Rationale: There is a critical need to develop robust, mechanistic strategies to identify patients at increased risk of cancer therapeutics–related cardiac dysfunction (CTRCD).

Objective: We aimed to discover new biomarkers associated with doxorubicin- and trastuzumab-induced CTRCD using high-throughput proteomic profiling.

Methods and Results: Plasma, echocardiograms, and clinical outcomes were collected at standardized intervals in breast cancer patients undergoing doxorubicin and trastuzumab cancer therapy. Thirty-one longitudinal plasma samples from 3 cases with CTRCD and 4 age- and cancer-matched controls without CTRCD were processed and analyzed using label-free liquid chromatography–mass spectrometry. From these analyses, 862 proteins were identified from case/control pairs 1 and 2 and 1360 proteins from case/control pair 3. Proteins with a >1.5-fold change in cases compared with controls with a \( P<0.05 \) either at the time of CTRCD diagnosis or across all time points were considered candidate diagnostic or predictive biomarkers, respectively. The protein that demonstrated the largest differences between cases and controls was immunoglobulin E, with higher levels detected at baseline and across all time points in controls without CTRCD as compared with matched CTRCD cases (\( P<0.05 \)). Similarly, in a validation study of 35 participants treated with doxorubicin and trastuzumab, high baseline immunoglobulin E levels were associated with a significantly lower risk of CTRCD (\( P=0.018 \)).

Conclusions: In patients receiving doxorubicin and trastuzumab, high baseline immunoglobulin E levels are associated with a lower risk of CTRCD. These novel findings suggest a new paradigm in cardio-oncology, implicating the immune system as a potential mediator of doxorubicin- and trastuzumab-induced cardiac dysfunction. (Circ Res. 2016;119:1135-1144. DOI: 10.1161/CIRCRESAHA.116.309004.)

Key Words: cardiomyopathy ■ CTRCD ■ immune mediators ■ label-free quantitation ■ plasma biomarkers ■ proteomics

Doxorubicin and trastuzumab (Herceptin) are used widely in the treatment of breast cancer, are highly effective, and have led to important survival gains.\(^1\) However, these agents carry a substantially increased risk of cardiovascular morbidity and mortality. Doxorubicin-induced cardiac dysfunction occurs in 9% of treated patients at dosages of 250 mg/m\(^2\).\(^2\) Trastuzumab, a highly effective humanized monoclonal antibody used in the treatment of Her2 (ErbB2)-positive breast cancer, also causes significant declines in left ventricular ejection fraction (EF), resulting in potential delays or cessation of necessary therapy.\(^3,4\) When anthracyclines and trastuzumab are used in combination, up to 18% of patients develop cancer therapeutics–related cardiac dysfunction (CTRCD) and 2% to 4% develop severe, symptomatic heart failure.\(^5\)

There is an important need to identify patients at increased risk of developing CTRCD, particularly before the development of overt disease. Cardiovascular biomarkers have been widely studied as potential tools to risk-stratify patients; however, many of these markers are neither specific nor sensitive for diagnosing CTRCD or for predicting which patients are at increased cardiovascular risk. Moreover, our lack of mechanistic understanding...
of the underlying pathophysiology of doxorubicin and trastuzumab cardiac dysfunction has hindered the development of newer markers. As such, discovery proteomics represents a unique opportunity to identify novel pathways and biomarkers.

Traditionally, there have been several hurdles that have hindered advances in proteomics discovery. These include the great complexity of the plasma or serum proteome; the presence of a few high-abundant proteins; the wide dynamic range of protein concentrations; and the molecular heterogeneity of many proteins. However, considerable advances have been made in liquid chromatography–mass spectrometry–based biomarker discovery and validation methods over the past several years that have substantially reduced these barriers. Now, rigorous and powerful proteomic-based technologies can be used to successfully discover and validate new biomarkers.

The overall purpose of this study was to apply current proteomics techniques to discover new circulating biomarkers that can be used in diagnosis and risk prediction in CTRCD. In breast cancer patients undergoing therapy with doxorubicin and trastuzumab, we hypothesized that patterns of change over time in protein markers would differ between participants who experience CTRCD and those who do not and specifically sought to discover markers that demonstrated significant changes at the time of CTRCD diagnosis (diagnostic markers) and before the onset of CTRCD (predictive markers). To determine the answer to this question, we used a cohort of well-phenotyped breast cancer patients undergoing doxorubicin and trastuzumab therapy with longitudinal blood sampling coupled to cardiac dysfunction outcomes, derived from clinical and quantitative echocardiography data. We first performed a matched case–control discovery study in longitudinal samples and then validated our most promising biomarker in a cohort analysis of baseline samples using independent assays.

Methods

Study Population
Cases and controls were selected from the CCT study (Cardiotoxicity of Cancer Therapy), an ongoing, National Heart, Lung, and Blood Institute–funded prospective longitudinal cohort study of women with breast cancer recruited from the Rena Rowan Breast Cancer Center at the Abramson Cancer Center at the University of Pennsylvania (Philadelphia, PA). The primary inclusion criteria were women at least 18 years of age diagnosed with breast cancer and prescribed doxorubicin or trastuzumab therapy. The only exclusion criterion was pregnancy. Cases and controls all received doxorubicin (240 mg/m²) and cyclophosphamide followed by paclitaxel and trastuzumab, the latter as per standard prescribing algorithms.

At baseline, before initiation of chemotherapy, and at each follow-up visit, each participant provided detailed clinical data via standardized questionnaires. Clinical data were verified via review of medical records. Blood samples were obtained at baseline, during doxorubicin, after doxorubicin completion, and every 6 weeks during trastuzumab. Transthoracic echocardiograms were performed at standardized intervals, and participants underwent an echocardiogram at baseline, after doxorubicin completion, and every 3 months during trastuzumab therapy (Figure 1A). This study was approved by the University of Pennsylvania Institutional Review Board, and all participants provided written informed consent.

Transthoracic Echocardiography
Transthoracic echocardiograms were acquired by a dedicated sonographer team at an Intersocietal Accreditation Commission laboratory according to a specific protocol at baseline and standardized time intervals. Two-dimensional images were acquired using Vivid 7 or E9 machines (GE Healthcare, Milwaukee, WI).

Figure 1. Study cohort and experimental approach. A, Blood draw and echocardiography protocol for patients who were treated with doxorubicin and trastuzumab therapy. * denotes when transthoracic echocardiograms were performed. ‡ denotes when blood samples were collected. B, Strategy for proteomic discovery and validation of candidate biomarkers. Steps highlighted in orange were performed using longitudinal plasma samples (including baseline). Step highlighted in blue was performed using only baseline plasma samples. IgE indicates immunoglobulin E; and MS, mass spectrometry.
Echocardiograms were quantitated at the University of Pennsylvania Center for Quantitative Echocardiography (Philadelphia, PA). Quantification was performed using the Tomtec Cardiac Performance Analysis (Unterschleissheim, Germany). Apical 4-chamber left ventricular end-diastolic volume and end-systolic volume were calculated using the Simpson’s method of discs as recommended by the American Society of Echocardiography and were used to derive left ventricular EF.6

Identification of Cancer Therapy–Related Cardiac Dysfunction Cases and Controls

Of the participants who experienced CTRCD, as defined as cardiac dysfunction with a reduction in EF by ≥10% from baseline to an absolute value of <50% at any subsequent visit, 3 were selected for proteomics analyses. Cases also had to experience symptoms of heart failure as adjudicated by a cardiologist, and all were started on cardiac medications.

Controls were selected based on the lack of significant EF change during the entire duration of follow-up (<10% absolute change in EF, and EF>50%) and were matched to the cases based on specific selection criteria. These criteria for matching included age (±10 years), hormonal status, cancer stage, and race. In one instance, a patient meeting all of the criteria was not available. For this reason, 2 controls were selected for case 2 (Table 1).

Plasma Sample Collection, Time Points, and Processing

For all participants, blood was collected from venipuncture in the presence of EDTA, and this plasma was processed at 3353 rpm for 20 minutes, aliquoted, and stored at −80°C. Longitudinal plasma samples for each case and control were selected for the proteomics study to evaluate changes over time in the proteome between cases and controls. In total, 31 samples from 3 cases and 4 controls in the cohort of participants receiving doxorubicin followed by trastuzumab were selected for discovery analyses. Samples used in the discovery analysis were derived from the following time points: before any chemotherapy, during chemotherapy, at the time of CTRCD diagnosis, and after the CTRCD diagnosis (Figure 1A). Time points for the matched controls were selected to match the case specimens. Samples used in subsequent validation analyses were derived from baseline only.

Proteomics Discovery and Data Analysis

The 31 longitudinal case and control plasma samples used for discovery were processed using a 3-dimensional plasma proteome analysis strategy previously developed by the Speicher laboratory7 (Methods in the Online Data Supplement; Online Figure I). Briefly, 80 μL aliquots of plasma samples were depleted of 20 abundant plasma proteins using a ProteoPrep20 Immunodepletion Column (Sigma-Aldrich, St Louis, MO) and run for a short distance (2 cm) on 1D sodium dodecyl sulfate gels. Three lanes representing the immunodepleted fraction from 10 μL of original plasma were run for each sample, and each lane was sliced into twenty 1-mm slices. Corresponding slices for the triplicate lanes of each sample that represented a total of 30 μL original plasma were combined and digested with trypsin.

Each set of case/control samples was analyzed in a separate label-free liquid chromatography–mass spectrometry/mass spectrometry experiment. The first 2 case/control longitudinal sample sets were analyzed on an LTQ Orbitrap XL (Thermo Scientific, Waltham, MA) mass spectrometer, and the third case/control sample set was analyzed on a Q Exactive Plus mass spectrometer (Thermo Scientific). Both instruments were equipped with Nano-Acquity (Waters, Milford, MA) pumps and a column heater maintained at 40°C. Tryptic digests were injected onto a UPLC Symmetry trap column (180 μm ID×2 cm packed with 5 μm C18 resin; Waters), and peptides were separated by reverse phase high-pressure liquid chromatography on a BEH C18 nanocapillary analytic column (75 μm ID×25 cm, 1.7 μm particle size, Waters) at a flow rate of 200 nL/min. Solvent A was Milli-Q (Millipore, Billerica, MA) water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The 85-minute gradient used for samples analyzed on the Orbitrap XL was as previously described,7 while a slightly extended 95-minute gradient consisting of 5% to 30% B over 75 minutes, 30% to 80% B over 10 minutes, 80% B for 10 minutes, before returning to 5% B over 1 minute was used for analysis of samples on the Q Exactive Plus. The high-pressure liquid chromatography, peak retention times, and mass spectrometer were carefully monitored throughout each experiment to ensure that performance was within tight tolerances to facilitate comparisons of liquid chromatography–mass spectrometry signals.

Raw mass spectrometric data were processed by MaxQuant software (Version 1.4.1.2) as previously described8,9 (Methods in the Online Data Supplement; Online Data Supplement). The 31 longitudinal sample sets were analyzed on an LTQ Orbitrap XL (Thermo Scientific, Waltham, MA) mass spectrometer, and the third case/control sample set was analyzed on a Q Exactive Plus mass spectrometer (Thermo Scientific). Both instruments were equipped with Nano-Acquity (Waters, Milford, MA) pumps and a column heater maintained at 40°C. Tryptic digests were injected onto a UPLC Symmetry trap column (180 μm ID×2 cm packed with 5 μm C18 resin; Waters), and peptides were separated by reverse phase high-pressure liquid chromatography on a BEH C18 nanocapillary analytic column (75 μm ID×25 cm, 1.7 μm particle size, Waters) at a flow rate of 200 nL/min. Solvent A was Milli-Q (Millipore, Billerica, MA) water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The 85-minute gradient used for samples analyzed on the Orbitrap XL was as previously described,7 while a slightly extended 95-minute gradient consisting of 5% to 30% B over 75 minutes, 30% to 80% B over 10 minutes, 80% B for 10 minutes, before returning to 5% B over 1 minute was used for analysis of samples on the Q Exactive Plus. The high-pressure liquid chromatography, peak retention times, and mass spectrometer were carefully monitored throughout each experiment to ensure that performance was within tight tolerances to facilitate comparisons of liquid chromatography–mass spectrometry signals.

Table 1. Clinical Characteristics of Case/Control Pairs Used in Discovery Proteomics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Race</th>
<th>Nodal Status</th>
<th>Hormone Status</th>
<th>Breast Cancer Side</th>
<th>Baseline EF, %</th>
<th>Nadir EF, %</th>
<th>CTRCD Timing, days</th>
<th>Cardiac Medication</th>
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</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>48</td>
<td>Cauc</td>
<td>N1</td>
<td>ER+</td>
<td>Right</td>
<td>57</td>
<td>37</td>
<td>238</td>
<td>Enalapril, Carvedilol</td>
</tr>
<tr>
<td>Ctrl 1</td>
<td>40</td>
<td>Cauc</td>
<td>N1</td>
<td>ER+</td>
<td>Right</td>
<td>63</td>
<td>55</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Case 2</td>
<td>46</td>
<td>Cauc</td>
<td>N3C</td>
<td>ER−</td>
<td>Right</td>
<td>53</td>
<td>42</td>
<td>226</td>
<td>Enalapril, Carvedilol</td>
</tr>
<tr>
<td>Ctrl 2A</td>
<td>43</td>
<td>Asian</td>
<td>N2</td>
<td>ER−</td>
<td>Left</td>
<td>58</td>
<td>53</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ctrl 2B</td>
<td>53</td>
<td>Cauc</td>
<td>N2</td>
<td>ER+</td>
<td>Left</td>
<td>50</td>
<td>45</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Case 3</td>
<td>43</td>
<td>Asian</td>
<td>N0</td>
<td>ER−</td>
<td>Right</td>
<td>53</td>
<td>38</td>
<td>167</td>
<td>Lisinopril, Carvedilol</td>
</tr>
<tr>
<td>Ctrl 3</td>
<td>36</td>
<td>Cauc</td>
<td>N0</td>
<td>ER−</td>
<td>Left</td>
<td>61</td>
<td>52</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

CTRCD indicates cancer therapeutics-related cardiac dysfunction; Ctrl, control; EF, ejection fraction; and ER, estrogen receptor.
in the Online Data Supplement). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE (Proteomics Identifications Database) partner repository, with the data set identifier PXD004058.

Validation of Findings

Luminex Assay
To verify our most promising proteomics discovery findings, singleplex immunoglobulin E (IgE) and multiplex IgG1, IgG2, IgG3, IgG4, IgA, and IgM Isotyping assays were performed using Luminex kits (Millipore) according to the manufacturer’s protocol (Figure 1B). The 31 longitudinal plasma samples from the 3 case/control pairs were diluted 1:50 or 1:16000 for IgE and Isotyping kits, respectively, and added in duplicate on 96-well plates. Multiplex assays (Millipore) for T-helper cells (Th1- and Th2-associated cytokines, interferon-γ, interleukin [IL]-4, IL-5, IL-6, IL-10, IL-13, GM-CSF [granulocyte-macrophage colony-stimulating factor], tumor necrosis factor-α, IL-2, IL-12, IL-1β, IL-7, IL-8, IL-17A, IL-21, IL-23, macrophage inflammatory protein [MIP]-1α, MIP-1β, MIP-3α, fractalkine, ITAC [IFN-inducible T-cell-chemoattractant]) were also performed to further evaluate changes in IgE-associated immune markers. Beads were read using a MAGPIX instrument (Millipore), and data were analyzed with Milliplex Analyst software (version 5.1; Methods in the Online Data Supplement).

Plasma Samples From Healthy Participants
To define the immunoglobulin levels among healthy participants, 13 plasma EDTA samples were obtained from healthy female donors at the University of Pennsylvania (n=5) and the Wistar Institute (n=8). Participants were without evidence of comorbid conditions, including cardiovascular or oncological disease. Plasma samples were processed and stored in an identical manner to the cancer cohort and tested on the platforms as detailed earlier.

Standard Colorimetric ELISA Assay on Baseline Samples From the Doxorubicin and Trastuzumab Cohort and Normal Healthy Participants
Sandwich ELISA assays (Affymetrix eBioscience, San Diego, CA) were used to measure human IgE, IgG1, and IgG4 plasma levels at baseline (before treatment) for the entire cohort of 35 participants who received doxorubicin and trastuzumab, plus 13 normal female plasma donors (Figure 1B; Methods in the Online Data Supplement). ELISA plates were coated with each respective primary anti-human monoclonal antibodies, and assays were performed according to the manufacturer’s instructions.

Statistical Methods
In 2 separate discovery analyses, we sought to identify diagnostic and predictive biomarkers. Diagnostic biomarkers were defined as those proteins exhibiting a significant change in protein level at the same time as the onset of CTRCD. Candidate diagnostic biomarkers were selected by initially considering rate of change for individual cases; specifically, the level of a given protein was significantly higher or lower (2-tailed Student t test P value <0.05 and fold change >1.5) at one or more pre-CTRCD time points compared with time points after diagnosis of CTRCD.

Predictive biomarkers were defined as those that exhibited overall significant differences between case and control at baseline or at all time points; that is, there was a consistent difference between case and control starting at baseline and persisting throughout the study. Significantly changed proteins were defined as having both >1.5-fold change between the average of all time points for each case and control pair and a Student t test P value < 0.05.

For Luminex validation studies, differences between case and control samples were calculated at baseline using a 2-tailed Student t test. For the ELISA data derived from the 35 baseline samples, the nonparametric Wilcoxon rank-sum test was used to compare baseline IgE levels from individuals in the doxorubicin/trastuzumab cohort who developed CTRCD with those individuals who did not develop CTRCD. Logistic regression models were then used to determine the associations between baseline levels of biomarkers and odds of CTRCD. Baseline IgE levels and IgE/IgG1 ratios were transformed on the log2 scale for these analyses. Area under the receiver operating characteristic curves were calculated to assess the discriminative ability of each biomarker. The biomarker cut point at which the optimal sensitivity and specificity could be achieved was also calculated. Statistical significance was set at P<0.05 for all analyses.

Results
Patient Characteristics of Discovery Cohort
Cases and controls selected for the proteomics discovery analyses had the following characteristics as detailed in Table 1. All participants received a regimen containing doxorubicin (240 mg/m²), cyclophosphamide, followed by paclitaxel and trastuzumab for 4 cycles, followed by 1 year of trastuzumab therapy. All participants also received radiotherapy. No participants had any history of cardiovascular disease or risk factors before cancer therapy.

In the cases, CTRCD occurred between 167 and 238 days. Moreover, cases all complained of heart failure symptoms, including dyspnea on exertion and fatigue, and were all started on cardiac medications after diagnosis, including angiotensin-converting enzyme-inhibitors and beta blockers. Controls did not have any evidence of significant or sustained declines in EF (ie, CTRCD) or symptoms of heart failure. Timeline plots of clinical assessment of cardiac function by EF and the relationship to analyzed plasma fractions are shown in Figure 2.

Proteomics Biomarker Discovery
As previously noted, case/control pairs 1 and 2 were analyzed using an Orbitrap XL mass spectrometer, whereas case/control 3 was analyzed on a Q Exactive Plus instrument when this newer, higher performance instrument became available. Approximately 862 proteins were identified from case/control 1 and 2 plasma proteomes, whereas analysis of case/control 3 resulted in the identification of 1360 proteins. The increased depth of analysis achieved in case/control 3 was seen as potentially valuable because most of the additional ≈500 proteins
Diagnostic biomarkers were expected to be those proteins that showed an increase or decrease in protein abundance specifically associated with the timeframe of CTRCD development. Surprisingly, although several proteins exhibited large changes in abundance over time, none of these protein changes were significantly associated with onset of CTRCD.

Predictive biomarkers were proteins that exhibited differences in the level between case and control either before treatment (baseline, at the time of first plasma collection) or at all time points for cases and controls (p<0.05). The 6 best scoring candidate predictive biomarkers are summarized in the heatmap of Figure 3A. Longitudinal trends were also evaluated for each patient analyzed in the discovery experiments. Interestingly, the 3 proteins with the largest overall case/control differences (Figure 3B) were either consistently lower in all case time points as compared with matched controls, that is, IgE, or higher in cases as compared with matched controls, that is, dopamine β-hydroxylase and cathepsin S. We focused on baseline IgE for validation because it showed the largest differences, from 5- to 58-fold, between cases and controls (Figure 3A).

Validation of Baseline IgE as a Biomarker of Cardiac Dysfunction

Our most promising biomarker, IgE, was identified in the proteome comparisons based on the epsilon chain C region of immunoglobulin. IgE is the lowest abundance immunoglobulin in plasma, with concentrations in the low ng/mL range. Importantly, unlike other immunoglobulins, IgE was not a target of the antibody column used to deplete abundant proteins. Both the heat maps and the longitudinal trend plots show that this protein was consistently low at all time points in all 3 cases in our discovery proteomics analyses (Figure 3A and 3B).

To verify the discovery results for IgE and to determine whether the observed lower IgE levels in cases were indicative of a general suppression of the immune system, we quantitated all immunoglobulin subtype levels using a multiplexed Luminex assay platform in the 3 cases and 4 matched controls. These results are displayed graphically in Figure 4, which details baseline immunoglobulin data only, and in Online Figure IIB, which includes data from all time points. There was no evidence of a general immunosuppression in cases either at baseline (Figure 4B) or throughout therapy in the longitudinal plasma samples (Online Figure IIB). Importantly, the IgE differences at baseline (Figure 4A) and the longitudinal trends observed in the proteome discovery experiments (Online Figure IIA) were verified using this independent Luminex assay. When baseline levels in cases and controls were compared, there was a highly significant difference between groups, with controls being higher (P=0.01). An additional interesting observation that arose from these initial validation experiments is that IgG4 levels were also low at baseline for all cases (Figure 4B). To further investigate the role of the immune system in doxorubicin and trastuzumab CTRCD, we also evaluated IgE-related Th1 and Th2 cytokine profiles for cases and controls. Interestingly, several IgE-related cytokines, such as IL4, IL5, IL17, and fractalkine, were also elevated in controls as compared with cases at baseline (Online Figure III).

Based on these results, a more comprehensive evaluation of IgE, IgG1, and IgG4 levels was performed by assaying the baseline plasma samples of all 35 participants in the doxorubicin/trastuzumab cohort (Table 2). Conventional colorimetric ELISA assays were used for this second set of validation experiments. The results demonstrated that baseline IgE levels were significantly higher (P=0.018) in participants who did not experience CTRCD (mean 498.8±401.0 ng/mL; median 389.3 ng/mL with range 60.5–1392.1) compared with those who suffered from CTRCD (mean 234.9±285.9 ng/mL; median 167 ng/mL with range 23.2–1059.2), suggesting a potential protective role for elevated IgE at baseline in cancer patients undergoing cardiotoxic therapy (Figure 5A). In this cohort, 9 of the 35 participants had a history of allergies or asthma or
were taking allergy medications. However, there was no relationship between allergy and baseline IgE levels, and the distribution of participants with a history of allergies or asthma was similar across participants with and without CTRCD.

We also analyzed samples from a cohort of healthy female volunteers without asthma or allergies and without evidence of any comorbidity, including cardiovascular disease or cancer, to determine IgE levels in normal individuals (mean 179.6±231.7 ng/mL; median 97.0 ng/mL [range 36.9–853.8]). This comparison did not reveal any significant difference between cases who developed CTRCD with normal individuals (P=0.99). In contrast, controls who did not experience CTRCD were significantly higher in comparison to noncancer, healthy female volunteers (P=0.007). These results suggest that cancer patients who did not experience CTRCD had elevated IgE levels at baseline that may offer a cardioprotective benefit.

IgG1 and IgG4 levels were not significantly different between individuals with or without CTRCD, nor was the ratio between IgE and IgG4 (P>0.05 for all; Figure 5B). However, when the ratio of IgE/IgG1 was evaluated, the differences in patients who experienced CTRCD compared with those who did not were most pronounced (P=0.008). Similarly, logistic regression models of IgE and the IgE/IgG1 ratio demonstrated a significant association with high IgE levels and a decreased probability of cardiac dysfunction. Each doubling of IgE was associated with an odds ratio of 0.52 (95%
confidence interval 0.31, 0.90; \( P = 0.018 \)) for the development of CTRCD. For the ratio of IgE/IgG1, each doubling was associated with an odds ratio of 0.63 (95% confidence interval 0.43, 0.92; \( P = 0.017 \)). Moreover, area under the receiver operating characteristics curve analyses suggest strong discriminative ability with IgE and IgE/IgG1, with areas under the receiver operating characteristic curves of 0.73 and 0.76, respectively (Figure 6). We also evaluated the sensitivities and specificities at various cut points of IgE and IgE/IgG1. For IgE, a measure of 188.5 ng/mL had a combined sensitivity of 69% and specificity of 68%. The IgE/IgG1 ratio at a cut point of \( 2.05 \times 10^{-5} \) demonstrated the highest combined sensitivity of 75% and specificity of 74%.

Overall, these findings suggest that elevated IgE or IgE/IgG1 levels before doxorubicin and trastuzumab therapy may have a protective effect against cardiac dysfunction in patients undergoing this therapy.

**Discussion**

In this study, we used state-of-the-art proteomic profiling techniques to uncover potential new biomarkers for doxorubicin and trastuzumab CTRCD. Using a carefully phenotyped cohort of breast cancer patients, we discovered differences in baseline circulating IgE levels between patients who have doxorubicin- and trastuzumab-induced cardiac dysfunction compared with those who do not. These novel findings present a new paradigm in CTRCD and implicate a specific, understudied component of the immune system as a potential mediator of doxorubicin- and trastuzumab-induced cardiac dysfunction.

Few studies have quantitated circulating IgE levels in humans, and the results have often been conflicting. Some have noted differences according to demographics, with decreases in IgE with age and increased levels in males,\(^{15}\) and associations between IgE-associated markers and an increased risk of breast cancer, as compared with controls.\(^{16}\) The main established roles
of IgE are in the defense against parasitic diseases and pathogenesis of allergic diseases. Upstream, control of IgE expression occurs primarily through 2 subtypes of CD4+ T-helper cells: Th1 and Th2. Th1 and Th2 have counter-regulatory effects because cytokines secreted by Th1 cells, such as interferon-γ inhibit Th2 cytokine production and vice versa. Th2 cells secrete several interleukins, including IL-4, IL-5, IL-6, IL-10, and IL-13. IL-4 and IL-13 are important for B-cell isotype switching to IgE and IL-4-dependent IgE synthesis. Downstream, mast cells are hematopoietic inflammatory cells that are stimulated by IgE.

We postulate that IgE levels may represent some type of dysregulation between Th1 and Th2. In an exploratory analysis of T-helper cell cytokines and chemokines, several Th2 cytokines also showed trends where baseline levels were higher in controls compared with cases who developed CTRCD (Online Figure III). Patients with high IgE levels at baseline may have a more Th-2-skewed T cell subset composition or a tendency to make a greater Th-2 response.

Of note, a large body of literature supports a role for the immune system in maintaining myocardial homeostasis in heart failure. Immune mediators influence myocyte hypertrophy, fetal gene expression, and myocyte loss through apoptosis, and patients with heart failure have been reported to have higher levels of Th1, as opposed to Th2-associated cytokines. However, dysregulation of the inflammatory response has been shown to be both detrimental as well as beneficial to cardiac repair in response to various stressors, emphasizing the dual nature of the inflammatory system. In animal models, experimental induction of the Th1 response in mice led to worse left ventricular stiffness, dilation, and fibrosis. Moreover, mast cell–deficient mice, presumably indicative of an impaired acute inflammatory response, demonstrated improved survival and less myocardial necrosis in the setting of viral myocarditis. Conversely, in radiation-induced cardiac injury, emerging data

Table 2. Clinical Characteristics of Validation Cohort (N=35)

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>N (%) or Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45 (39, 57)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>21 (60)</td>
</tr>
<tr>
<td>Black</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Other or unknown</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Breast cancer side</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>16 (46)</td>
</tr>
<tr>
<td>Right</td>
<td>14 (40)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>5 (14)</td>
</tr>
<tr>
<td>Breast cancer nodal status</td>
<td></td>
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<tr>
<td>N0</td>
<td>15 (43)</td>
</tr>
<tr>
<td>N1</td>
<td>13 (37)</td>
</tr>
<tr>
<td>N2</td>
<td>5 (14)</td>
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<tr>
<td>N3</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Breast cancer hormonal status</td>
<td></td>
</tr>
<tr>
<td>ER+</td>
<td>18 (51)</td>
</tr>
<tr>
<td>Left-sided radiotherapy</td>
<td>16 (46)</td>
</tr>
<tr>
<td>Baseline EF, %</td>
<td>55 (54, 59)</td>
</tr>
<tr>
<td>Cancer therapeutics–related cardiac dysfunction, %</td>
<td>18 (51)</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; and ER, estrogen receptor.

Figure 5. ELISA validation for baseline IgE, IgG4, IgG1 levels and ratios in the doxorubicin and trastuzumab cohort plasma.

A. Standard sandwich ELISA results for baseline (before treatment) plasma for all 35 participants in the doxorubicin/trastuzumab cohort.

B. Ratios of the immunoglobulins assayed. No CTRCD indicates participants treated with doxorubicin and trastuzumab who did not develop cancer therapy–related cardiac dysfunction. CTRCD indicates participants treated with doxorubicin and trastuzumab who were diagnosed with cancer therapeutics–related cardiac dysfunction. Wilcoxon rank-sum test was used, and corresponding P values are reported. In each graph, the bars represent the median and interquartile range. The dotted line is the threshold observed, which reaches the highest specificity and sensitivity for IgE or IgE/IgG1. Ig indicates immunoglobulin.
also support a protective role of the immune system in the cardiovascular system. Here, mast cell–deficient mice demonstrated worse cardiac injury when exposed to radiation as compared with controls, suggesting that the absence of mast cells results in worsened cardiac remodeling in this setting. Although IgE levels in this setting are unknown, it is biologically plausible that mast cell–deficient mice also have low IgE levels. Our data strongly suggest the need for further study of IgE and the immune system in the pathogenesis of cancer therapy–induced cardiac dysfunction, and in particular, IgE-deficient animal models could shed important insight into this area.

Although initially counterintuitive, it is tempting to speculate that high IgE levels in cancer patients, before exposure to cardiotoxic therapies, results in a beneficial, protective response, with improvement of cardiac myocyte survival and stabilization of cardiac function with exposure to various stressors, including doxorubicin, trastuzumab, or possibly even chest radiation therapy. It is, thus, also possible that low circulating IgE levels in the cancer setting result in or reflect a lack of the ability of the cardiovascular system to appropriately compensate in response to cardiac stress and injury induced by these therapies. It is interesting to note that the breast cancer patients who are at increased risk of developing cardiac dysfunction had IgE levels similar to healthy donors and would suggest that elevation of IgE through unknown mechanisms results in cardioprotection and possibly greater cardiac reserve. Perhaps, patients with low IgE levels may also have a more susceptible, anergic state. Clearly, additional research is needed to characterize the potential role of IgE in cancer therapy–associated cardiac dysfunction in greater detail, including the time course and duration of IgE-associated immune system activation and subsequent downstream mediators.

For the specific application of assessing risk of cardiac dysfunction in response to breast cancer therapies, the current results suggest that IgE and IgG1 levels could be measured before initiation of the therapeutic regimen as a method for stratifying patients for cardiac dysfunction risk. Those participants with low IgE and low IgE/IgG1 ratios seem to be at higher risk of developing cardiac dysfunction and might be more closely monitored for development of cardiac dysfunction. Further studies are needed to determine whether baseline or longitudinal monitoring of IgE, IgG1, and possibly other Ig subtypes could confer any benefit in identifying risk or onset of cardiac dysfunction.

We note that using powerful proteomic technology, we identified at least 2 additional interesting candidate markers, dopamine β-hydroxylase and cathepsin S. Given the lack of robust high throughput assays for verification of these proteins, we were unable to validate these findings. However, future studies will focus on the development of appropriate assays for further evaluating these candidate biomarkers.

Although the identification of baseline IgE and IgE/IgG1 ratios as predictive biomarkers of risk for CTRCD in breast cancer patients receiving doxorubicin and trastuzumab is intriguing, these findings need to be validated in additional patient populations, and mechanisms need to be further elucidated in basic studies. Similarly, the potential predictive value of these biomarkers for other cardiotoxic therapeutic regimens must be evaluated because it remains unclear if these findings represent a susceptibility in response to exposure to doxorubicin, trastuzumab—a humanized monoclonal IgG antibody, or possibly even radiation therapy. In summary, although IgE has not previously been directly implicated in CTRCD, our findings suggest that it may be indicative of a previously unknown mechanistic connection with cardiac dysfunction in breast cancer patients undergoing therapy with doxorubicin and trastuzumab.

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**Disclosures**

Dr Ky is a consultant for Roche Diagnostics. The other authors report no conflicts.
Anthracyclines, such as doxorubicin, when used in combination with trastuzumab are two commonly used breast cancer therapies that have led to improvements in cancer survival, but carry a significant risk of cardiac dysfunction. Early identification of subclinical cardiac dysfunction would enable the initiation of cardioprotective strategies, prevent the interruption or discontinuation of necessary cancer therapy, and reduce the development of potential subsequent symptomatic heart failure. Current proteomic-based technologies can be used to successfully discover and validate new biomarkers, and although there are several existing biomarkers for cardiac failure in other settings, there is no prior research in the area of blood biomarkers for doxorubicin and trastuzumab.

What New Information Does This Article Contribute?

- Baseline IgE or the IgE/IgG1 ratio may be promising new biomarkers in doxorubicin- and trastuzumab-induced cardiac dysfunction.
- The immune system is a potential mediator of cardiac dysfunction after such therapy.
Baseline Immunoglobulin E Levels as a Marker of Doxorubicin- and Trastuzumab-Associated Cardiac Dysfunction
Lynn A. Beer, Andrew V. Kossenkov, Qin Liu, Eline Luning Prak, Susan Domchek, David W. Speicher and Bonnie Ky

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Proteomics Discovery

The 31 case and control plasma samples were processed using a 3-dimensional plasma proteome analysis strategy previously developed by the Speicher laboratory (Supplemental Figure I). Specifically, 80 µl aliquots of plasma samples were depleted of 20 abundant plasma proteins using a ProteoPrep20 Immunodepletion Column (Sigma-Aldrich, St. Louis, MO), a highly specific antibody column that removes approximately 95% of total plasma protein, enabling higher plasma volumes to be loaded onto a gel for downstream analyses. Plasma samples were filtered through a 0.22 µm microcentrifuge filter and injected onto the immunodepletion column. The flow-through fractions containing unbound proteins were collected, pooled, and precipitated overnight with 200 proof ethanol that was prechilled to -20°C. Ethanol supernatants were carefully removed and protein pellets were frozen and stored at -20°C. Frozen protein pellets were thawed briefly, resuspended in SDS-PAGE sample buffer, reduced and alkylated, and run for a short distance (2 cm) on 1-D SDS gels. Three lanes representing the immunodepleted fraction from 10 µl of original plasma were run for each sample, and each lane was sliced into twenty 1-mm slices. Corresponding slices for the triplicate lanes of each sample that represented a total of 30 µl original plasma were combined and digested with trypsin.

Each set of case/control samples was analyzed in a separate label-free LC-MS/MS experiment. The first two case/control longitudinal sample sets were analyzed on an LTQ Orbitrap XL (Thermo Scientific, Waltham, MA) mass spectrometer and the third case/control sample set was analyzed on a Q Exactive Plus mass spectrometer (Thermo Scientific). Both instruments were equipped with Nano-Acquity (Waters, Milford, MA) pumps and a column heater maintained at 40°C. Tryptic digests were injected onto a UPLC Symmetry trap column (180 µm i.d. x 2 cm packed with 5 µm C18 resin; Waters), and peptides were separated by reverse phase-high pressure liquid chromatography (RP-HPLC) on a BEH C18 nanocapillary analytical column (75µm i.d. x 25 cm, 1.7 µm particle size, Waters) at a flow rate of 200 nL/min. Solvent A was Milli-Q (Millipore, Billerica, MA) water containing 0.1% formic acid, and Solvent B was acetonitrile containing 0.1% formic acid. The 85 minute gradient used for samples analyzed on the Orbitrap XL was as previously described; i.e., peptides were eluted at 200 nL/min using an ACN gradient consisting of 5–28% B over 42 min, 28–50% B over 25.5 min, 50–80% B over 5 min, 80% B for 4.5 min before returning to 5% B over 0.5 min. The column was re-equilibrated using 5% B at 400 nl/min for 20 min before injecting the next sample. A slightly extended 95 minute gradient consisting of 5-30% B over 75 min, 30-80% B over 10 min, 80%B for 10 min, before returning to 5% B over 1 min was used for analysis of samples on the Q Exactive Plus. The HPLC, peak retention times, and mass spectrometer were carefully monitored throughout each experiment to ensure that performance was within tight tolerances in order to facilitate comparisons of LC-MS signals.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD004058.

Data Processing and Analysis
Raw mass spectrometric data were processed by MaxQuant software (Ver. 1.4.1.2)\(^3\) and the "match between runs" option was enabled to match identifications across samples.\(^5\) Peak lists were searched against the human Uniprot database with a full tryptic constraint using the Andromeda search engine.\(^6\) Carbamidomethyl cysteine was set as a fixed modification and methionine oxidation and N-terminus acetylation were set as variable modifications. Common expected contaminants including keratins and trypsin and a decoy database, produced by reversing the sequence of each protein, were appended to the forward database. Criteria for high confidence peptide/protein identifications included a false discovery rate (FDR) set to 1% for proteins and peptides, and removal of proteins identified by a single peptide. Relative abundance of each protein across all samples in an experiment was determined using the label-free quantitation option of MaxQuant.\(^3\) The software sums intensity of each full MS scan across all identified peptide peaks associated with a given protein. Protein identifications were filtered using Perseus software (http://www.perseus-framework.org) to remove reverse hits, contaminants, and proteins identified only by modified peptides or low confidence single peptide identifications. In addition, prior to statistical analysis, Perseus was used impute missing data points by creating a Gaussian distribution of random numbers to simulate the distribution of low signal values (imputation width=0.3, shift=1.8).\(^7\)

Due to different instruments and slightly different gradients used while analyzing cases/controls 1and 2 (Orbitrap XL, 85 minute gradient) and case/control 3 (Q Exactive Plus, 95 minute gradient), we were unable to analyze the entire proteomes from all three case and control pairs in a single quantitative MaxQuant comparison. Hence, we analyzed each case/control pair individually and combined the results for interpretation and comparison. Specifically, identical protein groups across experiments were matched by their Uniprot accession numbers, and statistical tests, further described below, were performed on normalized and logarithmic intensities within each case and control pair. Heat maps of protein intensity z-scores and longitudinal trends were used to select the best markers which displayed increased or decreased protein levels between cases and controls.

ELISA Assays

Luminex

To verify our most promising proteomics discovery findings, singleplex IgE and multiplex IgG1, IgG2, IgG3, IgG4, IgA and IgM Isotyping assays were performed using Luminex kits (Millipore) according to the manufacturer’s protocol. In general, 96-well plates were washed with 50µl assay buffer. All washes were removed with gentle blotting on paper towels. Next, the 31 longitudinal plasma samples from the 3 case/control pairs were diluted 1:50 or 1:16,000 for IgE and Isotyping kits, respectively, and 50µl of each standard, control, or plasma sample was added in duplicate on the plates. Next, 25 µL of pre-mixed antibody-immobilized beads was added to each well and plates were incubated with agitation on a shaker on for 1 h at room temperature. After incubation, liquid was removed and the plate was washed twice with 200 µL of wash buffer. Following washes, 25 µL of anti-human kappa and lambda- phycoerythrin was added into each well and incubated for 1hr at room temperature. Finally, the liquid was removed and 150 µL of sheath fluid was added to each well. The beads were resuspended on a shaker for 1 min and read on using a MAGPIX instrument (Millipore) and data were analyzed with Milliplex Analyst software (version 5.1). The instrument was programmed to collect at least 50 beads per analyte and raw data were measured as mean fluorescence intensity (MFI).

The high sensitivity T-cell 21-plex panel (Millipore) was also used to evaluate T-helper cell cytokines and chemokines (e.g. IFN\(\gamma\), IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, TNF-\(\alpha\), IL-2,
IL-12, IL-1β, IL-7, IL-8, IL-17A, IL-21, IL-23, MIP-1α, MIP-1β, MIP-3α, Fractalkine, ITAC). 96-well plates were first washed with 200 µl of assay buffer and 50 µl of each standard and control were added to their respective wells. Next, 25 µl of the longitudinal plasma samples from the three case/control pairs was added in duplicate to sample wells containing 25 µl of assay buffer. 25 µL of pre-mixed antibody-immobilized magnetic beads were added to each well followed by overnight incubation at 4°C. Plates were washed three times with 200 µl of wash buffer and 50 µl of detection antibodies were added per well and incubated for 1hr at RT. 50 µl of streptavidin-phycoerythrin was added to the wells and incubated for an additional 30 min. Finally, plates were washed three times with 200 µl wash buffer, 150 µL of sheath fluid was added to each well, and the resuspended beads were read on a MAGPIX instrument, as described above.

**Standard Colorimetric ELISA Assay**

Sandwich ELISA assays (Affymetrix eBioscience, San Diego, CA) were used to measure human IgE, IgG1 and IgG4 plasma levels at baseline (prior to treatment) for the entire cohort of 35 patients who received doxorubicin and trastuzumab, plus 13 normal female plasma donors (Figure 1B). 96-well ELISA plates were coated with each respective primary anti-human monoclonal antibodies diluted in PBS and incubated overnight at 4°C followed by overnight blocking with PBS containing 0.5% Tween 20 and 5% BSA. Plates were washed twice with PBS, 0.05% Tween 20 after blocking and each subsequent step. Plasma samples were diluted 1:10 (IgE), 1:2000 (IgG1), or 1:1000 (IgG4) in PBS containing 0.1% Tween 20 and 1% BSA and were added in triplicate to the plates and incubated for 2 hrs at RT. Horseradish peroxidase (HRP)-conjugated secondary anti-human monoclonal antibody was added to plates and incubated for 1 hr at RT. The plates were then developed by adding tetra 3,30,5,50-tetramethylbenzidine (TMB) substrate and incubated for 20 min at RT. Finally, the reaction was stopped by adding 2N sulfuric acid and plates were read at 450 nm.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. Proteomics Discovery Scheme. Experimental protocol for 3-D label-free quantitative biomarker discovery. “Top-20” depletion followed by SDS-PAGE and LC-MS/MS were used to compare longitudinal plasma samples from patients treated with doxorubicin and trastuzumab who were diagnosed with cancer therapeutics-related cardiac dysfunction (Cases) with their matched controls. MaxQuant label-free quantitation software was used to identify changes between the two groups.

Supplemental Figure II. Longitudinal Plots of Immunoglobulin Subtypes Using Luminex Assays. Longitudinal trends of IgE (A.) and other Ig subtypes (B.) measured in 31 samples from the discovery case (red) and control (blue) pairs. Timepoints for each analyzed plasma draw are noted by symbols.

Supplemental Figure III. Baseline Levels of T-helper Cell Cytokines and Chemokines Using Luminex Assays. A. Baseline measurements of the 21 analytes measured in the 3 case and 4 control samples from the proteomics discovery. Two-tailed Student's t-test p-value and fold change between averages are reported; heat map indicates highest values in red, lowest values in blue, and mid-range values in white for each analyte; zeroes indicate sample was below the detection limit of the assay; fold change >2.0 highlighted in green; *= because all case values were zero, average control value is reported for IL2. B. Histograms for select analytes from panel A having p value <0.25 and fold change >2.0.
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SUPPLEMENTAL REFERENCES


