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Supplemental Appendix for:
Ventricular-Arterial Coupling in Breast Cancer Patients Following Treatment with Anthracycline-Containing Adjuvant Chemotherapy
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Supplemental Appendix

Exclusion Criteria

Exclusion criteria included: 1) treatment with (neo) adjuvant trastuzamab, 2) evidence of cancer recurrence, 3) clinically diagnosed cerebrovascular, neurological, respiratory, metabolic disease, or diabetes, 4) absolute or relative contraindications to exercise,[1] (e.g uncontrolled hypertension, high degree atrioventricular block, malignant arrhythmia etc.) 5) <18 years and >80 years of age, 6) current smoker, or 7) BMI of $\geq$35 kg/m^2.

Exercise Screening

At visit one subjects performed a cardiopulmonary exercise test (CPET) to symptom limitation on an upright cycle ergometer (Ergoline 800S, SensorMedics, Yorba Linda, CA) with expired analysis (Sensormedics Vmax 29C, SensorMedics, Yorba Linda, CA) to: 1) screen for any contraindications to exercise, 2) determine peak oxygen consumption (VO_{2peak}) and 3) determine individual exercise intensities for the exercise echocardiographic tests. The test protocol was performed according to ATS guidelines,[1] but modified for cancer populations.[2] After stable resting metabolic values were achieved, subjects cycled unloaded for two minutes before the load was increased by 10-15 W.min^{-1} until symptom-limitation. The intensity increases were determined from the predicted maximal workload and the subject's activity level with the intention of making tests last 8-12 minutes in duration. During each test, oxyhemoglobin saturation measured by pulse oximetry (Radical 7, Maximo, Irvine, CA) and heart rates measured by electrocardiography (CardioSoftTM, GE Healthcare, Waukesha, WI) were monitored continuously. Blood pressure was measured using manual sphygmomanometery performed every two minutes. Exertional dyspnea and leg discomfort were also evaluated every two minutes using the modified Borg scale.[3]

Echocardiographic Measurements of Systolic and Diastolic Function and Analysis

Ultrasound measurements of end-diastolic volume (EDV) and end-systolic volume (ESV) of the left ventricle were made at rest and during steady state (heart rate within 5 beats/min) upright cycling exercise using a commercially available ultrasound system (IE33, Phillips, Netherlands). 2D-transthoracic echocardiographic images were made in the apical four and apical two chamber views to determine left ventricular EDV and ESV by the modified Simpson's rule.[4] Stroke volume was calculated as EDV – ESV and ejection fraction was measured as stroke volume / EDV. A minimum of 3 measurements for each image were analyzed and averaged for the calculation of each cardiac variable. The time length of each image 'bin' acquired varied between 2-4 cardiac cycles.

A discontinuous protocol where patients were given 5-10 minutes rest between exercise at each intensity (rest, 25%, 50% and 75% W_{max} as determined by the cardiopulmonary exercise test) was utilized so that subjects could maximize their ability to maintain steady state exercise for longer while holding a stable body position, which allowed more optimal image acquisition. Blood pressure was measured at rest and when the subject reached steady state using manual sphygmomanometry. Blood pressure measurements were made at least twice to confirm the accuracy of the measurement. To assess diastolic filling parameters at rest, the velocity of mitral valve flow was evaluated in the apical four-chamber view by positioning the sample volume at the level of the tips of the mitral leaflets. Using this technique, the peak early diastolic flow velocity (E), the deceleration time from E, the peak late diastolic flow velocity, and the duration of A velocity were measured. Ea and Ees were evaluated using echocardiographic measures. Ea was calculated as the ratio of end-systolic pressure (ESP) to stroke volume (SV).[5] ESP was determined by the equation 0.9 x systolic blood pressure which has previously been validated in humans.[6] Ees was calculated using the single beat technique,[7] which has been used previously during exercise.[8-10] Ees was determined by the following equation: $[E\text{es} = (\text{DBP} - [E_{Nd} \times \text{SBP} \times 0.9]) / (\text{SV} \times E_{Nd\ (est)})]$, where $E_{Nd\ (est)}$ is the estimated normalized LV elastance at the onset of ejection, and DBP and SBP are diastolic and systolic blood pressures, respectively. $E_{Nd\ (est)}$ was determined by the following equation: $[E_{Nd\ (est)} = 0.0275 - 0.165 \times \text{LVEF} + 0.3656 \times (\text{DBP/ESP}) + 0.515 \times E_{Nd\ (avg)}$. $E_{Nd\ (avg)}$ was determined by the following seven-term polynomial function: $E_{Nd\ (avg)} = \sum a_i \times t_{Nd}^{\ i}$ (i=0), where a_i are (0.35695, −7.2266, 74.249, −307.39, 684.54, −856.92, 571.95, −159.1) for i=0 to 7, respectively. The value of t_{Nd} was determined by the ratio of pre-ejection period (R wave → flow onset) to total

systolic period (R wave → end-flow), with the time at onset and termination of flow defined non-invasively from the aortic Doppler waveform.

Measurement of Arterial Stiffness using Applanation Tonometry and Carotid IMT Measurement and Analysis

Conduit regional arterial stiffness was assessed non-invasively by measuring central (carotid-femoral flow pulse wave velocity (PWV)) and peripheral (carotid-radial) PWV using hand held-tonometers (SPT-301 Millar Instruments, Houston, TX). All measures were made on the right side unless lymph nodes had been removed on the right side during breast cancer treatment, in which case the left arm was used. Carotid and femoral waveforms, followed by carotid and radial artery waveforms, were recorded simultaneously with the tonometers applied directly to the skin over the area of greatest pulsation. Twenty consecutive reproducible beats, determined by the technician through a consistent pressure waveform (shape and relative magnitude), were collected simultaneously at both sites, with a concurrent electrocardiograph to obtain R-R intervals. Pulse distance was determined using anthropometric measuring tape centrally by subtracting the distance from carotid measurement to sternal notch from the distance from sternal notch to femoral pulse measurement, and peripherally by subtracting the distance from carotid measurement to sternal notch from the distance from sternal notch to the radial measurement. This technique was used as it has been shown to have the best agreement with aortic PWV measured invasively using cardiac catheterization.[155] PWV was then determined for each by taking the pulse transit time / distance.

Local arterial stiffness was assessed at the carotid artery to approximate central artery stiffness. Carotid artery blood pressure waveforms were obtained using the hand-held tonometer positioned over the greatest pulsation on the right carotid artery, while concurrent carotid images were obtained using B-mode ultrasound positioned over the left carotid artery ~2 cm proximal to the bifurcation of the external and internal carotid arteries. Participants also had a continuous blood pressure measurement (Finometer - Finapres Medical Systems B.V.; Amsterdam, The Netherlands) and electrocardiograph for simultaneous recordings of brachial blood pressure and R-R intervals. Ten consecutive carotid and brachial waveforms were collected and analyzed using commercially available software (LabChart v7, National Instruments, Austin, Texas) with 10 complete heart cycles collected with the ultrasound machine. Continuous blood pressure was calibrated to manual blood pressure if a discrepancy existed between the two measures. In this case, manual blood pressure pre and post collection was averaged and used to manually calibrate the finometer throughout the test. Maximal and minimal lumen diameters were calculated using edge detection software as described below. Carotid arterial compliance, distensibility and ß-stiffness index (an index of arterial compliance adjusted for distending pressure) were calculated as described elsewhere.[11,12]

Carotid IMT was measured using a high-resolution ultrasound (Vivid-q, GE, Fairfield, CT, USA). Subjects were measured in the supine position with a slight hyperextension of the neck and at a 45° lateral flexion away from the side being scanned using a 8 MHz high frequency linear array transducer. Images were taken of the far wall, 1 cm proximal to the carotid bulb. The IMT at end diastole (1 frame prior to the R-interval) of 10 successive beats was recorded and averaged.

Brachial Artery Endothelial Dependent and Independent Vasodilation and Analysis

Endothelial function was evaluated using ultrasound measures of flow mediated endothelial dependent vasodilation (FMD) of the brachial artery. On arrival to the laboratory the subject laid supine in a dimly lit, temperate room for 15 min before measurements were made. The ultrasound probe was orientated over the brachial artery and positioned to obtain a clear arterial blood velocity signal with no interference from adjacent venous flow. All measurements were made above the antecubital fossa of the right arm using a 10-Mhz multifrequency linear array transducer and a high-resolution ultrasound machine (T3000L; Terason, Burlington, MA). This position has been shown to be primarily nitric oxide (NO) dependent, and is recommended by recent published guidelines.[13] Measures were made in the left arm if lymph nodes had been removed during breast cancer treatment. The duplex ultrasound machine allowed for simultaneous acquisition of diameter and pulse-wave velocity Doppler velocity signals, which is essential for quantifying shear stress and time to peak flow and dilation.[13] Reactive hyperaemia (FMD) was induced by inflation of a blood pressure cuff around the forearm to ~50 mmHg above resting systolic blood pressure for 5 minutes. The diameter of the brachial artery and blood flow velocity was assessed continuously for 1 minute before and for 3 min following cuff deflation using specialized

custom-designed 'gold-standard' edge detection and wall tracking software independent of investigator bias.[14,15] This software determined peak diameter from the 30 Hz of mean diameter data derived according to the methods described below. After 30 min of rest to allow arterial diameter to return to baseline, another 3-min of baseline recordings was made before a sublingual 400-µg spray dose of nitroglycerine, or glyceryl trinitrate (GTN), with images recorded for a further 10 min to measure endothelial independent vasodilation of the brachial artery. Each subject was asked if they had a history of headaches or migraines. Only those with no history received GTN.

Carotid and Brachial Artery Post-Analysis

An individual blind to group allocation performed all ultrasound analysis. Semi automated edge detection software (GE Healthcare, Echopac Dimensions, Version 110.1.2) was used in post–analysis of the carotid arterial wall to determine CIMT. An ROI of approximately 150 data points was used for each sample, and determination of the near and far wall were determined by identifying changes in pixel intensity (described below). Maximum and minimum diameters for the carotid artery were calculated as described below for brachial artery diameters. Diameters from each frame collected over 10 cardiac cycles were exported to an excel document, where 10 maximum and 10 minimum diameters were selected and averaged.

In order to perform post-test analysis of the brachial artery, video images from the ultrasound machine were taken and stored as a DICOM file. A region of interest (ROI) was drawn on both the B-mode image and Doppler strip scales to allow for automatic calibration of diameter and velocity, respectively. Then, a second ROI was drawn around an optimal area of the B-mode image, which generated a pixel-generated algorithm that identified the angle-corrected near and far-wall lines for every pixel column within the ROI. The algorithm identified the edge of the artery by determining the point where the pixel intensity changed most rapidly using a Rake routine by scanning from bottom to top (and vice versa) on both the near and fall wall. The ROI selection was chosen manually and was based primarily on clarity of the image throughout the entire image capture, which usually contained between 200-300 diameter measures per frame, and occurred at 30 frames per second. An ROI was also placed around the entire Doppler trace, which automatically tracked in real time the velocity tracing at 30Hz. Diameter measures were then synched with velocity measures at 30 Hz to calculate blood flow and shear rate. Erroneous data was manually identified and deleted from both the B-mode and Doppler trace. This was only completed when it was clear that the software was not picking up the wall of the artery by confirming on the B-mode image the diversion of the generated line from the arterial wall. Subjects were removed from analysis if this occurred for significant portions of the test.

The time to peak diameter (in seconds) was calculated from the point of cuff deflation to maximum diameter. Calculation of FMD and time to peak were therefore observer-independent and based on standardized algorithms applied to the data, which had undergone automated edge-detection and wall tracking. The post-deflation shear rate stimulus responsible for the endothelium-dependent FMD was acquired from simultaneously acquired velocity and diameter measurement at 30 Hz. Shear rate area under the curve was calculated using the Reiman Sum Technique and calculated shear up to time of peak dilation. Shear rate was calculated as 4 x peak velocity divided by average diameter. The analysis software determined both of these automatically. Peak FMD was also adjusted for baseline diameter.[16] In brief, changes in diameter were log scaled and entered into an ANCOVA with group as a fixed factor and Dbase (logarithmically-transformed) as a covariate. The adjusted mean diameter changes were then back-transformed to provided adjusted values.

Additional Statistical Analysis

Sub-group analysis was performed by stratifying subjects into three groups using a tertile split for the magnitude change in Ea (ΔEa) from rest to 75% W_{max}, similar to that previously reported in healthy individuals [17]. As Ea was not different between survivors and controls, groups were collapsed and tertiles were determined on the full data set (n=60). Cardiovascular responses were then compared between survivors and controls based on 1) a large increase in Ea (ΔEa>0.57 mmHg/mL); 2) a small increase in Ea (0.03 mmHg/mL<ΔEa<0.57 mmHg/mL); and 3) no change or a decrease in Ea (ΔEa<0.03 mmHg/mL). To examine within group differences by change in Ea, the Kruskal-Wallis Test for comparing non-parametric continuous data for more than two independent groups was used, and Wilcoxon Rank Sum Tests were used to examine pairwise differences when appropriate.

Supplemental References

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