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# Breast cancer diagnosis is associated with relative left ventricular hypertrophy and elevated endothelin-1 signaling

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## Abstract

**Background:** The survival rates of women with breast cancer have improved significantly over the last four decades due to advances in breast cancer early diagnosis and therapy. However, breast cancer survivors have an increased risk of cardiovascular complications following chemotherapy. While this increased risk of later occurring structural cardiac remodeling and/or dysfunction has largely been attributed to the cardiotoxic effects of breast cancer therapies, the effect of the breast tumor itself on the heart prior to cancer treatment has been largely overlooked. Thus, the objectives of this study were to assess the cardiac phenotype in breast cancer patients prior to cancer chemotherapy and to determine the effects of human breast cancer cells on cardiomyocytes.

**Methods:** We investigated left ventricular (LV) function and structure using cardiac magnetic resonance imaging in women with breast cancer prior to systemic therapy and a control cohort of women with comparable baseline factors. In addition, we explored how breast cancer cells communicate with the cardiomyocytes using cultured human cardiac and breast cancer cells.

**Results:** Our results indicate that even prior to full cancer treatment, breast cancer patients already exhibit relative LV hypertrophy (LVH). We further demonstrate that breast cancer cells likely contribute to cardiomyocyte hypertrophy through the secretion of soluble factors and that at least one of these factors is endothelin-1.

**Conclusion:** Overall, the findings of this study suggest that breast cancer cells play a greater role in inducing structural cardiac remodeling than previously appreciated and that tumor-derived endothelin-1 may play a pivotal role in this process.

**Keywords:** Breast cancer, Endothelin-1, Cardiac hypertrophy

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## Background

Breast cancer survival rates have improved over the last four decades largely as the result of advances in screening leading to earlier diagnosis and improved anti-cancer therapy [1, 2]. Since cytotoxic chemotherapies continue to be central in the treatment of breast cancer and patients generally live longer after the diagnosis and treatment of their cancer, the short- and long-term effects of these therapies on the rest of the body are becoming a focus of research [3–5]. A well-characterized and potentially lethal side effect of many breast cancer chemotherapies is cardiotoxicity [3–5]. As such, considerable research effort has focussed on reducing this cardiotoxicity as these treatments are key to many curative intent regimens [3–6]. Based on the well-established cardiotoxic effects of many chemotherapies used in breast cancer treatment, the prevailing theory is that the drugs trigger a dose-dependent left ventricular (LV) remodeling that can, in the worst cases, progress to heart failure and death [3–5]. Given that monitoring of cardiac structure/function in women diagnosed with breast cancer is often not initiated until after the initiation of chemotherapy, it is currently unknown if the breast tumor itself also contributes to cardiac remodeling independently from the chemotherapy.

Breast tumor cells produce a number of soluble factors that regulate the crosstalk between cancer cell subpopulations within the breast tumor [7]. Most of these factors are not only crucial for the proliferation and progression of the tumor but also for the metastasis of breast cancer cells [8]. Among these secreted factors, endothelin-1 (ET-1) has garnered attention as a vital factor for the growth, progression and metastasis of breast cancer cells [9]. ET-1 (a 21-amino acid peptide) is produced by breast cancer cells via proteolytic cleavage of a large biological precursor molecule, big ET-1 (a 38-amino acid peptide), which is mediated by endothelin converting enzyme (ECE) [10–12]. In addition to the effect of ET-1 on cancer progression [9], ET-1 is known to contribute to other cardiovascular complications in non-cancer conditions including hypertension, left ventricular hypertrophy (LVH) and heart failure [13–16]. In agreement with this, the circulating levels of ET-1 are increased in patients with cardiac diseases as well as in animal models of cardiac hypertrophy [17–19]. In addition, inhibition of ET-1 signaling pathway reduces LVH and improves heart function in several models of heart failure suggesting a crucial role of ET-1 signaling in cardiac remodeling [19–21]. Thus, regardless of the role that ET-1 plays in breast tumor progression, there is potential that elevating breast cancer-derived ET-1 levels may contribute to breast tumor-induced LVH.

Based on the above information, the purpose of this study is to characterize LV structure and function in

patients with breast cancer prior to receipt of cancer therapy. In addition, we aimed to investigate how breast cancer tumors communicate with the cardiomyocyte to induce cardiac remodeling and examine if ET-1 signaling could be the mechanism to explain LVH in breast cancer patients.

## Methods

### Materials

Human cell lines were purchased from American Type Cell Culture ((ATCC), Manassas, VA). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA), or Thermo Fisher Scientific (Waltham, MA), and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were developed using Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). ET-1 and big ET-1 assay kits were purchased from R&D system (Minneapolis, MN).

### Retrospective cohort analysis

This retrospective investigation involved 28 female patients with newly diagnosed breast cancer recruited from a tertiary care cancer hospital approximately 45 days after surgery and prior to any systemic therapy for cancer (Cross Cancer Institute, Edmonton, Alberta, Canada). A detailed medical history was recorded at the time of recruitment and those with hypertension, diabetes, or prior cancer treatments were excluded. All participants underwent anthropometric measurements (height, weight). Body mass index (BMI) and body surface area (BSA) were calculated for each patient. Blood pressure values were obtained on the same day that the non-contrast cardiac magnetic resonance imaging (MRI) scan examination was performed. A control cohort of women with comparable baseline factors including age, BMI, BSA and health history ( $n = 17$ ) were included.

### MRI protocol

All participants underwent a non-contrast cardiac magnetic resonance imaging (MRI) scan on a 1.5 T magnet (Siemens Healthcare, Erlangen, Germany). Image acquisition included steady-state free precession cines for cardiac structure and function and image analysis was performed by a single user using commercial software (Syngo, Siemens Healthcare). Left ventricular (LV) volumes and mass were calculated from a short axis stack of cines using a method of disks approach and were indexed to body surface area. The papillary muscles were excluded from LV mass and included in LV volume measures.

### Blood sample collection

All participants also had venipunctures at the time of their scan and their blood samples were stored in a 4 °C fridge. Following approval from our institutional review board, individual samples were accessed and separated by centrifugation at 3000 rpm for 10 min at room temperature and the plasma was frozen at – 80 °C.

### Cell culture and treatment

Human breast cancer MCF7 cells (ATCC HTB-22), T47-D (ATCC HTB-133), ZR75–1 (ATCC CRL-1500), MDA-MB-231 (ATCC HTB-2) and human left ventricular cardiomyocyte RL-14 cells (ATCC PTA-1499) were used in this study. Cells were grown in 75 cm<sup>2</sup> tissue culture flasks at 37 °C under a 5% CO<sub>2</sub> humidified environment. Human left ventricular cardiomyocyte RL-14 cells were maintained in DMEM/F-12, with phenol red supplemented with 12.5% fetal bovine serum, 20 Ml-glutamine, 100 IU/ml penicillin G and 100 µg/ml streptomycin [22–24]. The breast cancer cell lines, MCF7 and T47-D are hormone receptor positive breast cancer phenotype [25, 26], whereas ZR75–1 is a hormone receptor positive and human epidermal growth factor receptor 2 (HER2) positive cell line [26]. In contrast, MDA-MB-231 is a triple negative breast cancer cells [25].

At 80% confluence, 8 ml of serum-free media (DMEM/F-12) was conditioned with breast cancer cells in T-75 flask (cell number count was ~ 6 × 10<sup>6</sup>) for 48 h, collected and centrifuged (500 xg, 5 min) [27]. Cardiomyocytes were washed twice with phosphate-buffered saline (PBS), and then incubated with 2 mL of breast cancer conditioned medium for 24 h with or without 1 µg/ml ET-1 monoclonal primary antibody (MA1–10818, Thermo Fisher Scientific) or 0.25 µM ET-1 receptor blocker, BQ-123 (ab141005, Abcam). Synthetic ET-1 (250 pM) (E7764, Sigma Aldrich) was used as a positive control. Control cardiomyocytes were treated with identical non-conditioned medium used for culturing the breast cancer cells. Cell culture-based experiments were independently replicated 4–6 times.

### Preparation of fractionated conditioned medium

Millipore Amicon Ultracel-30 K 15 ml tubes with 30 kDa filters (Fisher Scientific, Ottawa, Ontario, Canada) was used to fractionate the MCF7 breast cancer conditioned medium into 2 fractions, the lower filtrate containing proteins of less than 30 kDa and the upper concentrate containing proteins greater than 30 kDa as described previously [28].

### RNA extraction, cDNA synthesis and quantification of mRNA expression by quantitative real-time polymerase chain reaction (real time-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen®, Carlsbad, CA, USA) as described previously [29,

30]. Quantification of gene expression was performed by real time-PCR LightCycler® 480 white 384-well reaction plates in the LightCycler® 480 System (Roche Life Science), as described previously [29, 30].

### Cell surface area measurement

In order to measure cell surface area, cardiomyocytes were fixed with 4% paraformaldehyde, and stained with wheat-germ-agglutinin (WGA) conjugated with Alexa fluor 488, then mounted with ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). Individual cell surface area was measured using ImageJ (NIH).

### Immunoblot analysis

Cardiomyocyte lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting was performed as described previously [30]. Briefly, proteins from each treated group were diluted with 3x loading buffer (0.1 M Tris (hydroxymethyl) aminomethane (Tris)-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 1.5% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol), boiled and loaded onto a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 120 V for 2 h and separated proteins were transferred to Trans-Blot nitrocellulose membrane (0.45 µm) in a buffer containing 25 mM Tris–HCl, 192 mM glycine, and 20% (v/v) methanol. Nitrocellulose membranes from the protein transfer were blocked overnight at 4 °C in a solution containing 5% skim milk powder, 2% BSA and 0.5% Tween-20 in Tris-buffered saline (TBS) solution (0.15 M NaCl, 3 mM KCl, 25 mM Tris-base). After blocking, the membranes were washed 6 times for 1 h with TBS-Tween-20 before being incubated with a primary antibody (0.2 µg/ml) for 2 h at room temperature in TBS solution containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with peroxidase conjugated secondary antibody was carried out in blocking solution for 1 h at room temperature.

The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare, Mississauga, ON). Chemiluminescent detection was performed on FUJI medical X-ray film (# 4741019291, FUJIFILM Corporation, Japan) using OPTIMAX X-Ray Film Processor (# 11704 0–1411-1934, PROTEC GmbH & Co. KG, Germany). The images were then scanned using HP Scanjet 4890 photo scanner. The intensity of the protein band was semi-quantified relative to the signals obtained for β-ACTIN protein, using ImageJ® image processing program (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij>). Pre-stained protein ladder (Precision Plus Protein Kaleidoscope, Cat # 161–0375, BIO-RAD, CA, USA) was loaded, electrophoresed and

transferred to Trans-Blot nitrocellulose membrane (0.45  $\mu\text{m}$ ) along with the samples as described above. As our pre-stained protein ladder does not react with the anti-body, we used autofluorescence tape (Radtape Plus, Cat # RADP200, Diversified Biotech, Inc.) to mark the molecular weight of the protein ladder bands close to our targeted protein.

#### ET-1 and big ET-1 assays

ET-1 and big ET-1 concentrations in conditioned media of breast cancer cells and human plasma were determined by immunoassay (R & D Systems).

#### Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (version 7.01) (GraphPad Software, Inc., La Jolla, CA). The Kolmogorov–Smirnov test was used to assess the normality of distribution of each parameter. One-way analysis of variance (ANOVA) followed by Tukey–Kramer or Dunnett post hoc multiple comparison test, unpaired two-tailed *t*-test for normally distributed data or Mann–Whitney U test for non-normally distributed data were carried out to assess which treatment group(s) showed a significant difference from the control group. Multiple *t*-test comparisons were adjusted using Bonferroni method. The correlations between ET-1 or Big ET-1 levels and LV parameters were determined using Pearson's correlation coefficient. A probability value obtained less than 0.05 is considered significant.

## Results

### Demographic and clinical parameters in patients with breast cancer and their healthy controls

BSA, BMI and health history for the patients with breast cancer and a control cohort of women are shown in Table 1. While breast cancer patients demonstrated an increased heart rate, systolic and diastolic blood pressure values did not differ between the observed groups (Table 1).

### Patients with breast cancer exhibit relative LVH prior to chemotherapy

Cardiac MRI scans revealed that women with breast cancer prior to chemotherapy demonstrated signs of cardiac remodeling evidenced by the significantly increased indexed LV mass, indexed LV end-diastolic volume (LVEDV) as well as indexed LV end-systolic volume (LVESV) compared to the control cohort women (Fig. 1a, b, c). In contrast, there were no differences in LV ejection fraction (LVEF) or LV stroke volume (Fig. 1d, e) between the observed groups suggesting that patients with breast cancer can be characterized by a relative LVH with normal systolic function. While there were no differences in LVEF between breast cancer patients and the control cohort of women (Fig. 1d), 4 patients

demonstrated LVEF < 55% which might occur as result of different progressive levels of LVH due to breast cancer.

### Human breast cancer conditioned medium induces hypertrophy in human cardiomyocytes

Given that cancer was the only independent, overt clinical parameter differing between the observed groups (Table 1), we hypothesized that breast tumors cause direct detrimental alterations in cardiomyocytes that induce LVH. To test this, we incubated human LV cardiomyocytes with conditioned medium of human breast cancer MCF7 cells as described in Materials and Methods (Fig. 2a), then measured cardiomyocyte size, which is indicative of cardiomyocyte hypertrophy [31]. Of interest, we observed that conditioned medium from breast cancer MCF7 cells significantly increased cardiomyocyte size compared to cells without conditioned medium (Fig. 2b, c). The pro-hypertrophic effect of breast cancer conditioned medium was confirmed by the significantly increased pro-hypertrophic marker,  $\beta$ -myosin heavy chain ( $\beta$ -MHC) (Fig. 2d) and the significantly decreased phosphorylated eukaryotic elongation factor-2 (p-eEF2) (Fig. 2e, f and Supplementary Fig. 1A, B, C), which is indicative of elevated eEF2 activity and increased protein synthesis [31]. Together, our findings indicate that breast tumors may directly induce cardiomyocyte hypertrophy and suggest a breast cancer cell-released soluble factor may be responsible for this effect.

### Breast cancer cells secrete ET-1

To identify the breast cancer cell-released soluble factor that induces cardiomyocyte hypertrophy, we fractionated breast cancer conditioned medium utilizing Millipore Amicon Ultracel-30 K tube filtration and screened the elute fractions for inducing hypertrophy in human cardiomyocytes. Surprisingly, the hypertrophic effect was located in the fraction with molecular weight above 30 kDa (Fig. 2g, h), which is much larger than the size of humoral factors including pro-inflammatory cytokines and growth factors (Fig. 2g, h).

Since proteome analysis has already identified a number of soluble factors secreted from breast cancer conditioned medium [32–34], we used these findings to hypothesize that ET-1 is one of the candidate soluble factors that could contribute to the hypertrophic changes observed in cardiomyocytes treated with conditioned medium of human breast cancer cells. Indeed, ET-1 has direct detrimental effects on the cardiovascular system and it is known as a pro-hypertrophic factor [19, 21, 35]. Consistent with our hypothesis, the active fraction of MCF7 breast cancer conditioned medium was highly enriched with ET-1 (Fig. 2i).

**Table 1** Demographic and clinical parameters in patients with breast cancer and a control cohort of women with comparable baseline factors

	Healthy (n = 17)	Cancer (n = 28)	P value
Age, years (mean ± SD)	59 ± 10	53 ± 10	0.15
Height, cm (mean ± SD)	166 ± 8	164 ± 6	0.25
Weight, kg (mean ± SD)	75 ± 17	75 ± 19	0.89
Body Surface Area, m <sup>2</sup> (mean ± SD)	2.0 ± 0.2	1.8 ± 0.2	0.81
Ideal Body Surface area, m <sup>2</sup> (mean ± SD)	2.0 ± 0.1	1.6 ± 0.1	0.72
Body Mass Index, kg/ m <sup>2</sup> (mean ± SD)	27 ± 6	28 ± 7	0.26
Hypertension (%)	0	0	NA
Diabetes (%)	0	0	NA
Receptor Status, n (%)			
Estrogen receptor/Progesterone receptor	NA	26 (93%)	NA
Human epidermal growth factor receptor-2	NA	9 (32%)	NA
Triple negative	NA	1 (4%)	NA
Laterality of Cancer, n (%)			
Left	NA	12 (43%)	NA
Right	NA	14 (50%)	NA
Bilateral	NA	2 (7%)	NA
Pathologic Cancer Stage, n (%)			
1	NA	12 (43%)	NA
2	NA	13 (46%)	NA
3	NA	3 (11%)	NA
Heart Rate, beat per minute (mean ± SD)	69 ± 10	80 ± 13	0.006
Systolic Blood Pressure, mmHg (mean ± SD)	126 ± 15	130 ± 15	0.37
Diastolic Blood Pressure, mmHg (mean ± SD)	75 ± 8	72 ± 10	0.32

### Breast cancer conditioned medium induces hypertrophy in human cardiomyocytes through the ET-1 signaling pathway

To determine whether ET-1 is responsible for the pro-hypertrophic effect of breast cancer cells, we treated human ventricular cardiomyocytes with conditioned medium of breast cancer MCF7 cells with or without the inclusion of neutralizing antibody against ET-1. Interestingly, we found that the neutralizing antibody against ET-1 blocked breast cancer conditioned medium-induced increases in cardiomyocyte size and abolished the upregulation of the pro-hypertrophic marker,  $\beta$ -MHC (Fig. 3a, b, c). These data indicate that ET-1 is required for the pro-hypertrophic effect of breast cancer conditioned medium.

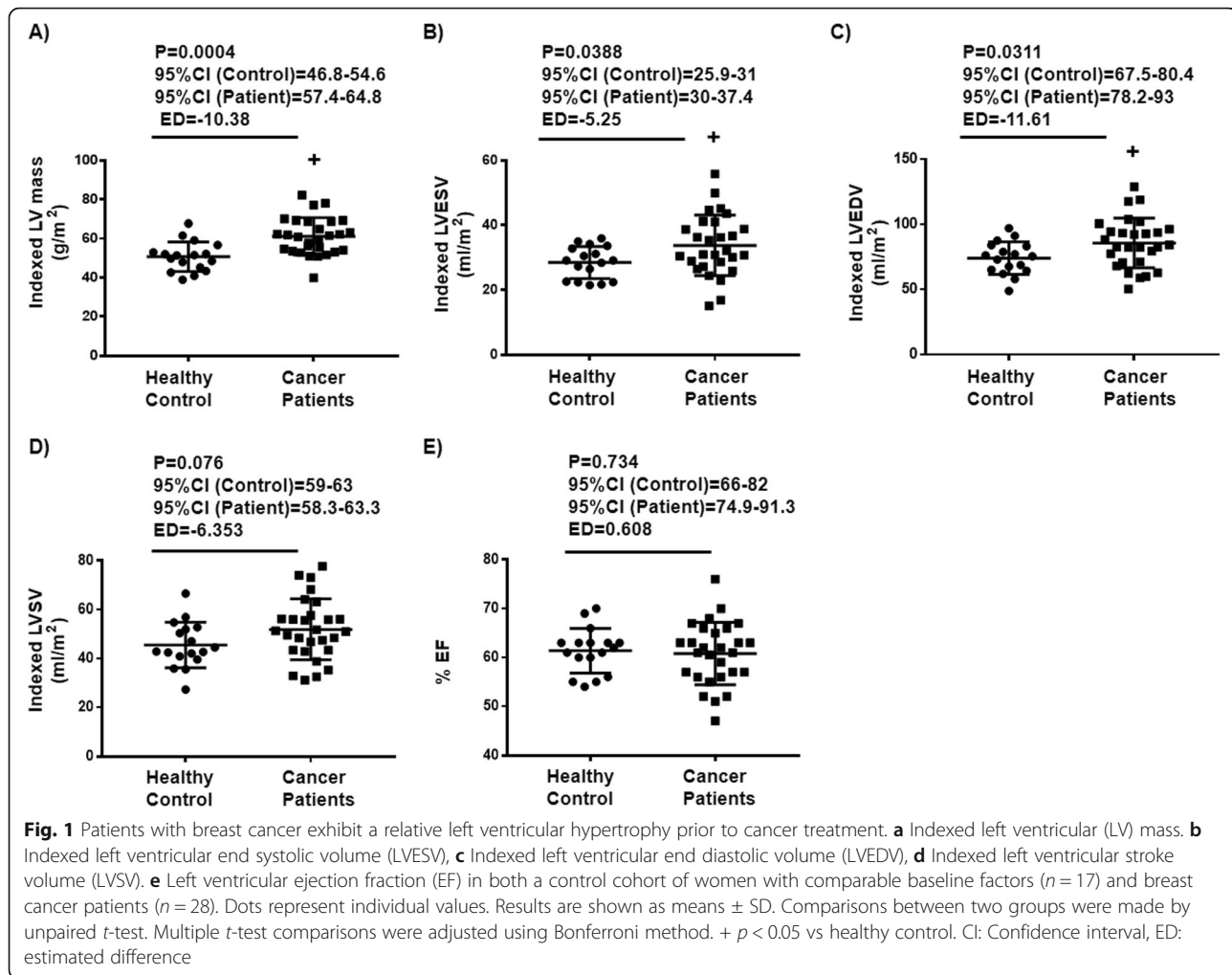
Because ET-1 induces cardiomyocyte hypertrophy through activation of ET type A receptor (ET<sub>A</sub> receptor) [21], we examined whether breast cancer conditioned medium-induced cardiomyocyte hypertrophy was dependent on the activation of ET<sub>A</sub> receptors. To do this, we incubated human ventricular cardiomyocytes

with conditioned medium of breast cancer MCF7 cells with or without the ET<sub>A</sub> receptor blocker, BQ-123. We observed that BQ-123 repressed breast cancer conditioned medium-induced increases in cardiomyocyte size (Fig. 3d, e). Overall, these findings indicate that conditioned medium of breast cancer MCF7 cells induce cardiomyocyte hypertrophy through an ET-1-dependent signaling pathway.

### Conditioned medium of other human breast cancer cells that secrete ET-1 also induce cardiomyocytes hypertrophy

We further examined whether the pro-hypertrophic effect observed in response to conditioned medium of breast cancer MCF-7 cells is also induced by conditioned medium of other human breast cancer cells that secrete ET-1, such as T47-D and ZR75-1 cells (Table 2) [34]. To do this, we incubated human ventricular cardiomyocytes with conditioned medium of breast cancer T47-D and ZR75-1 cells and compared these findings to the effects observed using synthetic ET-1 as a positive control. Our results showed that synthetic ET-1 and conditioned





medium of breast cancer T47-D and ZR75-1 cells significantly increased cardiomyocyte size and the pro-hypertrophic marker,  $\beta$ -MHC (Fig. 3f, g, h). In contrast, conditioned medium of other human breast cancer cells that do not secrete ET-1, such as MDA-MB-231 cells (Table 2) [34], did not induce cardiomyocyte hypertrophy (Fig. 3i, j). Our data are consistent with previous reports showing that other triple negative breast cancer cells such as BT549 and MDA-MB-435s had no secretion of ET-1, suggesting that triple negative breast cancer cells generally have no secretion of ET-1 [34]. Together, these data confirm that ET-1 is a soluble factor that induces the pro-hypertrophic changes of cardiomyocytes in response to conditioned medium of breast cancer cells that secrete ET-1.

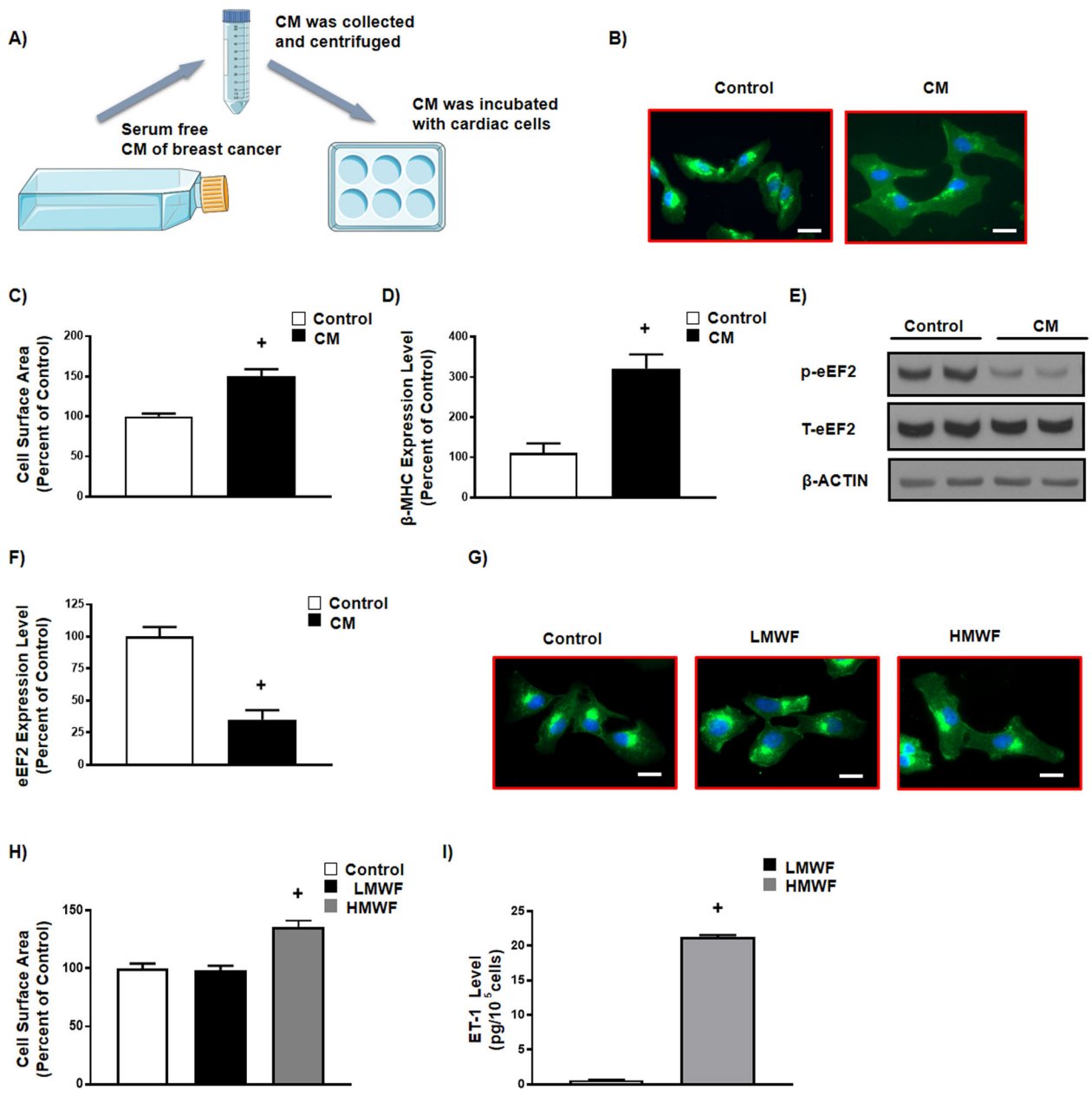
#### High circulating levels of ET-1 in breast cancer patients with a relative LVH

Since ET-1 is implicated as a crucial factor for the development of cardiomyocyte hypertrophy in response to

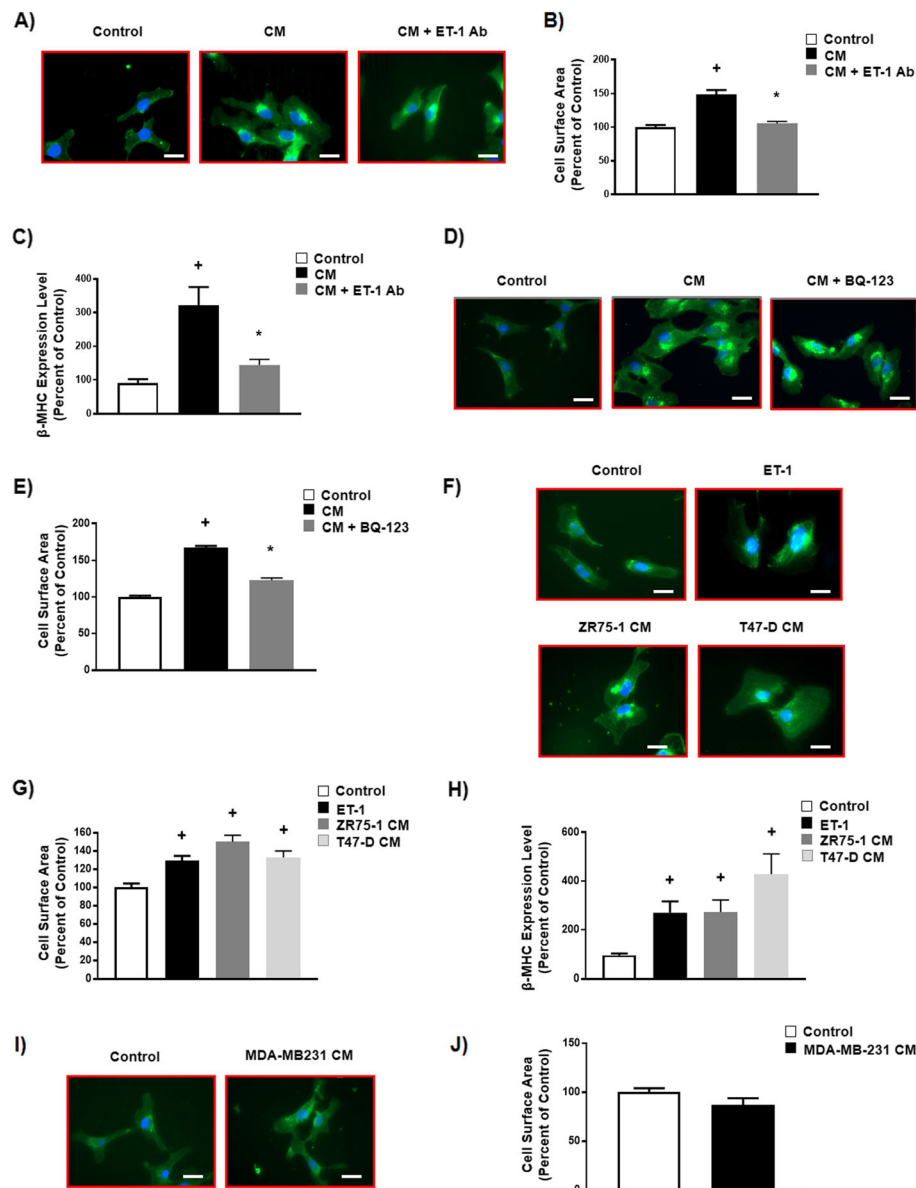
conditioned medium of certain breast cancer cells, we measured circulating ET-1 in our cohort of breast cancer patients and the control cohort of women. Of interest, we found that breast cancer patients demonstrated higher circulating level of ET-1 compared with the control cohorts, suggesting that ET-1 may contribute to a relative LVH in breast cancer patients (Fig. 4a). However, we could not find a significant correlation between ET-1 and a relative LVH in our breast cancer patients (Fig. 4b, c, d, e), which is not surprising since ET-1 has short half-life in the systemic circulation [20].

#### High circulating level of big ET-1 in breast cancer patients with a relative LVH

Given that big ET-1 is more stable than ET-1 [36] and that increased plasma levels of ET-1 are credited mainly to an increase in the level of big ET-1 [20], we measured big ET-1 in breast cancer patients with a relative LVH and controls. We found that our breast cancer patients with a relative LVH exhibited significantly higher



**Fig. 2** Human breast cancer conditioned medium induces hypertrophy in human left ventricular cardiomyocytes. **a** Schematic of conditioned medium (CM) treatment in human left ventricular cardiomyocytes. **b** Representative images of cardiomyocytes stained with WGA (green) and DAPI (blue), scale bar = 50  $\mu$ m. **c** Quantification of cell surface area in cardiomyocytes treated with either regular serum free medium or breast cancer MCF7 CM ( $n = 70$  per group). **d**  $\beta$ -myosin heavy chain ( $\beta$ -MHC) mRNA levels that were normalized to  $\beta$ -ACTIN in cardiomyocytes treated with either regular serum free medium or breast cancer MCF7 CM ( $n = 6$ ). **e** Lysates from cardiomyocytes were immunoblotted with antibodies against phosphorylated eukaryotic elongation factor 2 (p-eEF2), total-eEF2 and  $\beta$ -ACTIN, full blot is provided in supplementary file. **f** Quantification of protein expression levels in cardiomyocytes treated with either regular serum free medium or breast cancer MCF7 CM ( $n = 4$  per group). **g** Representative images of cardiomyocytes stained with WGA (green) and DAPI (blue), scale bar = 50  $\mu$ m. **h** Quantification of cell surface area in cardiomyocytes treated with either regular serum free medium or low molecular weight fractionated medium (LMWF, < 30 kDa) or high molecular weight fractionated medium (HMWF, > 30 kDa) ( $n = 70$  per group). **i** Quantification of endothelin-1 (ET-1) levels in cardiomyocytes treated with fractionated breast cancer MCF7 CM ( $n = 6$  per group). Results are shown as means  $\pm$  SEM. Comparisons between two groups were made by unpaired *t*-test, whereas comparisons between three groups were made by one-way ANOVA with a Tukey Kramer's post hoc multiple comparison test. +  $p < 0.05$  vs its own control group. Figure 2A was modified from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Generic License. <http://smart.servier.com/>



**Fig. 3** Breast cancer conditioned medium induces hypertrophy in human cardiomyocytes through endothelin-1 signaling pathway. **a** Representative images of cardiomyocytes stained with WGA (green) and DAPI (blue), scale bar = 50 μm, **b** Quantification of cell surface area in cardiomyocytes treated with either regular serum free medium or breast cancer MCF7 CM with or without the antibody against ET-1 (n = 70 per group). **c** β-MHC mRNA levels that were normalized to β-ACTIN in cardiomyocytes treated with either regular serum free medium or breast cancer MCF7 CM with or without antibodies against ET-1 (n = 6 per group), **d** Representative images of cardiomyocytes stained with WGA (green) and DAPI (blue), scale bar = 50 μm, **e** Quantification of cell surface area levels in cardiomyocytes treated with either regular serum free medium and breast cancer MCF7 CM with or without BQ-123 (n = 70 per group), **f** Cardiomyocytes were stained with WGA (green) and DAPI (blue), scale bar = 50 μm, **g** Quantification of cell surface area in cardiomyocytes treated with regular serum free medium, synthetic ET-1, breast cancer ZR75-1 CM or breast cancer T47D CM. **h** β-MHC mRNA levels that were normalized to β-ACTIN in cardiomyocytes treated with regular serum free medium, synthetic ET-1, breast cancer ZR75-1 CM or breast cancer T47D CM (n = 6 per group). **i** Representative images of cardiomyocytes stained with WGA (green) and DAPI (blue), scale bar = 50 μm, **j** Quantification of cell surface area in cardiomyocytes treated with regular serum free medium or triple negative breast cancer MDA-MB-231 CM. Results are shown as means ± SEM. Comparisons between two groups were made by unpaired t-test, whereas comparisons between three groups were made by one-way ANOVA with a Tukey Kramer's post hoc multiple comparison test. + p < 0.05 vs its own control group. \* p < 0.05 vs its CM

circulating levels of big ET-1 compared with control women (Fig. 4f), demonstrating that the endothelin system is activated in the breast cancer patients with a

relative LVH. Of interest, big ET-1 levels correlated positively with indexed LV mass (Fig. 4g) and LVESV (Fig. 4h) in the breast cancer patients. On the other



**Table 2** The level of endothelin-1 in conditioned medium of breast cancer cell lines

Cell Line	ET-1, pg/ml/10 <sup>5</sup> cells/48 h (mean ± SEM)
MCF7	21.24 ± 0.82
ZR75-1	126.03 ± 11.95
T47-D	254.62 ± 5.91
MDA-MB-231	Non-detected

hand, while a significant inverse relationship was obtained with LVEF (Fig. 4j), we could not find a significant correlation between big ET-1 and LVEDV (Fig. 4i). Together, these data suggest that cardiac remodeling in breast cancer patients can, in part, be attributed to the activation of endothelin system.

Notwithstanding the previous finding, while pT1 and pT2/3 stage patients demonstrated signs of cardiac remodeling evidenced by the significantly increased indexed LV mass (Fig. 5a), big ET-1 levels significantly increased and correlated positively with indexed LV mass in pT2/3 but not pT1 stage patients (Fig. 5b, c, d) suggesting that the activation of ET-1 signaling is more pronounced in advanced stages of breast cancer.

## Discussion

In an interesting and exciting new discovery, we show that even prior to systemic therapy for breast cancer, patients diagnosed with breast cancer exhibit LV remodeling characterized by relative hypertrophy. We also provide evidence suggesting that breast tumors communicate directly with cardiomyocytes to induce this phenotype. Our findings support the hypothesis that the tumor itself can have a detrimental effect on the heart [37]. This notion is important as breast cancer survivors are at high risk for cardiac disease [6, 38]. Considering the fact that risk stratification of cancer survivors is problematic [39], understanding the pathogenesis of the cardiovascular risk associated with a breast cancer diagnosis may improve the identification of susceptible patients.

Given the fact that; 1) patients diagnosed with breast cancer exhibit LV remodeling, and 2) conditioned medium of breast cancer cells induces hypertrophy in human cardiomyocytes, we postulated that breast tumor cells secrete soluble factors that cause detrimental alterations in important signaling pathways in cardiomyocytes that induce relative LVH. While breast cancer cells secrete a number of pro-inflammatory cytokines and growth factors that contribute to the progression and metastasis of cancer [32], our data show that none of these factors are implicated in the development of cardiomyocyte hypertrophy in response to breast tumor

cells. Thus, these findings indicate that the observed cardiomyocyte hypertrophy is not due to inflammation and/or the secretion of small molecules such as pro-inflammatory cytokines and growth factors, suggesting that the cardiomyocyte response is due to active peptides secreted by the breast cancer cells.

ET-1 is an active peptide that has garnered much attention over the last decade as an important factor for the progression and metastasis of breast cancer [33]. Interestingly, ET-1 is also known as a pro-hypertrophic factor [21] and its circulating level is increased in patients with LVH and heart failure [19, 35]. Thus, it is likely that ET-1 could be one of the candidate soluble factors that govern the hypertrophic changes of cardiomyocytes. Consistent with this notion, only conditioned medium of human breast cancer cells that secrete ET-1 were able to induce cardiomyocyte hypertrophy. In addition, this hypertrophy was abolished by either an ET-1 antibody or an ET-1 receptor blocker indicating that the induction of cardiomyocytes hypertrophy by conditioned medium from breast tumor cells is mediated through the ET-1 signaling pathway.

In order to examine whether the effect observed in the cell culture model holds true in the clinical setting, we measured the circulating levels of ET-1 in our breast cancer patients and their controls. While the circulating levels of ET-1 was higher in our breast cancer patients compared to control women, we could not find a significant correlation between LV mass and ET-1 within the patient group. The lack of a correlation might be attributed to the fact that ET-1 is unstable as it is degraded in the circulation or is rapidly taken up by the vasculature [20]. Thus, it is likely that measured levels of circulating ET-1 from a distal appendage might underestimate the amount of ET-1 located in closer proximity to the tumor. This is in agreement with the notion that absolute ET-1 levels in the blood may be an inaccurate indicator of endothelin system activation [40]. Nevertheless, the significantly increased circulating level of ET-1 in our breast cancer patients suggests that ET-1 contributes to LV remodeling in breast cancer patients [19].

Big ET-1 is more stable than ET-1 as it has a longer half-life and it is cleared slowly from the circulation [36, 41]. Thus, big ET-1 is considered to be a sensitive measure of endothelin system activation [41]. Given that the high circulating level of ET-1 is mainly attributed to the release of big ET-1 [36, 41], we measured big ET-1 in our breast cancer patients with relative LVH and healthy control women. Interestingly, the high levels of big ET-1 in our breast cancer patients was positively correlated with LV volume and mass. These findings not only confirm that the activation of endothelin system may play a role in mediating relative LVH in breast cancer patients but also indicate that big ET-1 is a sensitive biomarker that may help clinicians

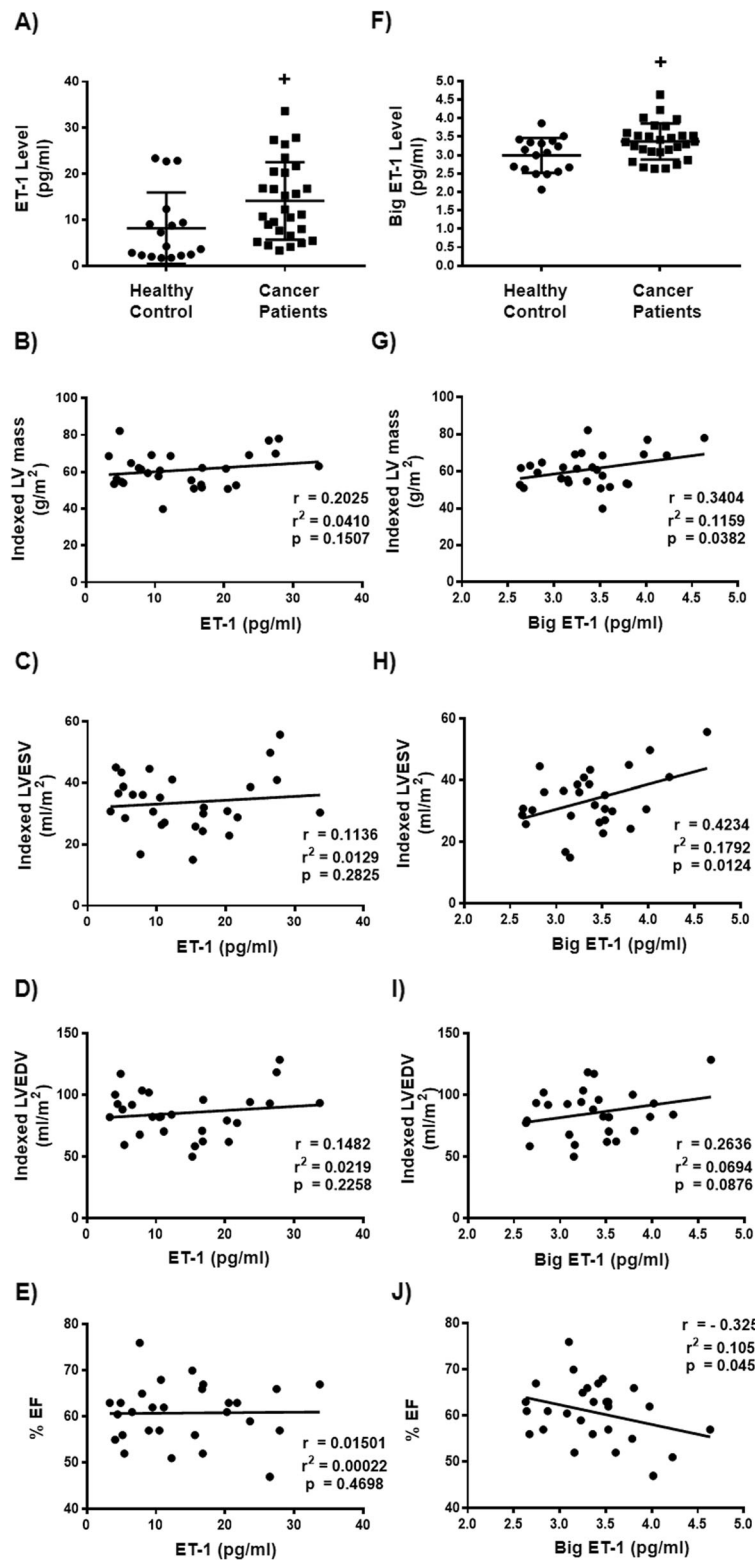


Fig. 4 (See legend on next page.)

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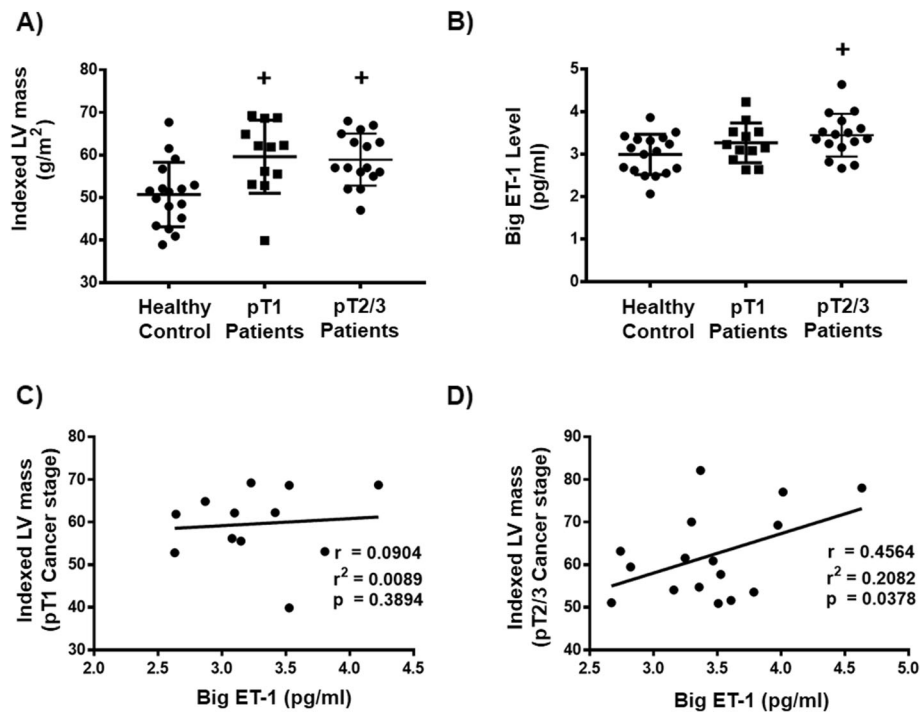
**Fig. 4** High circulating level of endothelin-1 and big endothelin-1 in patients with breast cancer with relative left ventricular hypertrophy. **a** Quantification of endothelin-1 (ET-1) levels in both breast cancer patients ( $n = 28$ ) and a control cohort of women with comparable baseline factors ( $n = 17$ ). **b** Correlation between ET-1 concentration and indexed left ventricular (LV) mass, **c** Correlation between ET-1 concentration and indexed left ventricular end systolic volume (LVESV), **d** Correlation between ET-1 concentration and indexed left ventricular end diastolic volume (LVEDV), **e** Correlation between ET-1 concentration and left ventricular ejection fraction (LVEF) in breast cancer patients ( $n = 28$ ). **f** Quantification of big ET-1 levels in both breast cancer patients ( $n = 28$ ) and the control cohort ( $n = 17$ ). **g** Correlation between big ET-1 concentration and indexed LV mass, **h** Correlation between big ET-1 concentration and indexed LVESV, **i** Correlation between big ET-1 concentration and indexed LVEDV, **j** Correlation between big ET-1 concentration and LVEF in breast cancer patients ( $n = 28$ ). Dots represent individual values. Results are shown as means  $\pm$  SD. Comparisons between two groups were made by unpaired *t*-test. \*  $p < 0.05$  vs healthy control. The correlations between ET-1 or Big ET-1 levels and LV parameters was determined using Pearson's correlation coefficient

identify patients at higher risk for subsequent cardiac remodelling. In agreement with our results, plasma big ET-1 is increased in patients with heart failure or cardiomyopathy and is significantly correlated with the extent of cardiac remodeling [36, 41, 42]. Thus, plasma big ET-1 is recommended as a prognostic biomarker of hypertrophic cardiomyopathy due to endothelin system activation [42].

## Conclusion

Our results indicate that even prior to cancer treatment, breast cancer patients already exhibit relative LVH. We show that breast tumor cells may contribute to

cardiomyocytes hypertrophy through the secretion of soluble factors and that one of these major factors is ET-1. While we have determined the level of ET-1 and big ET-1 in breast cancer patients, this may not reflect the actual levels of these factors produced by tumors as 24 of 28 of our breast cancer patients had tumour resection approximately 45 days prior to the cardiac MRI measure. Nevertheless, our data provided insight into a potential mechanism that may involve breast cancer cell-secreted ET-1 causing cardiomyocyte cell growth. Thus, given the fact that LVH is strongly linked to increased risk of heart failure in clinical studies [42],



**Fig. 5** Circulating level of big endothelin-1 correlated with pT stage of breast cancer patients with relative left ventricular hypertrophy. **a** Indexed left ventricular (LV) mass in pT1 stage breast cancer patients ( $n = 12$ ), pT2/3 stage breast cancer patients ( $n = 16$ ) and a control cohort of women with comparable baseline factors ( $n = 17$ ). **b** Big ET-1 concentration in pT1 stage breast cancer patients ( $n = 12$ ), pT2/3 stage breast cancer patients ( $n = 16$ ) and a control cohort of women with comparable baseline factors ( $n = 17$ ). **c** Correlation between big ET-1 concentration and indexed LV mass in pT1 stage breast cancer patients ( $n = 12$ ), **d** Correlation between big ET-1 concentration and indexed LV mass in pT2/3 stage breast cancer patients ( $n = 16$ ). Dots represent individual values. Results are shown as means  $\pm$  SD. Comparisons between three groups were made by one-way ANOVA with a Dunnett post hoc multiple comparison test. + $p < 0.05$  vs healthy control. The correlations between Big ET-1 levels and indexed LV mass in pT stage of breast cancer patients was determined using Pearson's correlation coefficient

targeting ET-1 signaling pathway and LVH may help minimize the susceptibility of patients with breast cancer to cardiovascular disease and ultimately improve their survival.

An important limitation of the current study is that we used a relatively small number of patients and healthy control women and thus, these findings should be confirmed in a larger cohort of patients and a control cohort with comparable baseline factors. Although we found that ET-1 signaling is increased in our breast cancer patients, we have not determined the level of residual tumor in these patients. Nonetheless, circulating ET-1 level might not accurately reflect the amount of ET-1 located in closer proximity to the tumor. Moreover, we have not explored the effect of other factors such as a stress-related increase in catecholamines on LVH and ET-1 signaling in our breast cancer patients. Lastly, additional studies should explore the clinical implications of relative LVH and increased ET-1 signaling in breast cancer patients during cancer treatment and beyond.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s12885-020-07217-1>.

**Additional file 1 Supplementary Fig. 1.** The original, full blot for phosphorylated eukaryotic elongation factor-2 (p-eEF2), total-eEF2 and  $\beta$ -ACTIN.

## Abbreviations

ATCC: American Tissue Culture Collection; BMI: Body mass index; BSA: Body surface area; CM: Conditioned medium; DAPI: 4',6-diamidino-2-phenylindole; EF: Ejection fraction; ECE: Endothelin converting enzyme; ET-1: Endothelin-1; HER2: Human epidermal growth factor receptor 2; HMWF: High molecular weight fractionated medium; LV: Left ventricular; LVH: Left ventricular hypertrophy; LVEDV: Left ventricular end diastolic volume; LVESV: Left ventricular end systolic volume; LVSV: Left ventricular stroke volume; LMWF: Low molecular weight fractionated medium; MRI: Non-contrast cardiac magnetic resonance imaging; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; p-eEF2: Phosphorylated eukaryotic elongation factor 2; WGA: Wheat-germ-agglutinin;  $\beta$ -MHC:  $\beta$ -myosin heavy chain

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## Authors' contributions

The authors read and approved the final manuscript. Participated in research design: ZHM, JRBD, DIP, JRM, EP, GS. Conducted experiments: ZHM, ASA, ST, DIP. Performed data analysis: ZHM, JRBD, DIP, MF. Wrote or contributed to the writing of the manuscript: ZHM, JRBD, DIP, GS, JRM, EP, AOSE, MF.

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## Availability of data and materials

The data used or analyzed during this study are included in this article and available from the corresponding author upon reasonable request.

## Ethics approval and consent to participate

Ethics approval was obtained from the University of Alberta Biomedical Panel and the Alberta Cancer Board, and written informed consent was obtained from study participants.

## Consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

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