An Initial Evaluation of Human Plasma cMLC-1 as a Potential Protein Biomarker for Trastuzumab-Induced Cardiotoxicity, Breast Cancer Screening and Progression

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Abstract

Background
Trastuzumab is a targeted therapy for human epidermal growth factor receptor 2 (HER2)-positive breast cancer. However, trastuzumab-induced cardiotoxicity (TIC) has been reported as a single agent or combined with anthracycline. Methods such as biomarkers for early TIC detection is not available. Blood-based protein biomarker prostate-specific antigen (PSA) for diagnosis, screening, prediction of response to therapy, and disease progression has revolutionized management and outcome of prostate cancer. Nevertheless, no blood biomarkers exist for breast cancer diagnosis or screening.

Methods
We evaluated for the first time the potentials of cardiac myosin light chain 1 (cMLC-1) as a biomarker to predict TIC, screen breast cancer and monitor breast cancer progression. Plasma cMCL-1 was measured quantitatively using enzyme-linked immunosorbent assays (ELISA). Archived paired plasma samples collected before and after trastuzumab treatment from 15 HER2+ patients with or without cardiotoxicity, recently collected unpaired plasma samples from 79 breast cancer patients (40 HER2+, 39 HER2-) and 46 healthy donors were tested for cMLC-1 levels.

Results
We found that elevated plasma level of cMLC-1 is associated with cardiotoxicity in 43% of trastuzumab-treated patients. In addition, we demonstrated that elevated plasma cMCL-1 is associated with breast cancer. The cutoff cMLC-1 concentration is estimated to be 44.99 ng/mL with a sensitivity of 59.49% (95%CI: 48.47%-69.63%) and specificity of 71.74% (95%CI: 57.45%-82.68%). We also found that plasma cMCL-1 is more elevated in HER2- than in HER2+ breast cancer patients. As a result, improved sensitivity of 79.49% (95%CI: 64.47%-89.22%) with the specificity of 63.04% (95%CI:48.60%-75.48%) were obtained for cMLC-1 to predict HER2- breast cancer with the cutoff at 37.17 ng/mL. Moreover, we determined that cMLC-1 level was significantly higher in patients with metastatic breast cancer than in patients with non-metastatic breast cancer.

Conclusions
Here we report the first exploratory human study on the potential of cMLC-1 as a blood protein biomarker for predicting TIC. Additionally, we show our findings which shed light on and filled, to some extent, the gap of knowledge of the potential of cMLC-1 as a blood protein biomarker for screening breast cancer, especially for HER2- breast cancer, and disease progression of breast cancer.
Background

Breast cancer is one of the most common malignancies in the United States, with over 280,000 new cases expected in 2021 [1]. Approximately one in five women diagnosed with breast cancer worldwide will have an aggressive form of breast cancer with human epidermal growth factor receptor 2 (HER2) gene amplification or HER2 protein overexpression, known as HER2+ subtype [2]. Trastuzumab (sold under the brand name Herceptin®) is a humanized monoclonal antibody specifically targeting HER2 that is used to treat both early- and late-stage HER2+ breast cancer. When started before or after surgery to treat early disease, the drug is administered every 21 days for a total of one year. For advanced breast cancer, treatment is typically given as long as the patient continues to derive clinical benefit [3]. Trastuzumab is typically prescribed as a single agent or in combination with standard chemotherapy regimens such as anthracyclines. However, trastuzumab treatment is associated with cardiac dysfunction, which manifests as a decrease in left ventricular ejection fraction (LVEF) and heart failure [4–6]. Trastuzumab-induced cardiotoxicity (TIC) has been reported to occur in up to 7% of patients when used as a single agent [7]. When combined with an anthracycline, however, cardiotoxicity increases dramatically to up to 27% of patients [7]. Screening for TIC involves an echocardiogram approximately every 3 months, however, a reduction in LVEF is associated with late-phase cardiac failure. Thus, biomarkers of TIC are needed for earlier detection and better management of critical early cardiac alterations that occur in cancer patients receiving trastuzumab.

Troponin-I (TnI) is considered as a sensitive and specific biomarker in the diagnosis of myocardial infarction. However, it is not sensitive and specific for the diagnosis of early stage of TIC [8]. There are no clinically approved biomarkers that can be used to predict the cardiac dysfunction induced by trastuzumab.

Cardiac myosin light chain-1 (cMLC-1, also known as myosin essential light chain (ELC)), the peripheral blood encoded by the MYL3 gene, is a part of the myosin complex with an important role in cardiac muscle contraction [8, 9]. Impaired integrity of damaged or injured cardiomyocytes leads to release of cMLC-1 from the myocardium into circulation [10–12]. Previously, we investigated the possibility of cMLC-1 as a potential biomarker for TIC in mice. Using echocardiography, we found that trastuzumab significantly reduced LVEF [8]. Importantly, this reduced LVEF was associated with elevated levels of serum cMLC-1 in mice [8]. The initial purpose of this study was to validate this finding in humans. We tested the hypothesis that plasma cMLC-1 level is associated with TIC in a cohort of patients using plasma samples collected before and after trastuzumab treatment.

Interestingly, while we were establishing the method to test quantitively cMLC-1 level in plasma collected from normal donors (n = 11) and breast cancer patients, we found that cMLC-1 level was significantly lower in normal donors than in breast cancer patients, and that cMLC-1 level was higher in HER2- patients (n = 10) than in HER2+ (n = 10) patients. Inspired by these captivating, although very preliminary findings, we expanded not only the sample size of each group to confirm the initial data but also compared the difference of cMLC-1 level in metastatic vs non-metastatic patients.
Blood-based protein biomarkers for diagnosis, screening, prediction of response to therapy, and disease progression are already taking hold in the cancer world, namely, serum prostate-specific antigen (PSA) and carcinoembryonic antigen (CEA). PSA levels have been used as a prostate cancer biomarker screening, that has revolutionized the clinical management of the disease [13]. PSA has been used for monitoring response to therapy, follow-up after completion of therapy and biochemical recurrence [14]. CEA measurements have been mainly used as a biomarker to monitor colorectal carcinoma treatment, recurrence and metastasis to liver [15]. A blood-based protein biomarker to detect treatment complications or to screen for breast cancer is not currently available in standard oncology practice. This study evaluated for the first time - cMLC-1 protein level in human plasma as a potential biomarker using samples collected from HER2+ patients who were treated with trastuzumab and developed cardiotoxicity and those who did not, and non-metastatic or metastatic breast cancer patients with HER2+ (n = 40) and HER2- (n = 39), and healthy donors (n = 46).

Materials And Methods

Patients. Archived human plasma samples were collected at multiple time points from a relatively homogenous patient population. The cohort consisted of women with newly diagnosed breast cancer who underwent treatment with anthracyclines followed by taxanes and trastuzumab (n = 15). Of which, 7 patients subsequently developed cardiotoxicity and 8 patients did not (Tables 1, 2). Cardiotoxicity was defined using the Cardiac Review and Evaluation Committee for Trastuzumab (CREC) criteria as a decrease of more than 10% in the echocardiographic LVEF to a value of less than 55%. Women were monitored every 3 months. The plasma samples were already collected and banked under the Massachusetts General Hospital Institutional Review Board (IRB protocol 2006P000886).
| Patient | Time            | Mean                  | p value  |
|---------|-----------------|-----------------------|----------|
| #1      | baseline vs 3-month | 55.05 ± 6.37 vs 65.32 ± 2.72 | 0.230    |
| #1      | baseline vs 6-month | 55.05 ± 6.37 vs 75.57 ± 7.07 | 0.010    |
| #3      | baseline vs 3-month | 0.57 ± 0.74 vs 110.09 ± 26.94 | 0.001    |
| #3      | baseline vs 6-month | 0.57 ± 0.74 vs 91.63 ± 15.36 | 0.002    |
| #3      | 3-month vs 6-month | 110.09 ± 26.94 vs 91.63 ± 15.36 | 0.264    |
| #3      | 6-month vs 9-month | 91.63 ± 15.36 vs 51.16 ± 0.64 | 0.059    |
| #4      | baseline vs 6-month | 18.19 ± 1.45 vs 20.49 ± 2.02 | 0.076    |
| #4      | 3-month vs 6-month | 12.96 ± 0.61 vs 20.49 ± 2.02 | 0.035    |
| #5      | baseline vs 3-month | 26.57 ± 5.33 vs 18.42 ± 1.81 | 0.148    |
| #5      | baseline vs 6-month | 26.57 ± 5.33 vs 27.67 ± 1.13 | 0.410    |
| #5      | 3-month vs 6-month | 18.42 ± 1.81 vs 27.67 ± 1.13 | 0.024    |
| #5      | 3-month vs 9-month | 18.42 ± 1.81 vs 24.88 ± 1.41 | 0.053    |
Table 2
Comparisons of plasma cMLC-1 levels before and 3-month after trastuzumab treatment in breast cancer patients.

| Patient | Baseline   | 3-month     | p value | Diagnosed Cardiotoxicity |
|---------|------------|-------------|---------|--------------------------|
| #1      | 55.05 ± 6.37 | 65.32 ± 2.72 | 0.138   | NO                       |
| #2      | 17.6 ± 1.10  | 27.95 ± 3.38 | 0.050   | NO                       |
| #3      | 0.57 ± 0.74  | 110.09 ± 26.94 | 0.002   | YES                      |
| #4      | 18.19 ± 1.45 | 12.96 ± 0.61  | 0.040   | YES                      |
| #5      | 26.27 ± 5.33 | 18.42 ± 1.81  | 0.148   | NO                       |
| #6      | 209.4 ± 31.11| 298.7 ± 67.75 | 0.083   | YES                      |
| #7      | 78.82 ± 3.65 | 42.38 ± 0.61  | 0.005   | YES                      |
| #8      | 14.35 ± 0.11 | 119.4 ± 0.09  | 9.42E-07 | NO                       |
| #9      | 33.24 ± 3.56 | 28.67 ± 0.25  | 0.164   | YES                      |
| #10     | 0.22 ± 0.10  | 1.04 ± 0.75   | 0.194   | NO                       |
| #11     | 89.48 ± 13.69| 39.76 ± 2.82  | 0.035   | NO                       |
| #12     | 72.18 ± 5.82 | 60.25 ± 6.88  | 0.158   | NO                       |
| #13     | 48.96 ± 5.56 | 55.61 ± 8.05  | 0.283   | NO                       |
| #14     | 100.1 ± 28.01| 79.08 ± 19.40 | 0.217   | YES                      |
| #15     | 60.54 ± 17.20| 174.6 ± 49.23 | 0.018   | YES                      |

Patients with HER2+ (n = 40) and HER2- (n = 39) breast cancer were recruited from the Massachusetts General Hospital Cancer Center between March 2018 and January 2020 (Table 3). Based on existing clinical guidelines [16], we defined HER2+ as ≥ 2.0 amplified by Fluorescent In Situ Hybridization (FISH) as noted on the pathology report from the date of original diagnosis, or as 2–3+ by immunohistochemistry (IHC) if FISH was not available. Patients who were receiving trastuzumab as standard therapy were also included in the HER2+ cohort, even if IHC and FISH did not meet the criteria. Relevant clinical data such as LVEF and treatment history were extracted from electronic medical records. All studies were approved by the Dana Farber/Harvard Cancer Center Institutional Review Board (IRB protocol 13–416). Patients provided written informed consent for data collection, blood collection, and downstream analysis.
Table 3
Patient demographics and characteristics.

| Characteristic | HER2+  | HER2-  | Total  |
|---------------|--------|--------|--------|
|               | n = 40 | n = 39 | n = 79 |
| Number (%)    | Number (%) | Number (%) |
| Demographic characteristics | | | |
| Age (years, m+/s) | 54.5 (+/-12.7) | 59.1 (+/-9.6) | 56.9 (+/-11.39) |
| Race | | | |
| Black | 2 (5%) | 1 (2.6%) | 3 (3.8%) |
| Asian | 1 (2.5%) | 2 (5.3%) | 3 (3.8%) |
| White | 34 (85%) | 35 (89.7%) | 69 (87.3%) |
| Hispanic | 1 (2.5%) | 0 | 1 (1.3%) |
| Other | 2 (5%) | 1 (2.6%) | 3 (3.8%) |
| Clinical characteristics | | | |
| ER | | | |
| Positive | 32 (80%) | 34 (87.2%) | 66 (83.5%) |
| Negative | 8 (20%) | 5 (12.8%) | 13 (16.5%) |
| Unknown | 0 | 0 | 0 |
| PR | | | |
| Positive | 21 (52.5%) | 29 (74.4%) | 50 (63.3%) |
| Negative | 18 (45%) | 10 (25.6%) | 28 (35.4%) |
| Unknown | 1 (2.5%) | 0 | 1 (1.3%) |
| Histology | | | |
| Ductal | 31 (77.5%) | 23 (58.9%) | 54 (68.4%) |
| Lobular | 3 (7.5%) | 7 (17.9%) | 10 (12.7%) |
| Mixed | 4 (10%) | 2 (5.1%) | 6 (7.6%) |
| NOS | 2 (5%) | 7 (17.9%) | 9 (11.4%) |
| Metastatic | | | |
| Patients |        |        |        |
|----------|--------|--------|--------|
| Yes      | 22 (55%) | 30 (76.9%) | 52 (65.8%) |
| No       | 18 (45%)  | 9 (23.1%)  | 27 (34.1%)  |

**Treatment history**

| Radiation treatment |        |        |        |
|---------------------|--------|--------|--------|
| Yes                 | 28 (70%) | 32 (82.1%) | 60 (75.9%) |
| No                  | 12 (30%)  | 7 (17.9%)  | 19 (24%)   |

| Trastuzumab at blood collection |        |        |
|---------------------------------|--------|--------|
| Yes                             | 34 (85%) | -      | -      |
| No                              | 6 (15%)   | -      | -      |

**Other type of therapy at blood collection**

| CDK4/6 (single or in combo) | 1 (2.5%) | 21 (53.8%) | 22 (27.8%) |
| PIK3CA/mTOR (single or in combo) | 1 (2.5%) | 8 (20.5%) | 9 (11.4%) |
| Chemotherapy                  | 3 (7.5%) | 3 (7.7%) | 6 (7.6%) |
| Immunotherapy                 | 0       | 1 (2.6%) | 1 (1.3%) |
| Targeted therapy              | 1 (2.5%) | 5 (12.8%) | 6 (7.6%) |
| Endocrine therapy (single agent) | 0       | 5 (12.8%) | 5 (12.8%) |

Plasma from healthy donors (n = 46) was obtained commercially (Innovative Research, 46430 Peary Court, Novi, MI 48377).  

**Plasma collection.** Blood samples (10 mL) were collected in cfDNA BCT tubes (Streck Inc., La Vista, NE, USA) at an arbitrary time point coinciding with the patients’ clinical visits. Samples were stored ambient for up to 7 days and were centrifuged at 1,000 x g for 15 min at 2–8°C. In one instance, plasma was previously isolated from whole blood by double centrifugation at 1,600 x g for 10 min followed by 3,000 x g for 10 min. The resulting plasma was frozen at -80°C, and later thawed for analysis.

**Measurement of cMLC-1 by ELISA.** A sandwich ELISA was performed using the human cMLC-1 ELISA kit from MyBioSource (Cat# MBS2506936, San Diego, CA, USA) according to the protocol provided. A 1:10 plasma dilution was added into the anti-cMLC-1 antibody pre-coated well and incubated at 37 °C for 1.5 h. The plasma samples were then decanted. Next, 100 µl of biotinylated anti-cMLC-1 detection antibody working solution was added to each well, and incubated for 1 h at 37 °C. After decanting the solution, the wells were washed with the provided washing buffer 3 times. One hundred µl of horseradish peroxidase conjugated avidin (HRP-avidin) working solution was added to each well, and incubated for 30 min at 37 °C.
˚C. The non-bound HRP-avidin was removed by washing with the buffer 5 times. To generate the colorimetric signal, 90 µL of substrate reagent was added to each well. After incubation for 15 min, the enzymatic reaction was stopped with 50 µL stop solution. The optical density (OD) of each well was measured with a microplate reader (Epoch, BioTeck Instrument, Winooski, VT, USA) at a wavelength of 450 nm. In the same test, serial concentrations of standard cMLC-1 working solution (provided in the kit) were included. A four-parameter logistic curve on log-log equation was followed to draw the calibration curve. All samples were tested in triplicate.

To establish an appropriate detection method, each plasma at undiluted and 2-fold dilutions were tested to confirm that its cMLC-1 concentration was within the detectable range of the ELISA kit (0.625 to 40 ng/mL). Based on this titration experiment, we found that 1:10 dilutions were optimal to detect cMLC-1 concentrations that fit well within the standard curve. The calibration curve following a four-parameter logistic curve on log-log equation was: \( y = A_2 + (A_1 - A_2)/(1+(x/x_0)^p) \), where \( A_1 = 0.051 \), \( A_2 = 6.401 \), \( x_0 = 96.859 \), \( p = 0.887 \), \( R^2 = 0.996 \). All the plasma samples were tested at least 2 times with a duplicate of each sample, and the concentration of cMLC-1 was calculated according to the calibration equation.

**Statistical analysis.** Paired samples were compared with the paired \( t \) test. Area under the curve (AUC) was used to evaluate the clinical performance of the tests, and estimates of sensitivity, specificity, and predictive values were calculated and reported with 95 % confidence intervals (CI). The medians of foci intensity distributions were tested with the Mann-Whitney U test. We used one-way ANOVA for multiple samples. Data are expressed as mean ± SD of the number of biological replicates indicated in each figure legend. Values of \( p < 0.05 \) were considered significant.

**Results**

**Elevated plasma level of cMLC-1 is associated with cardiotoxicity in 43% of trastuzumab treated patients tested.** Preclinical data indicated that reduced LVEF was associated with elevated serum cMLC-1 in mice [8]. To investigate if this finding is clinically relevant in humans, (i.e., if plasma cMLC-1 level is associated with TIC in a cohort of patients) initially we tested archived plasma collected from women with newly diagnosed breast cancer who underwent treatment with anthracyclines followed by taxanes and trastuzumab (n = 5). Among these, 2 patients had subsequently developed cardiotoxicity and 3 patients had not. Plasma samples were collected at multiple time points (baseline, 3, 6, and 9 months) and were tested for cMLC-1 levels. Compared to baseline, cMLC-1 was increased in patient #3, who had developed cardiotoxicity, but unchanged in patient #4, who had also experienced cardiotoxicity. cMLC-1 remained unchanged in patients #1, #2 and #5, patients who had not developed cardiotoxicity (Fig. 1A, Table 1). The data were somewhat supportive of our hypothesis, however, not conclusive due to the limited number of patient samples tested. It is worth Nothing that, unlike the huge difference of cMLC-1 between 0 and 3 months in patient #3, the changes of cMLC-1 were small between 3 and 6 months in patients #1, #4 and #5. Additionally, there was no change between 3 and 6 months in patient #2, or between 6 and 9 months in any of the 5 patients (Fig. 1A, Table 1). Thus, we decided to test samples from an additional 10 patients (n = 15). We collected at baseline (prior trastuzumab treatment) and at 3 months after initiation.
of trastuzumab treatment. Three-month serum cMLC-1 measurements were significantly higher than baseline in cardiotoxicity patients #3, #6 and #15 (3/7, 43%) (Fig. 1B, Table 2). However, one non-cardiotoxicity patient #8 (1/8, 13%) had a higher cMLC-1 level at 3 months than at baseline (Fig. 1B, Table 2). Based on the data, the use of plasma cMLC-1 as a biomarker for TIC needs to be further investigated and validated to identify a cutoff to predict TIC with good sensitivity and specificity in a larger cohort of patients with and without cardiotoxicity following trastuzumab treatment.

**Elevated plasma cMCL-1 is associated with breast cancer.** There are currently no blood-based biomarkers approved for the detection of breast cancer. While we were establishing the assay to reliably measure cMLC-1 protein in human plasma, we unexpectedly observed that cMLC-1 level was significantly higher in breast cancer patients (n = 20) than normal donors (n = 10). Thus, we wanted to explore the possibility that cMLC-1 could be used as a biomarker for screening of breast cancer patients. To this end, we further tested plasma cMLC-1 levels in more patients with breast cancer (n = 79) and in more normal donors (n = 46). We found that cMLC-1 level was significantly higher in patients with breast cancer than in normal donors (63.18 ± 55.31 ng/mL vs 37.61 ± 35.39 ng/mL, p = 0.0006) (Fig. 2A). The receiver operator characteristic (ROC) curve analysis of breast cancer (HER2- and HER2+) vs normal donors determined area under curve (AUC) value of the logistic regression is 0.6791 (p = 0.0009). It shows the cutoff cMLC-1 concentration is at 44.99 ng/mL with a sensitivity of 59.49% (95%CI: 48.47%-69.63%) and specificity of 71.74% (95%CI: 57.45% -82.68%) (Fig. 2B). It is also noteworthy that cMLC-1 in normal donors did not vary across age or race groups (Figs. 2C, D, E), suggesting that age or race does not affect cMLC-1 level. Collectively, this finding suggests cMLC-1 may be a novel potential biomarker combined with other methods and/or biomarkers for breast cancer screening.

**Plasma cMCL-1 is noticeably higher in HER2- than in HER2+ breast cancer patients.** Next, we analyzed and compared plasma cMCL-1 to determine if it is a potential biomarker for subtyping breast cancer. We found HER2- patients (n = 39) had a borderline significantly higher level of cMLC-1 than HER2+ patients (n = 40) (73.22 ± 55.88 ng/mL vs 56.67 ± 52.34 ng/mL, p = 0.0578) (Fig. 3A). Thus, as expected, compared to normal donors, HER2- patients had 2.0-fold higher levels of cMLC-1 (73.22 ± 55.88 ng/mL vs 37.61 ± 35.39 ng/mL, p < 0.0001) (Fig. 3B). As a result, an improved sensitivity of 79.49% (95%CI: 64.47%-89.22%) with the specificity of 63.04% (95%CI:48.60%-75.48%) were reached for cMLC-1 to predict HER2- breast cancer with the cutoff at 37.17 ng/mL. In contrast, plasma cMLC-1 in HER2+ patients was increased, but to a lesser extent, compared to normal donors (56.67 ± 52.34 ng/mL vs 37.61 ± 35.39 ng/mL, p = 0.0549) and only reached a borderline statistical significance with the given sample size (Fig. 3C). ROC curve analysis of HER2- vs normal donors determined AUC value of the logistic regression is 0.7480 (p< 0.0001) (Fig. 3D). The data suggest that cMLC-1 may be better suited as a biomarker for HER2- than HER2+ disease or all breast cancer, in general.

**Plasma cMCL-1 is a potential biomarker for breast cancer progression.** We then wanted to assess if cMLC-1 levels differ in patients with or without metastasis. As shown in Table 3, 53 out of 79 patients (67%) had metastatic disease. We found that cMLC-1 level was higher in patients with metastatic breast cancer than in patients with early or locally advanced breast cancer, or non-metastatic breast cancer.
(75.96 ± 59.85 ng/mL vs. 43.41 ± 34.26 ng/mL, p = 0.0072) (Fig. 4A). The difference between HER2-metastatic versus non-metastatic patients did not show statistical significance (78.68 ± 60.78 ng/mL vs. 54.99 ± 28.54 ng/mL, p = 0.3657) (Fig. 4B). This finding may reflect the fact that out of all metastatic patients (n = 53), more patients (57.7%) were HER2- with high cMLC-1 levels while fewer patients (42.3%) were HER2+ with lower cMLC-1 levels (Table 3). In contrast, we found that patients with HER2+ metastatic disease had higher levels of cMLC-1 than non-metastatic HER2+ patients (72.26 ± 58.37 ng/mL vs. 37.62 ± 35.57 ng/mL, p = 0.0204) (Fig. 4C). Our results indicate that the cMLC-1 level may be associated with the progression of disease and may serve as a potential biomarker for metastasis as well as monitoring response to therapy.

**cMCL-1 in plasma is stable over time.** To evaluate the stability of plasma cMCL-1 over time, we assayed samples kept in -80°C freezer storage for 12, 19 and 24 months. We found plasma levels of cMCL-1 were consistent across all time points tested for a given patient or normal donor sample. Representative data are shown (Fig. 5), and indicate that cMLC-1 in plasma stored at -80°C is stable for at least 2 years. Its long-term stability will facilitate its use as a biomarker and in other clinical studies.

**Discussion**

Increasing evidence shows that treatment with trastuzumab is associated with significant cardiotoxicity [17–19]. Alarmingly, a decrease in LVEF has even been found in asymptomatic patients. Early identification of patients who are at risk for left ventricular dysfunction following trastuzumab therapy is essential for early initiation of cardioprotective measures. A blood-based biomarker for TIC could better serve as an ongoing surveillance strategy than the current system of echocardiographic LVEF measurement. Blood biomarkers could be potential tools to detect cardiac dysfunction early and predict TIC before an echo reveals LVEF reduction. However, to date, there is a lack of such biomarkers. Previous studies focused mainly on evaluating the potential of troponins, brain natriuretic peptide (BNP), N-terminal pro b-type natriuretic peptide (NT-proBNP) and high-sensitivity C-reactive protein (hs-CRP) as blood protein biomarkers to predict TIC. Sawaya et al. revealed an association between troponin I (also known as cardiac troponin I) levels at 3 months post-treatment with trastuzumab and development of cardiotoxicity at 6 months [20]. Later, Onitilo et al. reported that elevated hs-CRP, but not BNP, or troponin I, predicted decreased LVEF with a sensitivity of 92.9 % and specificity of only 45.7%. With such a high false positives rate, this assay does not reliably predict toxicity [21]. Recently, Zardavas et al. found that baseline (before trastuzumab treatment) troponin I and T were elevated in only 13.6% (56 of 412) and 24.8%(101 of 407) patients, respectively, and that these measurements were associated with significantly increased risk of reduced LVEF [22]. While these findings are encouraging, these efforts indicate that we are still at the beginning of the search for better biomarkers for early prediction and identification of TIC.

The finding that reduced LVEF following trastuzumab treatment was associated with elevated levels of serum cMCL-1 in mice has led to the present study [8]. Past studies have shown that circulating cMCL-1 protein was elevated in patients a few hours after acute myocardial infarction, and peaked on days 2 to 4 post infarction [23, 24]. In addition, serum levels of cMCL-1 and creatine kinase (CK) were measured in
serial samples from 49 patients with acute myocardial infarction. The results suggested that serum cMLC-1 is a better marker than CK in predicting LVEF changes [25]. This established association between serum cMLC-1 and LVEF provided the basis for investigating cMLC-1 as a non-invasive blood-based protein biomarker for early TIC prediction.

The results of initial phase of this research found that plasma cMLC-1 levels were elevated, with respect to baseline, in 4/15 trastuzumab treated breast cancer patients. Of these, 43% (3/7) of patients developed TIC and 13% of patients (1/8) did not develop TIC. The elevated plasma cMLC-1 was detected as early as 3-months post-treatment with trastuzumab. Due to the small sample size tested, the value of plasma cMLC-1 as a biomarker for early detection and prediction of TIC is not conclusive and should be further evaluated in a larger cohort of patients. For future studies, sample collection should be timed within the 3 month period (i.e. 1-week, 2-week, 1-, 2- and 3- months post-treatment with trastuzumab) in order to validate cMLC-1 as a biomarker for early detection or prediction of TIC [23, 24].

To date, the most effective means of early detection and screening involves mammography, a technique that has been approved and widely practiced since the 1980s [26]. Though mammography has undoubtedly improved outcomes for women with breast cancer—research estimates at least a 50% mortality reduction since becoming standard practice—this method is imperfect and presents its own challenges [27]. Yearly mammograms are recommended starting at age 40, which is often too late for women with some of the more aggressive forms of breast cancer. Additionally, due to the nature of the exam and the frequency at which it is required, attendance rates among women for their yearly mammograms vary, suggesting an additional layer of more accessible screening measures may help close the gap [28]. Finally, the false positive rate for mammography is alarmingly high. In the U.S., the 10-year false positive rate is 30%, and 50% of all women will receive a false positive result at some point [28]. The ideal solution for breast cancer screening is a blood-based biomarker that can complement or replace the flawed practice of mammography to overcome its shortcomings. A blood test is generally far easier to schedule, better tolerated by patients, and can be integrated into a routine clinical visit. Additionally, a blood test can be justifiably introduced earlier than age 40, as it will be less expensive for payers.

Currently, no blood biomarkers for breast cancer diagnosis or screening have been approved for clinical use. Our data suggest, however, that cMLC-1 may be a potential candidate as a biomarker for initial and/or combinational screening of women under 40 who are at high risk for breast cancer (Fig. 2). Plasma cMCL-1 would be more sensitive in predicting HER2- breast cancer as cMLC-1 is significantly higher in HER2- than HER2+ patients (Fig. 3). We anticipate that a larger sample size would result in a narrower 95% confidence interval for sensitivity and specificity to predict either breast cancer or HER2-breast cancer. In addition, plasma cMLC-1 is correlated with disease progression as it is higher in metastatic breast cancer than in non-metastatic breast cancer patients (Fig. 4). Therefore, cMLC-1 may also be clinically useful to monitor disease progression and monitor response to therapy. Moreover, since breast cancer has 4 major subtypes with different treatment courses and prognoses (luminal A, luminal B, HER2+, and triple negative), it is advisable to measure the cMLC-1 in each subtype of breast cancer to
validate the potential of cMLC-1 as a biomarker across subtypes for disease screening and therapy monitoring. The method described here to detect plasma cMLC-1 is fast and easy. Importantly, we demonstrated that plasma cMLC-1 is stable over time after storage in -80°C freezers for at least 2 years (Fig. 5).

Conclusion

The results of this investigation provide a sound basis for the novel and exciting further investigation of cMLC-1 as a blood protein biomarker for predicting TIC, screening for breast cancer (especially for HER2-breast cancer), evaluating disease progression, and monitoring treatment response. Furthermore, our study highlights the need to define the mechanisms(s) of how and why plasma cMLC-1 is elevated in breast cancer patients.

Abbreviations

cMLC-1
cardiac myosin light chain 1; HER2: epidermal growth factor receptor 2; LVEF: left ventricular ejection fraction; TIC: Trastuzumab-induced cardiotoxicity; TnI: Troponin-I; ELC: essential light chain; PSA: prostate-specific antigen; CEA: carcinoembryonic antigen; CREC: Cardiac Review and Evaluation Committee for Trastuzumab; FISH: Fluorescent In Situ Hybridization; IHC: immunohistochemistry; ELISA: enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; OD: optical density; AUC: area under the curve; CI: confidence intervals; SD: standard deviation; ROC: receiver operator characteristic; BNP: brain natriuretic peptide; NT-proBNP: N-terminal pro b-type natriuretic peptide; hs-CRP: high-sensitivity C-reactive protein; CK: creatine kinase

Declarations

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Not applicable

Authors’ contributions

XW, WW, SJI and MCS conceived the study and designed the experiments. LJ, YL and TS carried out the experiments. RA, CSW, KH and ANV collected patient sample collection and provided de-identified patient information. CJP and DEM supported the patient sample collection. RA and LJ analyzed and organized patient information. LY, RA, LJ and HZ analyzed the data. XW, LY and RA interpreted the data. XW, LY, RA and ZH wrote the manuscript. All the authors read and approved the submitted manuscript.

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Availability of data and materials

The data in the current study are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

All studies were approved by the Massachusetts General Hospital Institutional Review Board (IRB protocol 2006P000886) and the Dana Farber/Harvard Cancer Center Institutional Review Board (IRB protocol 13-416). Patients provided written informed consent for data collection, blood collection, and downstream analysis.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

A

B

Figure 1

Profile of plasma cMLC-1 levels in trastuzumab-treated breast cancer patients with or without cardiotoxicity. Plasma samples were collected at multiple time points as indicated. Each plasma sample was 1:10 diluted and tested to determine cMLC-1 concentration by ELISA. The mean ± SD of cMLC-1 in each sample is shown. Baseline: before trastuzumab treatment; 3, 6 and 9 months: time points after trastuzumab treatment (A). A total of 15 paired-plasma samples collected at before (baseline) and after 3-months trastuzumab treatment were measured for cMLC-1 (B). The mean ± SD of cMLC-1 in each sample is shown. The paired Student -t test was used to analyze the differences. * p<0.05; ** p <0.01; *** p <0.005 and **** p <0.001.
Plasma cMLC-1 level was significantly higher in breast cancer patients than normal healthy women. Each plasma sample was 1:10 diluted and tested to determine cMLC-1 concentration by ELISA. The mean ± SD of cMLC-1 in breast cancer patients (n=79) vs normal donors(n=46) is shown. The Mann-Whitney U test was used to analyze the difference. **** p=0.0006 (A). The receiver operator characteristic (ROC) graph of the logistic regression result was calculated by GraphPad Prism 8 to determine the relationship between sensitivity and specificity. The cutoff of cMLC-1 at (or higher) 49.55 ng/mL was chosen to reach a sensitivity of 59.49% (B). To determine the impact of age and race factors on cMLC-1 level, plasma samples from all normal donors with different ages and races (n=46) were analyzed and compared. The one-way ANOVA was used for difference among all indicated groups of age (p=0.8630) (C) and of race (p=0.138) (D). To ensure the data were accurate, cMLC-1 between “Hispanic” and “Caucasian” were analyzed by the Mann-Whitney U test (p=0.0988) (E) without the “black” group given its small size of samples (p=0.1338).
Plasma cMLC-1 level was higher in HER2- than HER2+ patients. The mean ± SD of cMLC-1 in HER- (n=39) vs HER2+ (n=40) is shown (p=0.0578) (A). Plasma cMLC-1 was much higher in HER2- patients than in normal donors (n=46) (p<0.0001) (B); plasma cMLC-1 was borderline significantly higher in HER2+ patients than in normal donors (p=0.0549) (C). The Mann-Whitney U test was used to analyze the above differences between every two groups. ROC curve analysis determined area under curve (AUC) value of the logistic regression is 0.7480 (p<0.0001), indicating cMLC-1 at (or higher) the cutoff of 37.17 ng/mL could predict HER2- breast cancer (D).
Figure 4

Plasma cMCL-1 was higher in metastatic than in non-metastatic breast cancer patients. The mean ± SD of cMLC-1 in metastatic (n=52) vs non-metastatic patients (n=27) is shown (p=0.0069) (A). Plasma cMLC-1 was not significantly different in HER2- metastatic (n=30) vs non-metastatic patients (n=9) (p=0.3657) (B). Plasma cMLC-1 was higher in HER2+ metastatic (n=22) vs non-metastatic patients (n=18) (p=0.0204) (C). The Mann-Whitney U test was used to analyze the above differences between every two groups.

Figure 5

Stability of plasma cMCL-1 was quantitatively determined. Plasma cMLC-1 was repeatedly tested for sample from patients and normal donors. Representative data using the same set of normal donors samples (n=10) stored at -80°C for 12, 19 and 24 months are shown. The one way ANAVA was used to test the difference (p=0.8737).