Inorganic Nitrate Supplementation Improves Diastolic Function in Cancer Survivors treated with Anthracycline Chemotherapy

by

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B.S., Kansas State University, 2018

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Kinesiology
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KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

Approved by:

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Abstract

Background: Cancer survivors treated with anthracycline-based chemotherapy have a high risk of developing anthracycline-induced cardiotoxicities, including cardiac abnormalities, endothelial dysfunction, and dilated cardiomyopathy. Notably, the imbalance of decreased nitric oxide (NO) production and increased reactive oxygen species has been shown to cause significant damage to cardiac tissue and mitochondria. Therefore, the aim of the current investigation was to determine if an inorganic dietary nitrate (NO_3^-) supplementation period could restore normal cardiac function in cancer survivors with a history of anthracycline chemotherapy. **Methods:** Ten cancer survivors, 9 with breast cancer and 1 with lymphoma, completed the experiment. Standard and Tissue Doppler echocardiography were used to assess LV and carotid artery function during systole and diastole at rest. **Results:** There were no differences in ventricular-arterial coupling ($p=0.10$), arterial stiffness ($p=0.38$) or strain of the LV ($p=0.49$). However, NO_3^- supplementation improved strain rate in early filling, early mitral septal wall annular velocity, and mitral A-wave velocity or late diastolic filling. **Conclusion:** Following NO_3^- supplementation, cancer survivors with a history of anthracycline chemotherapy showed significant improvements in diastolic function compared to placebo treatments. These findings add support to the literature of the therapeutic benefits of inorganic dietary NO_3^- supplementation on cardiovascular function in clinical populations.

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Chapter 1 - Introduction

Left ventricular (LV) dysfunction (1), endothelial dysfunction (38), and increases in arterial stiffness (5, 9) can occur within the months/years following receipt of anti-cancer anthracycline chemotherapy. Furthermore, this class of chemotherapy is known to promote unfavorable long-term subclinical cardiovascular complications in cancer survivors (5-10 years after receipt of anthracyclines) that contribute to the progression in elevated cardiovascular disease morbidity and mortality rates (33). Hence, there is a growing need to provide cancer survivors with secondary strategies that may improve key parameters of cardiovascular health.

Mechanistically, the pathophysiology of anthracycline-induced cardiotoxicity is associated with increased generation of reactive oxygen species (ROS) relative to antioxidant defenses (23, 28). This increase in ROS activity has multiple consequences, including increased lipid peroxidation, peroxynitrate ($ONOO^-$) formation resulting in cell injury and calcium dysregulation that over time results in ventricular systolic and diastolic dysfunction, endothelial dysfunction, and increased arterial stiffness (14, 24, 37). One important pathway for the generation of peroxynitrate is through endothelial nitric oxide synthase-dependent mechanisms (42), resulting in decreased nitric oxide (NO) bioavailability and cGMP signaling. Given that NO is a vital signaling molecule for ventricular and vascular function; in its' absence, individuals are at an increased risk of developing cardiovascular complications (50).

Recently, orally ingested inorganic nitrates (NO_3^-), which increase NO bioavailability via the nitrate-nitrite-nitric oxide pathway and high antioxidant capacity (22), have been explored as one potential therapeutic strategy in animals given anthracycline chemotherapy. Zhu et al. (2011)

demonstrated that CF-1 mice simultaneously receiving doxorubicin and NO_3^- supplementation showed significant preservation of LV contractility, ejection fraction, systolic and diastolic pressures compared to the doxorubicin-only controls (52). In addition, Xi et al. (2012) showed that animals treated with doxorubicin on NO_3^- supplementation had an inhibition of lipid peroxidation and H_2O_2 generation via the upregulation of an enzyme peroxiredoxin 3 (Prx3), which attenuates ROS production in the myocardial mitochondria (49).

Given these preclinical findings, NO_3^- supplementation may be a promising therapeutic agent for improving cardiovascular function in cancer survivors with a history of anthracycline chemotherapy. However, the efficacy of orally ingested inorganic NO_3^- for improving parameters of cardiovascular health in these patients is entirely unknown. In this context, we hypothesized that inorganic NO_3^- administration would improve standard 2D and Doppler-based indices of LV systolic and diastolic function, arterial stiffness, and ventricular-arterial coupling in human cancer survivors with a history of anthracycline chemotherapy treatment.

Chapter 2 - Methods

Patients were eligible for inclusion in the present study if they had a history of breast cancer or lymphoma and had received anthracycline chemotherapy as part of their cancer treatment. Patients were excluded from participation if they had any of the following: were diagnosed with overt cardiovascular disease, diabetes, kidney disease, high risk of kidney stones, hemochromatosis, were a current smoker, or were currently taking antioxidant supplements (e.g., Glutathione, Quercetin, Vitamin C, Resveratrol, Selenium, Vitamin E or Fish Oil). Throughout the study, patients were instructed to maintain their regular diet and refrain from mouthwash, chewing gum, and alcohol, as alcohol has been shown to decrease the reduction efficacy of the nitrate-nitrite-nitric oxide pathway (48). All procedures were approved by the Kansas State University Institutional Review Board for research involving human subjects, and all standards conformed to the *Declaration of Helsinki.*

This study was a prospective single-center, double-blind, randomized, placebo-controlled trial. All participants consumed 70 mL of concentrated nitrate-rich [NO_3^-] beetroot juice supplement (BR; BEET IT Sport, James White Drinks Ltd, Ipswich, UK) containing [12.9 mmol NO_3^-], or 70 mL nitrate-depleted placebo (PL: blackcurrant juice cordial with negligible NO_3^- content) once a day for 7 days (1-week daily dosing). Patients then entered a 7-10 day "washout" period before entering the opposing arm of the study (17). For all visits, the participants consumed the final juice supplement ~2 hours before the testing visit.

Experimental Procedures

Nitrate and Nitrite

Upon arrival to the laboratory, venous blood samples were drawn into lithium heparin vacutainers (Becton Dickenson, NJ) to evaluate circulating plasma nitrite [NO_2^-] levels post supplemental periods. Immediately following the blood draw, blood samples were spun and plasma was collected and frozen until analysis. [NO_2^-] analysis was performed within 30 minutes after the sample had thawed via chemiluminescence with an Ionic/Sievers NO analyzer (NOA 280i, GE, Boulder, CO) in duplicates following the instrument calibration (8).

2-Dimmensional and Tissue Doppler Echocardiography

Each participant was placed supine on an echocardiographic table that utilizes a unique tilt function to place the patients into the 45^{o} left-lateral decubitus position, while supporting the torso, hips, and legs. Three-lead electrocardiogram ECG was used to measure heart rate (HR). Following a 5 min acclimation period, transthoracic echocardiography was performed at rest by an experienced sonographer, according to the standards of the American Society of Echocardiography using a commercially available system (Vivid S6 BT12; GE Healthcare) with a 1.5-to 4.3-MHz phased array transducer. All echocardiography parameters were collected at rest and averaged over three cardiac cycles. LV volumes were quantified at end-systolic volume (ESV) and end-diastolic volume (EDV) via the perpendicular axis from the apex to the distal boundary of the mitral valve (MV) leaflets. LV ejection fraction (LVEF) was calculated as: LVEF = [(EDV-ESV)/EDV] x 100. Stroke volume (SV) and cardiac output (Q) were calculated as SV = ESV-EDV and Q = SV x HR, respectively. LV diastolic function was measured by

Doppler measurements of MV inflow from the apical 4-chamber view with the sample windows placed between the MV leaflets. From the MV inflow tracing, the peak velocities for early (E), and late (A) filling were obtained, E deceleration time was measured, and the E/A ratio was calculated. 2-D tissue Doppler velocity data for peak myocardial velocities in systole (Sm) and early (Em) filling were recorded in the apical 4-chamber view with a narrow sector and high frame rate by placing the sample window in the basal segment of the interventricular septum. LV longitudinal strain and strain rate were derived from 2-dimensional apical images using tissue Doppler imaging data as previously described (35). Longitudinal myocardial function was evaluated using peak systolic strain, peak systolic strain rate, and early filling strain rate in the basal segments of the interventricular septum (29).

Carotid Artery Stiffness

Cross-sectional carotid artery diameter was measured at systole and diastole on the distal wall of the carotid artery, roughly 2-3 cm below the bifurcation with a commercial two-dimensional ultrasound system (Logiq S8, GE Medical Systems, Milwaukee, WI) via a high-resolution phased array transducer operating at 10 MHz with simultaneous ECG recordings. All measurements were obtained in the supine position with the subjects head slightly tilted to the contralateral side. These images were recorded for ~8-10 seconds (8-10 cardiac cycles) and were stored for off-line analysis. Using the ultrasound system image analysis software, the maximal (i.e., end systole) and minimal (i.e., end diastole) luminal diameters were calculated from the distance between media-adventitia interfaces at each cardiac cycle by a blinded investigator (9). M-mode image quality was considered acceptable if the lumen-intima-media adventitia

interfaces were clearly identifiable. To calculate stiffness, the luminal diameters were time-aligned with simultaneous ECG measurements; thus, the stroke changes of the carotid artery and central blood pressure could be used to determine the stiffness of the vessel. Central waveform and pressures were calculated with the FinometerPro software, (BeatScope 1.1a Finapress Medical Systems) via the Modelflow method from the calibrated finger artery waveform. Arterial stiffness index was then calculated as: ($\beta = \ln(Ps/Pd)/[(Ds-Dd/Dd)]$. Ps and Pd reflect central aortic systole and diastole while Ds and Dd reflect carotid systole and diastole diameters.

Ventricular-Arterial Coupling

Ventricular-Arterial Coupling (VA-Coupling) was derived by the ratio of effective arterial elastance (E_a) to LV end-systolic elastance (E_{es}). E_a was calculated as end-systolic pressure/stroke volume (2), with end-systolic pressure calculated from the central aortic blood waveform (9). E_{es} was determined using the validated single beat method as previously described (27) (See Appendix A).

Statistical Analysis

Data were analyzed with commercially available statistical software package (Sigmaplot; version 12.5, Systat software, San Jose). Plasma NO_3^- values were compared between conditions as a paired-samples t-test. Differences between end points for the treatment and control measurements were compared with paired t-tests. A value of $P < 0.05$ was considered significant. Given the crossover design a modified intent-to-treat strategy was used, which only subjects who

completed both laboratory visits were included. The study was powered to detect differences between NO_3^- and placebo conditions when effect sizes >0.8 with 80% power and a nominal α level of 0.05.

Chapter 3 - Results

A total of 26 subjects were screened, with 11 subjects meeting the inclusion criteria and entering the study. One subject became ill during the second round of supplementation and withdrew herself from further testing. Therefore, 10 subjects were included in the final analysis.

Study Participants

The mean age of study participants was 58 ± 10.5 years, all of which were women. At the time of enrollment, patients had received their anthracycline-based chemotherapy treatment an average of 11.9 ± 9.2 years prior to the study. Seven patients were treated with a combination doxorubicin and cyclophosphamide with a mean cumulative dose of 385mg/m^2 of doxorubicin and 3947mg/m^2 of cyclophosphamide. One patient was concurrently on a combination of doxorubicin (600mg/m^2) and dacarbazine (900mg/m^2). One patient received a combination of epirubicin (740mg/m^2) and cyclophosphamide (1480mg/m^2). One patient received a combination of mitoxantrone (87mg/m^2) and cyclophosphamide (1350mg/m^2). Patients received anthracycline treatment for a mean duration of 4.7 ± 1.4 months.

Oral NO_3^- supplementation significantly increased plasma nitrite levels (2613 ± 754μM) compared to the nitrate-poor placebo supplementation (517 ± 870μM: p=0.0004). Compared to placebo, NO_3^- supplementation had no effect on resting HR (66 ± 8 bpm versus 67 ± 10 bpm; p=0.23), systolic blood pressure (133 ± 16 mmHg versus 130 ± 11 mmHg; p=0.21), or diastolic blood pressure (73 ± 8 mmHg versus 71 ± 8 mmHg; p=0.10)

Dimensions and LV Systolic and Diastolic Functions

As shown in Table 1, one week of NO_3^- treatment did not significantly alter LV ESV, EDV, or SV compared to placebo. LV ejection fraction was unchanged with NO_3^- treatment. Similarly, septal wall systolic velocity (Sm), systolic strain and strain rate showed no significance differences between the two conditions. LV diastolic function parameters, including septal Em and strain rate in early diastolic filling were suggestive of diastolic improvements following NO_3^- treatment. Significant improvements in septal wall early diastolic velocity (Em) were observed following NO_3^-treatment (Figure 1). Mitral valve late diastolic filling velocity was significantly increased following NO_3^- treatment. Likewise, LV longitudinal strain rate in early filling was significantly increased following NO_3^-supplementation compared to placebo (Figure 2).

Vascular Stiffness

Carotid artery systolic (124±14 mmHg versus 127±13 mmHg; p=0.21) and diastolic diameter (72±11 mmHg versus 73±6 mmHg; p=0.33) were not significantly different between the two conditions. Similarly, arterial pulse pressure (52±15 mmHg versus 58±16 mmHg; p=0.07) was not different resulting in a similar carotid artery β-stiffness index between the two conditions.

Ventricular-Arterial Coupling

Ea was not significantly different following NO_3^- supplementation compared to placebo (2.38 versus 2.48; p=0.30). Similarly, LV Ees was not significantly different between the two conditions (2.91 versus 2.83; p=0.39), which is consistent with measurements of LV systolic function. Taken together, ventricular-arterial coupling assessed as the ratio of Ea/Ees did not differ between NO_3^- supplementation and placebo conditions (0.82 versus 0.90; p=0.10).

Table 1

Table 1. Echocardiography

	Inorganic Nitrate	Placebo	Difference between Inorganic Nitrate and Placebo	P Value
EDV (ml)	92 ± 12.8	82 ± 7.4	10 ± 5.4	0.08
ESV (ml)	42 ± 7.4	35 ± 7.6	7 ± 0.2	0.11
SV (ml)	50 ± 9.9	47 ± 5.8	3 ± 4.1	0.15
MV Deceleration Time (ms)	265 ± 87.3	248 ± 76.6	17 ± 10.7	0.36
MV E-wave velocity (cm/s)	0.7 ± .17	0.7 ± .39	0 ± 0.22	0.45
MV A-Wave Velocity (cm/s)	0.6 ± .23	0.5 ± .17	0.1 ± .06	0.01
MV E/A Ratio	1.1 ± .19	1.1 ± .27	0 ± 0.08	0.22

mean±SD

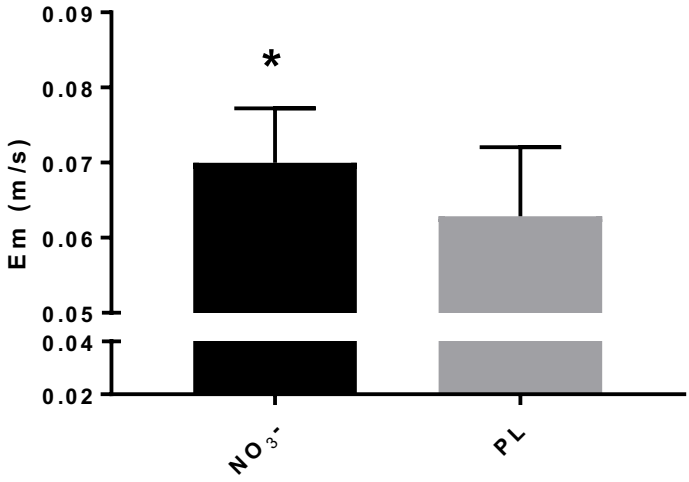

Figure 1 - Septal mitral annular velocity obtained in early diastole (Em) following NO_3^- and PL conditions. * Significantly different compared to placebo.

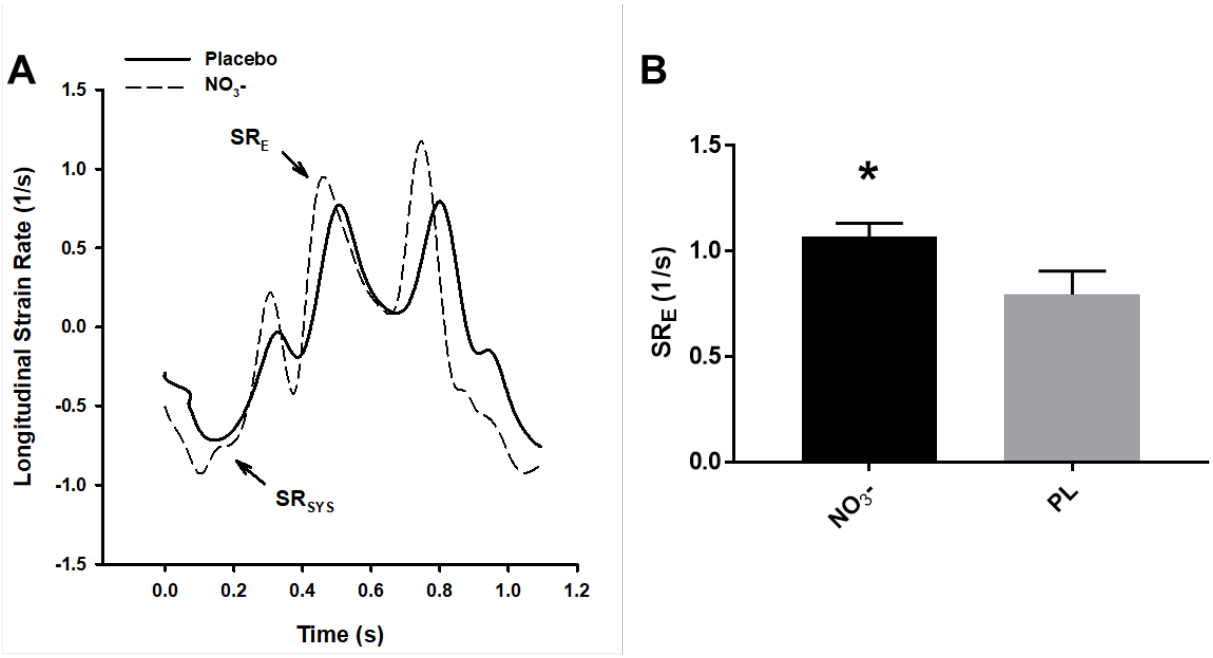

Figure 2 - Longitudinal strain rate of the left ventricle in a representative patient following NO_3^- treatment (A). Left ventricular strain rate during early diastole (SRE) following NO_3^- and placebo (PL). SRSYS, LV peak systolic strain rate. * Significantly different from placebo.

Chapter 4 - Discussion

The primary aim of this study was to examine the therapeutic effects of inorganic dietary nitrates on the cardiovascular function of cancer survivors with a history of anthracycline chemotherapy. To our knowledge, this was the first study to investigate the effects of inorganic dietary NO_3^- supplementation on the cardiovascular system in human cancer survivors. A critical finding of this study was that we demonstrated that NO_3^- supplementation significantly improved left ventricular diastolic function in cancer survivors. These findings suggest that therapeutic strategies that target NO bioavailability may provide secondary prevention of anthracycline-induced changes in left ventricular diastolic function (6). However, we did not observe significant improvements in left ventricular systolic function or vascular function with NO_3^- supplementation compared to a placebo supplementation.

Since the 1960's, anthracycline-chemotherapy (e.g. doxorubicin, epirubicin, and daunorubicin) has been a widely used anti-cancer agent for the treatment of several human cancers (39, 40). While this chemotherapy is extremely effective at eliminating the cancer cells, it is often coupled with problematic anthracycline-induced cardiotoxicities, including: asymptomatic systolic and diastolic dysfunction (6), heart failure (4), and increased aortic stiffness (5); therefore, accelerating the rate of early onset cardiovascular morbidity and mortality (16). Smith et al. confirmed a significantly greater risk of developing cardiotoxicities with anthracycline treatment compared to other non-anthracycline regimens (36) that occur in a dose-dependent manner (1, 47).

The most commonly used anthracycline, doxorubicin, has been the target for investigation into the underlying mechanisms of anthracycline-induced cardiotoxicity. In cardiac tissue, doxorubicin enters the cardiomyocyte through diffusion and is reduced via NADH dehydrogenase which causes direct damage to cellular membranes and organelles (e.g. mitochondria) by increased free radical production, decreased antioxidants, and sulfhydryl groups (28). The increased oxidative stress elicited by doxorubicin can alter cardiomyocytes by activating an apoptotic cascade, ultimately resulting in irreversible cardiac cell death (34, 39). Moreover, doxorubicin can disrupt sarcomere structure and impair energy metabolism and ion gradients (33). A review performed by Rashi et al. (2010) on cancer drugs and cardiotoxicity indicated that secondary metabolites produced by anthracycline metabolism inhibit Ca^{2+} release from the sarcoplasmic reticulum; thus, increasing the risk of developing LV dysfunction (13, 33).

Doxorubicin-induced cardiotoxicity reduces the production and bioavailability of NO via nitric oxide synthase (NOS) pathways (21). Specifically, in the animal model, Griffith and Stucher et al. demonstrated that doxorubicin is rapidly reduced in the presence of oxygen via endothelial nitric oxide synthase (eNOS) which progressively increases the amount of superoxide formation and decreased the amount of bioavailable NO (12, 46); therefore, increasing the overall amount of reactive oxygen species (ROS) and reduced NO-cGMP signaling. This imbalance of ROS will produces an increased amount of peroxynitrite which is known to cause ventricular dysfunction, calcium mishandling, and direct damage to DNA synthesis (12, 25, 49). Moreover, altered NO-cGMP signaling can significantly alter Ca^{2+} uptake through the sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2). Thus, strategies aimed at decreasing oxidative stress and increases nitric oxide bioavailability may attenuate the cardiotoxic effects of anthracycline chemotherapy.

Inorganic dietary NO_3^- supplementation elicits significant improvements in ventricular and vascular function with targeted NO production, via the nitrate-nitrite-nitric oxide pathway (21), and increased antioxidant capacity (22). Furthermore, substantial evidence suggests that inorganic nitrates also poses substantial antioxidant actions (49). As such, inorganic dietary nitrate supplementation acts as a cardioprotective agent against myocardial ischemic-reperfusion injury (3), hypertension (41), poor exercise performance (decrease O_2 demand) in both peripheral artery disease (18) and heart failure with preserved ejection fraction (51). Important to the present study, Zhu et al. demonstrated in mice that administering a nitrate supplementation before and after anthracycline treatment attenuated all anthracycline-induced cardiac dysfunction compared to those just receiving chemotherapy (52). Additionally, a significant preservation of mitochondrial function was observed, coupled with a decrease in ROS levels (52).

The present study expands on this previous work by exploring the benefits of inorganic dietary nitrate supplementation on human cancer survivors with a history of anthracycline chemotherapy. Notably, we established that one week of inorganic dietary nitrate supplementation significantly increased clinically relevant measurements of LV diastolic function. This is critical given the previous findings that significant LV diastolic dysfunction is associated with low doses of anthracycline (~200mg/m^2) that are below that known to elicit changes to contractile forces and systolic function (20).

Recent advances in non-invasive myocardial strain and strain rate imaging have allowed investigators to assess subclinical changes in parameters of systolic and diastolic deformation (7)

with greater sensitivity relative to traditional measurements of ejection fraction (40) or mitral valve velocities (45). As such, evaluation of LV strain and strain rate provide a non-invasive method to evaluate both changes in myocardial systolic and diastolic function with disease progression and the efficacy of potential therapeutic interventions (15, 26, 30).

Importantly, strain rate in the early filling phase of ventricular diastole has constantly been used as a clinically relevant measure of LV diastolic function (30). Pislaru et al. demonstrated in post myocardial infraction animals that strain rate during early and late diastole reflected abnormalities in relaxation and compliance due to diastolic pressure-wall thickness relations (32). Similarly, Park et al. revealed that diastolic strain rate is a more viable evaluation of diastolic function compared to mitral valve early and late velocities because it more accurately depicts regional diastolic function (31). As such, assessment of ventricular strain rate in early diastole provides valuable insight into LV diastolic function. Our findings demonstrate that NO_3^- supplementation increased LV longitudinal strain rate in early filling; therefore, highlighting the improvements in diastolic function in cancer survivors with a history of anthracycline chemotherapy.

In parallel with indices of myocardial strain, Doppler Tissue imaging provides additional information when evaluating changes in myocardial systolic and diastolic function with disease progression and following therapeutic intervention (11, 15). Specifically, early mitral septal wall annular velocity obtained in early diastole (Em) has been shown to provide clinically relevant measurement of LV diastolic function (44) that is directly related to LV relaxation rate (10). Importantly, Okada et al. established a strong correlation between septal Em and interventricular

longitudinal septal strain rate (r=0.75); thus, highlighting that these two measurements provide a synergistic evaluation of LV longitudinal myocardial relaxation and therefore a clinically applicable measurement of LV diastolic function (29). To the best of our knowledge, this is the first study to demonstrate improvements in diastolic function via strain rate imaging and Tissue Doppler myocardial velocities in cancer survivors treated with systemic anthracycline-chemotherapy.

Several experimental considerations warrant mention when interpreting the study findings. First, the total cumulative dose of anthracyclines (385 mg/m^2) administered to our patients potentially lessened the cardiotoxicity of this anti-cancer drug class. Volkova et al. (2011) reviewed the adverse side-effects of anthracycline treatment and noted an increased occurrence of clinically diagnosed cardiac systolic dysfunction in patients who received a cumulative dose of anthracyclines >550mg/m^2 (43). However, subclinical cardiotoxicity has been reported in cancer survivors who received < 550mg/m^2 (5), with LV diastolic dysfunction occurring with doses below 200mg/m^2 (20). Lastly, using submaximal exercise to evaluate cardiac function post nitrate supplementation could have provided additional insight into the beneficial effects of NO_3^- supplementation (41). However, Koelwyn et al. (2016) determined that the Ea (arterial elastance) portion of the ventricular-arterial coupling ratio in breast cancer survivors treated with anthracyclines was not different between rest and submaximal exercise; thus, aligning with our own results (19).

In conclusion, this is the first study, to our knowledge, to demonstrate an improvement in diastolic function in cancer survivors with a history of anthracycline chemotherapy in response

to inorganic dietary nitrate supplementation. The improvements in diastolic function suggest that therapeutic strategies that target NO bioavailability and ROS may be a clinically beneficial cardioprotective agent post-anthracycline administration.

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Appendix A - VA Coupling Calculations

The estimated normalized left ventricular elastance was calculated as:

$$E_{Nd(est)} = 0.0275 - (0.165 \times EF) + 0.3656 \times (P_d/P_{es}) + 0.515 \times E_{Nd(avg)}$$

where EF is ejection fraction, P_d is diastolic blood pressure, Pes is end-systolic pressure, and $E_{Nd(avg)}$ is calculated by a seven-term polynomial function:

$$E_{Nd(avg)} = \sum_{i=0} a_i \times t_{Nd}$$

where a_i for i = 0 are (0.35695, -7.2266, 74.249, -307.39, 684.54, -856.92, 571.95, -159.1), respectively and the value of t_{Nd} determined from the aortic Doppler waveform by by dividing the pre-ejection period (R wave to flow-onset) by the total systolic period (R-wave to end-flow). From these two equations left ventricular end-systolic elastance (Ees) was calculated as:

$$E_{es} = [P_d - (E_{Nd(est)} \times P_{es})]/[SV \times E_{Nd(est)}]$$