction, (C) fractional shortening, (D) E/A ratio, and (E) cardiac output. (F) Heart weight to tibia length ratio (HW/TL) (n = 13–15 per group). Values are represented as means ± SEM. Statistical significance of pairwise comparisons was determined by two-way ANOVA with Tukey’s HSD post-hoc tests (**p < .01, ***p < .001, ****p < .0001). Detailed statistical analysis is provided in Supplementary Table 4.
LV mass (mg) 107.926 ± 104.855
LVESEV (uL) 93.285 ± 90.844
LVEDV (uL) 50.860 ± 42.404
LVESV (uL) 1.125 ± 1.160
LVAW:s (mm) 1.089 ± 1.160
LVAW;d (mm) 0.889 ± 0.999
LVPW:s (mm) 0.882 ± 0.999
LVPW;d (mm) 0.672 ± 0.772
LVID;s (mm) 3.497 ± 3.432
LVID;d (mm) 4.513 ± 4.458

Table 1. Echocardiography-derived morphometry parameters after CSS in male C57BL/6N mice pre-exposed to DOX as juveniles. Values are presented as mean ± standard of the mean (SEM).

Discussion

It is increasingly recognized that suffering from cancer and undergoing cancer treatment has significant ramifications on patients’ lives (Schoormans et al., 2016; Schoormans, 2021) with up to 63% of childhood cancer survivors experiencing psychiatric diseases such as anxiety and depression (Brinkman et al., 2016; Prasad et al., 2015; Johannsdottir et al., 2017; Tremolada et al., 2016; Kuba et al., 2019). Furthermore, anthracycline-treated childhood cancer survivors are already at a higher risk for cardiovascular complications (van Dalen et al., 2011). In the general population, chronic psychosocial stress is associated with cardiovascular diseases, independent of other traditional risk factors (Rozanski et al., 1999). This led us to hypothesize thatjuvenile exposure to anthracyclines and adult-onset psychosocial stress could act synergistically to exacerbate cardiovascular complications (Blaes et al., 2021). We thus established a “two-hit” model in which young mice were administered DOX (Matsumura et al., 2018), paired to a validated model of CSS 5 weeks later upon reaching adulthood. To this end, we adopted our novel mouse model of juvenile DOX-induced latent cardiotoxicity (Matsumura et al., 2018), wherein DOX (4 mg/kg/week for 3 weeks) is administered to 5-week-old juvenile C57BL/6N mice (equivalent to ~10–12 in human years (Dutta & Sengupta, 2016)). This dosage regimen is equivalent to a human dose of 40 mg/m² (Reagan-Shaw et al., 2008), which is relevant to clinical doses used to treat hematologic malignancies in pediatric cancer patients (Kelly, 2012). Juvenile exposure to DOX caused modest cardiac atrophy, induced the gene expression of ANP a molecular marker of cardiotoxicity, and activated cardiac p38 MAPK (Matsumura et al., 2018). Echocardiographic measurements of cardiac function demonstrated that juvenile exposure to DOX had no effect on either systolic or diastolic function parameters (Matsumura et al., 2018; Maayah et al., 2021; Abdelgawad et al., 2021). Therefore, this approach models the clinical scenario of anthracycline-treated pediatric cancer patients who do not develop cardiac dysfunction immediately after completing their cancer treatment, but are left with “weaker” hearts predisposing them to cardiovascular risk factors later in life, in agreement with clinical studies (Leger et al., 2015; Tham et al., 2013; Çetin et al., 2018; Germanakis et al., 2006; Yazaki et al., 2018). Mice in the CSS model have been shown to develop sustained hypothalamic-pituitary-adrenal-axis activation, one of the hallmarks of chronic stress (Razzoli et al., 2014), anxiety and depression-like behavior (Razzoli et al., 2015, 2016; Sanghez et al., 2013). Notably, obesity resistance was also shown, if CSS is applied to morbidly diet-induced obese mice (Agrimi et al., 2019).

Our lab was the first to report that CSS causes persistent increase in MAP (Razzoli et al., 2020). In this study, we replicated CSS-induced hypertension in saline-treated mice. Strikingly, juvenile exposure to DOX abrogated the hypertensive response to CSS. DOX has been shown to lower blood pressure and increase heart rate in an adult rat model of DOX-induced cardiotoxicity (Rabelo et al., 2001). In addition, DOX-treated rats were less responsive to increasing doses of the vasocostrictive drug phenylephrine. Interestingly, these
alterations occurred in absence of a significant change in cardiac output (Rabelo et al., 2001). In another model, although DOX treatment elevated blood pressure, the vasoconstrictive response to phenylephrine was similarly blunted, implying a reduced cardiovascular reactivity to catecholamines (de Wildt et al., 1985). These preclinical observations are supported by clinical evidence of cardiovascular autonomic dysfunction in anthracycline-treated cancer survivors (Teng et al., 2021).

In our study, while juvenile DOX blunted the CSS-induced hypertension, it did not blunt the CSS-induced tachycardia. These results suggest that the effects of DOX in this study are mediated by changes in the vascular system, rather than the heart. In contrast to our current finding, we previously reported that juvenile exposure to DOX aggravated adult-onset ANGII-induced hypertension (Matsumura et al., 2018), implying that the impact of juvenile DOX exposure on blood pressure depends on the adult-onset hypertensive stimulus. In support of this notion, a recent study has shown that DOX impairs the vascular smooth muscle contractile response to phenylephrine, but not to ANGII (Boysman et al., 2021), suggesting the CSS-induced hypertension is likely mediated by catecholamines not ANGII.

We previously demonstrated that low doses of DOX (4 mg/kg/week for 3 weeks) did not cause cardiac dysfunction in young mice (Maayah et al., 2021; Abdelgawad et al., 2021), yet this regimen was sufficient to exacerbate ANGII-induced cardiac dysfunction (Matsumura et al., 2018; Agostinucci et al., 2022). The deterioration of cardiac function in DOX/ANGII-treated mice was associated with a significant increase in blood pressure (Matsumura et al., 2018). In contrast to these findings, neither DOX treatment, CSS, nor their combination adversely affected the cardiac function in this study. The preserved cardiac function in all experimental groups may be attributed to the observation that juvenile DOX exposure blunted the hypertensive response to CSS in clear contrast to the aggravated increase in blood pressure in the DOX/ANGII group. Interestingly, we have shown that ANGII but not catecholamines worsened cardiac dysfunction and exacerbated myocardial fibrosis following juvenile exposure to DOX (Agostinucci et al., 2022). These findings suggest that DOX-induced predisposition to cardiovascular risk factors is highly dependent on the pathologic stimulus imposed by a specific risk factor. There is paucity of studies reporting the effect of CSS on echocardiography-derived cardiac function. In one study, CSS caused modest cardiac dysfunction in healthy male C57BL/6J mice, but more severe cardiac dysfunction was precipitated in obese mice (Agrimi et al., 2019). Similarly, CSS did not significantly change cardiac function in wild-type mice, but it caused severe deterioration in cardiac function in mice with arrhythmogenic cardiomyopathy (Agrimi et al., 2020).

Anthracycline-induced subclinical cardiotoxicity is commonly associated with cardiac atrophy and thinning of the LV walls in childhood cancer survivors (Lipshultz et al., 2002, 2012, 2013; Mawad et al., 2020). We previously demonstrated that juvenile exposure to DOX blunted the adaptive hypertrophic response to both ANGII and isoproterenol in adult mice (Matsumura et al., 2018; Agostinucci et al., 2022). In this study, we also demonstrate that juvenile exposure to DOX abrogates the hypertrophic response to psychosocial stress, suggesting that exposure to DOX at a young age alters a fundamental mechanism of myocardial growth that prevents the adaptive hypertrophic response to a variety of stimuli. Several mechanisms have been proposed to delineate how DOX interferes with cardiac growth as discussed in (Chen et al., 2022). Future research is warranted to investigate the exact mechanism that mediates DOX-induced blunting of the hypertrophic response to CSS. The effect of CSS on features
of cardiac hypertrophy has been reported in a few studies only and results are somewhat inconsistent, including no hypertrophy in CD1 mice (Costoli et al., 2004) and the lack of echocardiography-derived indices of cardiac hypertrophy in non-obese C57BL/6J mice (Agrimi et al., 2019).

In this study, we demonstrate that neither juvenile exposure to DOX nor CSS caused significant cardiac fibrosis in naïve mice, but significant fibrosis was observed when the two hits were combined. The aggravated cardiac fibrosis in the DOX/CSS C57BL/6N mice was associated with a marked upregulation in multiple inflammatory and fibrotic markers including interleukin-6 and cyclooxygenase-2. Mechanistically, our data suggest that a pro-inflammatory state caused by the combined effect of DOX and CSS may have exacerbated cardiac fibrosis. CSS has been shown to cause modest but significant myocardial fibrosis in CD1 mice (Costoli et al., 2004), while other studies demonstrated that CSS was not sufficient to cause myocardial fibrosis in healthy mice, but it exacerbated myocardial fibrosis in obese mice (Agrimi et al., 2019) and in Dsg2<sup>mut/mut</sup> mice with arrhythmogenic cardiomyopathy (Agrimi et al., 2020). These discrepant results suggest that genetic background changes the individual

**Figure 6.** Inflammatory and fibrotic markers are upregulated following CSS in DOX-exposed mice. Male 5-week old C57BL/6N mice were administered DOX (4 mg/kg/week) or saline for 3 weeks and allowed to recover for 5 weeks prior to exposure to CSS. The mRNA expression of inflammatory markers (A) Cyclooxygenase-2 (Cox-2) and (B) Interleukin-6 (IL-6) and the fibrotic markers (C) Collagen 1α1 (Col1α1), (D) Transforming Growth Factor-beta-1 (TGF-beta1), and (E) Galectin-3 was determined by real-time PCR (n = 6 per group). Values are represented as means ± SEM. Statistical significance of pairwise comparisons was determined by two-way ANOVA with Tukey’s HSD post-hoc tests (*p < .05, **p < .01, ***p < .001, ****p < .0001). Detailed statistical analysis is provided in Supplementary Table 5.
predisposition to develop cardiac fibrosis in the presence of social stress exposure. The incidence and degree of cardiac fibrosis in anthracycline-treated childhood cancer survivors are also debated. Some studies report modest cardiac fibrosis in anthracycline-treated childhood cancer survivors (Mawad et al., 2020; Cheung et al., 2013), while other studies demonstrate lack of a statistically significant increase in cardiac fibrosis (Tong et al., 2019; Ylanen et al., 2013). The discrepancy in these clinical studies may have arisen from the divergent effects of different cardiovascular risk factors on the pathogenesis of latent anthracycline-induced cardiotoxicity in childhood cancer survivors. Therefore, more clinical research is needed to study the possible association between cardiac fibrosis and different cardiovascular risk factors, including psychosocial stress, in anthracycline-treated childhood cancer survivors.

In conclusion, we established, for the first time, a clinically relevant mouse model of juvenile DOX-induced latent cardiotoxicity that predisposes to adult-onset stress-induced adverse cardiac remodeling. Anthracyclines and psychosocial stress adversely affect multiple targets in the body; nevertheless, the cardiovascular system is particularly vulnerable to a detrimental synergy between these two pathologic stimuli. Accordingly, psychosocial stress should be taken into consideration in cardiovascular risk stratification of anthracycline-treated childhood cancer survivors.

Acknowledgments

Experiments using the NanoDrop 8000 and ABI 7900 HT were done with staff support at the University of Minnesota Genomics Center. Experiments using the Vevo 2100 echocardiography system were done with staff support at the University of Minnesota Imaging Center. Processing of heart tissues for histopathological analysis was performed with staff support at the Comparative Pathology Shared Resource, University of Minnesota Masonic Cancer Center. The stress and radiotelemetry experiments were conducted at the Physiology Core, University of Minnesota. We would like to thank Pilar Ariza for performing the surgeries, and Jan Pierre Pallais for help with the social stress experiments.

Disclosure statement

The authors declare that they do not have any financial or non-financial conflicts of interest concerning this manuscript.

Funding

This work was supported by the National Heart, Lung, and Blood Institute (NHLBI) grant R01HL151740 (B.N.Z. and A.B.), the National Institutes of Health’s National Center for Advancing Translational Sciences, grant UL1TR002494 (B.N.Z.), the American Cancer Society Institutional Research Grant #CON00000061006 (B.N.Z.), and the Minnesota Partnership for Biotechnology and Medical Genomics #18.04 (A.B.). I.Y.A. is supported by the Bighley Graduate Fellowship from the College of Pharmacy, University of Minnesota.

Notes on contributors

Marianne K.O. Grant, BA is a Senior Researcher in the Zordoky lab, University of Minnesota College of Pharmacy. Her current work focuses on cancer therapy-induced cardiovascular complications.

Maria Razzoli, PhD is a Senior Scientist in the Bartolomucci lab and the behavioral/metabolic specialist of the Physiology Core of the University of Minnesota Medical School. Her current work focuses on rodent stress models and induced behavioral and physiological alterations, including stress accelerated aging phenotypes.

Ibrahim Y. Abdelgawad, BSc is a PhD Candidate in the Zordoky lab, University of Minnesota College of Pharmacy. His current work focuses on cancer therapy-induced senescence.

Rachel Mansk, BA is a Research Scientist in the Bartolomucci lab and a graduate student at the University of Minnesota School of Public Health. Rachel’s research focuses on the social determinants of health.

Davis Seelig, DVM, PhD is Associate Professor, Department of Veterinary Clinical Sciences, University of Minnesota College of Veterinary Medicine and Director of the Comparative Pathology Shared Resource at the University of Minnesota Masonic Cancer Center. His research focuses on comparative and experimental pathology.

Alessandro Bartolomucci, PhD is Professor, Ancel Keys Biomedical Scholar in Physiology and Metabolism, and Director of the Physiology Core at the Department of Integrative Biology and Physiology, University of Minnesota Medical School. His laboratory is primarily interested in metabolic physiology, stress physiology and social determinants of health and aging.

Beshay N. Zordoky, PhD is Associate Professor of Experimental and Clinical Pharmacology, University of Minnesota College of Pharmacy. His current research interests include cancer therapy-induced cardiovascular complications and therapy-induced senescence.

Data availability statement

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

References

Abdelgawad, I. Y., Grant, M. K. O., Popescu, F. E., Largaespada, D. A., & Zordoky, B. N. (2021). Doxorubicin paradoxically ameliorates tumor-induced inflammation in young mice. International Journal of Molecular Sciences, 22, 9023. https://doi.org/10.3390/ijms22169023

Agostinucci, K., Grant, M. K. O., Seelig, D., Yucel, D., van Berlo, J., Bartolomucci, A., Dyck, J. R. B., & Zordoky, B. N. (2022). Divergent cardiac effects of angiotensin II and isoproterenol following juvenile exposure to doxorubicin. Frontiers in Cardiovascular Medicine, 9, 742193. https://doi.org/10.3389/fcvm.2022.742193

Agrimi, J., Scalco, A., Agafonova, J., Williams III, L., Pansari, N., Keceli, G., Jun, S., Wang, N., Mastorci, F., Tichnell, C., Murray, B., James, C. A., Calkins, H., Zaglia, T., Paolocci, N., & Chelko, S. P. (2020). Psychosocial stress hastens disease progression and sudden death in mice with arrhythmogenic cardiomyopathy. Journal of Clinical Medicine, 9, 3804. https://doi.org/10.3390/jcm9123804

Agrimi, J., Spalletti, C., Baroni, C., Keceli, G., Zhu, G., Caragnano, A., Matteucci, M., Chelko, S., Ramirez-Correa, G. A., Bedja, D., Casieri, V., Di Lascio, N., Scalco, A., Beltrami, A. P., Paolocci, N., Caleo, M., & Lionetti, V. (2019). Obese mice exposed to psychosocial stress display cardiac and hippocampal dysfunction associated with local brain-derived neurotrophic factor depletion. EBioMedicine, 47, 384–401. https://doi.org/10.1016/j.ebiom.2019.08.042

Arifin, H., Azanan, M. S., Abd Ghasar, S. S., Oh, L., Lau, K. H., Thirunavakarasu, T., Sedan, A., Ibrahim, K., Chan, A., Chin, T. F., Liew, F. F., Jeyamogan, S., Rosli, E. S., Baharudin, R., Yap, T. Y., Skinner, R.,
Costoli, T., Bartolomucci, A., Graiani, G., Stilli, D., Laviola, G., & Sgoifo, A. (2004). Effects of chronic psychosocial stress on cardiac autonomic responsiveness and myocardial structure in mice. American Journal of Physiology Heart and Circulatory Physiology, 286(6), H2133–2140. https://doi.org/10.1152/ajpheart.00869.2003

Dadomo, H., Sanghez, V., Di Cristo, L., Lori, A., Ceresini, G., Malinge, I., Parmigiani, S., Palanza, P., Sheardown, M., & Bartolomucci, A. (2011). Vulnerability to chronic subordination stress-induced depression-like disorders in adult 129SvEv male mice. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 35(6), 1461–1471. https://doi.org/10.1016/j.pnpbp.2010.11.016

de Wildt, D. J., de Jong, Y., Hillen, F. C., Steenbergen, P. A., & van Hoesel, Q. G. (1985). Cardiovascular effects of doxorubicin-induced toxicity in the intact Lou/M Wsl rat and in isolated heart preparations. The Journal of Pharmacology and Experimental Therapeutics, 231(1), 234–240.

Dutta, S., & Sengupta, P. (2016). Men and mice: Relating their ages. Life Sciences, 152, 244–248. https://doi.org/10.1016/j.lfs.2015.10.025

Ehrhardt, M. J., Muloonay, D. A., Li, C., Baassiri, M. J., Bjornard, K., Sandlund, J. T., Brinkman, T. M., Huang, I. C., Srivastava, D. K., Niss, K. K., Robison, L. L., Hudson, M. M., & Krull, K. R. (2018). Neurocognitive, psychosocial, and quality-of-life outcomes in adult survivors of childhood non-Hodgkin lymphoma. Cancer, 124(2), 417–425. https://doi.org/10.1002/ajc.31019

Faber, J., Wingert, A., Neu, M. A., Henninger, N., Eckerle, S., Munzel, T., Lackner, K. J., Beutel, M. E., Blettner, M., Rathmann, W., Peters, A., Meisinger, C., Linkohr, B., Neuhäuser, H., Kaatsch, P., Spix, C., Schneider, A., Merzenich, H., Panova-Neova, M., Prochaska, J. H., & Wild, P. S. (2018). Burden of cardiovascular risk factors and cardiovascular disease in childhood cancer survivors: Data from the German CVSS-study. European Heart Journal, 39(17), 1555–1562. https://doi.org/10.1002/ehj3.10026

Ford, D. E., Mead, L. A., Chang, P. P., Cooper-Patrick, L., Wang, N. Y., & Klag, M. J. (1998). Depression is a risk factor for coronary artery disease in men: The precursors study. Archives of Internal Medicine, 158(13), 1422–1426. https://doi.org/10.1001/archinte.158.13.1422

Germanakis, I., Kalimani, M., Parthenakis, F., Nikitovic, D., Stiakaki, E., Patrianakos, A., & Vardas, P. E. (2006). Correlation of plasma N-terminal pro-brain natriuretic peptide levels with left ventricle mass in children treated with anthracyclines. International Journal of Cardiology, 108(2), 212–215. https://doi.org/10.1016/j.ijcard.2005.05.006

Johanssottir, I. M. R., Hamre, H., Fossa, S. D., Loge, J. H., Drolsum, L., Lund, M. B., Nordsetten, L., & Kiserud, C. (2017). Adverse health outcomes and associations with self-reported general health in childhood lymphoma survivors. Journal of Adolescent and Young Adult Oncology, 6(3), 470–476. https://doi.org/10.1089/jayao.2017.0018

Kamphuis, M. H., Kalmijn, S., Tijhuis, M. A., Geerlings, M. I., Giampaoli, S., Nissen, A., Grobbée, D. E., & Kromhout, D. (2006). Depressive symptoms as risk factor of cardiovascular mortality in older European men: The Finland, Italy and Netherlands Elderly (FINE) study. The Journal of Clinical Endocrinology & Metabolism, 91(1), 3–13. https://doi.org/10.1210/jc.2005-0713

Kiviväki, M., Virtanen, M., Elavoino, M., Kouvonen, A., Vaananen, A., & Vahtera, J. (2006). Work stress in the etiology of coronary heart disease—a meta-analysis. Scandinavian Journal of Work, Environment & Health, 32(6), 431–442. https://doi.org/10.5271/sjweh.1049

Kuba, K., Esser, P., Mehnert, A., Hinz, A., Johansen, C., Lordick, F., & Gotze, H. (2019). Risk for depression and anxiety in long-term survivors of childhood leukemia. European Journal of Cancer, 124,21–35. https://doi.org/10.1016/j.ejca.2019.11.008

Kelly, K. M. (2012). Management of children with high-risk Hodgkin lymphoma. British Journal of Haematology, 157(1), 3–13. https://doi.org/10.1111/j.1365-2141.2011.08975.x

Kiviväki, M., Virtanen, M., Elavoino, M., Kouvonen, A., Vaananen, A., & Vahtera, J. (2006). Work stress in the etiology of coronary heart disease—a meta-analysis. Scandinavian Journal of Work, Environment & Health, 32(6), 431–442. https://doi.org/10.5271/sjweh.1049

Kuba, K., Esser, P., Mehnert, A., Hinz, A., Johansen, C., Lordick, F., & Gotze, H. (2019). Risk for depression and anxiety in long-term survivors of hematologic cancer. Health Psychology, 38(3), 187–195. https://doi.org/10.1037/hea0000713

Leger, K., Slone, T., Lemler, M., Leonard, D., Cochran, C., Bowman, W. P., Bashore, L., & Winick, N. (2015). Subclinical cardioactivity in childhood cancer survivors exposed to very low dose anthracycline therapy. Pediatric Blood & Cancer, 62(1), 123–127. https://doi.org/10.1002/pbc.25206
Physiology, Biochemistry, and Pharmacology, 47(2), 851–863. https://doi.org/10.1159/000490077

Simas, B. B., Nunes, E. A., Crestani, C. C., & Speretta, G. F. (2018). Cardiovascular and metabolic consequences of the association between chronic stress and high-fat diet in rats. Stress (Amsterdam, Netherlands), 21(3), 247–256. https://doi.org/10.1080/10253890.2018.1437413

Teng, A. E., Noor, B., Ajijola, O. A., & Yang, E. H. (2021). Chemotherapy and radiation-associated cardiac autonomic dysfunction. Current Oncology Reports, 23(2), 14. https://doi.org/10.1007/s11912-020-01013-7

Tham, E. B., Haykowsky, M. J., Chow, K., Spavor, M., Kaneko, S., Khoo, N. S., Pagano, J. J., Mackie, A. S., & Thompson, R. B. (2013). Diffuse myocardial fibrosis by T1-mapping in children with subclinical anthracycline cardiotoxicity: Relationship to exercise capacity, cumulative dose and remodeling. Journal of Cardiovascular Magnetic Resonance, 15, 48. https://doi.org/10.1186/1532-429X-15-48

Tong, X., Li, V. W., Liu, A. P., So, E. K., Chan, Q., Ho, K. K., Yau, J. P., Cheuk, D. K., Cheung, Y. F., & Ng, M. Y. (2019). Cardiac magnetic resonance T1 mapping in adolescent and young adult survivors of childhood cancers. Circulation: Cardiovascular Imaging, 12, e008453. https://doi.org/10.1161/CIRCIMAGING.118.008453

Tremolada, M., Bonichini, S., Basso, G., & Pillon, M. (2016). Post-traumatic stress symptoms and post-traumatic growth in 223 childhood cancer survivors: Predictive risk factors. Frontiers in Psychology, 7, 287. https://doi.org/10.3389/fpsyg.2016.00287

van Dalen, E. C., Raphael, M. F., Caron, H. N., & Kremer, L. C. (2011). Treatment including anthracyclines versus treatment not including anthracyclines for childhood cancer. Cochrane Database Systematic Review, 19(1):CD006647.

Vandecruys, E., Mondelaers, V., De Wolf, D., Benoit, Y., & Suys, B. (2012). Late cardiotoxicity after low dose of anthracycline therapy for acute lymphoblastic leukemia in childhood. Journal of Cancer Survivorship, 6(1), 95–101. https://doi.org/10.1007/s11764-011-0186-6

Vuotto, S. C., Krull, K. R., Li, C., Oeffinger, K. C., Green, D. M., Patel, S. K., Srivastava, D., Stovall, M., Ness, K. K., Armstrong, G. T., Robison, L. L., & Brinkman, T. M. (2017). Impact of chronic disease on emotional distress in adult survivors of childhood cancer: A report from the Childhood Cancer Survivor Study. Cancer, 123(3), 521–528. https://doi.org/10.1002/cncr.30348

Wilcox, R. R. (1987). New statistical procedures for the social sciences: Modern solutions to basic problems. L. Erlbaum Associates.

Yazaki, K., Takahashi, K., Shigemitsu, S., Yamada, M., Iso, T., Kobayashi, M., Akimoto, K., Tamaichi, H., Fujimura, J., Saito, M., Nii, M., & Shimizu, T. (2018). In-depth insight into the mechanisms of cardiac dysfunction in patients with childhood cancer after anthracycline treatment using layer-specific strain analysis. Circulation Journal, 82(3), 715–723. https://doi.org/10.1253/circj.CJ-17-0874

Yeh, E. T., & Vejpongsa, P. (2015). Subclinical cardiotoxicity associated with cancer therapy: Early detection and future directions. Journal of the American College of Cardiology, 65(23), 2523–2525. https://doi.org/10.1016/j.jacc.2015.04.012

Ylanen, K., Poutanen, T., Savikurki-Heikkila, P., Rinta-Kiikka, I., Eerola, A., & Vettenranta, K. (2013). Cardiac magnetic resonance imaging in the evaluation of the late effects of anthracyclines among long-term survivors of childhood cancer. Journal of the American College of Cardiology, 61(14), 1539–1547. https://doi.org/10.1016/j.jacc.2013.01.019