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CLINICAL TRIAL

Circulating adipose stromal cells as a response biomarker in phase II energy balance trials of obese breast cancer survivors and high-risk women

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Abstract

Purpose Circulating adipose stromal cells (CASC) are thought to be increased in obesity and facilitate angiogenesis, and tumor metastases.

Methods CASC were identified from buffy coat peripheral blood mononuclear cells (PBMCs) by flow cytometry as $CD34^{bright}CD31^{-}$ $CD45^{-}$ and CASC frequency was compared to adiposity measures in 33 women at increased risk for breast cancer. Feasibility of CASC as a response biomarker for a diet and exercise intervention in ten breast cancer survivors was then explored.

Results For 33 high-risk women, median CASC frequency was 9.7 per million PBMCs and trended positively with body mass index, fat mass index (FMI), and percent android fat. Correlation was significant when BMI was dichotomized at > versus < 35 kg/m^2 ($p = 0.02$). For ten breast cancer survivors with a median BMI of 37 kg/m^2, median CASC frequency was 16.4 per million PBMCs. In univariate analyses, change in BMI, total fat and visceral fat were significantly correlated with change in CASC frequency. On multivariate analysis, change in visceral adipose had the strongest association with change in CASC frequency ($p < 0.00078$).

Conclusions The association between the reduction in visceral adipose tissue and the decrease in frequency of circulating adipose stromal cells suggests that the latter might be a useful biomarker in clinical trials of obese breast cancer survivors undergoing a weight loss intervention.

Keywords Breast cancer · Obesity · Risk biomarkers

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Introduction

Obesity as defined by a body mass index (BMI) of > 30 kg/m^2 is associated with an increased risk of breast cancer in post-menopausal women [1] as well as an increased risk of cancer-related mortality after a breast cancer diagnosis [2, 3]. Although outcomes for severely obese women with BMI > 35 kg/m^2 are uniformly poorer than those for normal weight women, outcomes for overweight and mildly obese breast cancer survivors (BMI between 25 and 35 kg/m^2) have recently been observed to be superior to their normal weight counterparts [4]. This phenomenon called the obesity paradox is likely due in part to the lack of close correlation of BMI in the 25–35 kg/m^2 range with adipose amount or function, or the protective factors of muscle mass and fitness [4] as well as failure to consider smoking, co-morbidities, and adipose dysfunction in normal weight

women [5]. Assessment of total and compartmental fat and lean mass along with functional measures are likely to be more accurate than BMI alone in predicting the metabolic and pro-carcinogenic consequences of obesity [6–8].

Adipose stromal cells (ASCs) increase with increasing fat mass and are a source of pro-inflammatory cytokines and aromatase leading to increased estrogen production. Preclinical studies suggest that ASCs from both subcutaneous and visceral adipose can promote cancer cell division and migration [9, 10], but visceral ASCs are a particularly good source of pro-angiogenic factors [11]. Once in the circulation, circulating adipose stromal cells (CASCs) with the aid of chemokine receptors home to tumor sites where they promote angiogenesis and establishment of metastases [12–15]. CASC have been characterized by flow cytometry as $CD34^{bright}CD31^{-}$ $CD45^{-}$. This trio of antigen expression identifies primitive stromal cells (CD34 bright) while excluding hematopoietic and endothelial cells [13, 16]. CASC frequency is extremely low; reported as being at the level of detection of 10 per million (0.001%) peripheral blood mononuclear cells (PBMCs) in cancer-free non-obese individuals; whereas CASC frequency of up to 300 per million (0.03%) PBMCs has been observed in cancer-free obese individuals with BMI > 30 kg/m^2 [17]. While there is an observable difference in CASC frequency in obese versus non-obese individuals, no functional differences in CASCs have been observed in obese versus non-obese individuals [12]. In a small cross-sectional study of 24 breast cancer survivors, CASC frequency was higher in obese (CASC frequency of 0.77% with mean BMI of 36.1 kg/m^2) than in non-obese women (0.004% with mean BMI of 27.1 kg/m^2) [18]. No overall reduction in CASC frequency was reported in 13 obese survivors after a 6-month exercise program; but those who did have a reduction in body fat as measured by change in skinfold thickness had decreased CASC frequency [18]. This suggested to us that CASC might be a useful functional marker linking fat mass change and risk of breast cancer or cancer recurrence.

Dual-energy X-ray absorptiometry (DXA) can be used to accurately measure lean and fat mass as well as compartmental fat. Total fat mass, fat mass index (kg fat/m^2 height), and visceral adipose have been reported as superior to BMI in predicting metabolic abnormalities associated with cancer and cardiovascular risk [19–22]. Visceral adipose tissue (VAT) along with ectopic fat are primarily responsible for the chronic inflammation and metabolic dysfunction that often accompanies obesity [23–25]. VAT is traditionally measured by computed tomography (CT) or magnetic resonance but can be approximated by DXA with newer software programs. DXA gives similar assessments and measures of change over time as CT with less expense and discomfort [21, 22].

We report here the result of a pilot study exploring the association between CASC frequency and body composition in 33 women at increased risk of breast cancer with varying BMI; as well as before and after a 3-month diet and exercise intervention in ten obese breast cancer survivors.

Methods

Participants

Participants were recruited through the University of Kansas Medical Center's Breast Cancer Prevention and Survivorship Research Center, via protocols approved by an institutional review board. Prior to the initial procedure, all potential study participants were given oral and written information regarding the studies including risks and benefits and signed a consent (HSC# 4601 or Study00004575—registered in clinicaltrials.gov as NCT00291096 and NCT0296374, respectively).

Cross-sectional study in high-risk women

Thirty-three women (13 pre-menopausal and 20 post-menopausal) at increased risk for development of breast cancer who were taking part in an ongoing prospective risk biomarker assessment study comprised the high-risk cohort. Women were risk eligible on the basis of Tyer–Cuzick predicted 10-year risk of twice that of the average woman in their age group [26, 27]. Women had height and weight measured for BMI as well as waist circumference in a hospital gown. Body composition was assessed with DXA (GE Lunar Prodigy™) and measures included total and lean mass, total fat mass, and percent android and gynoid fat mass. Software available on the DXA GE Lunar Prodigy™ did not calculate visceral adipose tissue.

Diet and physical activity intervention in breast cancer survivors

Ten post-menopausal obese sedentary breast cancer survivors had CASC assessment performed as part of a diet and physical activity pilot study designed to determine whether older obese sedentary breast cancer survivors could reliably achieve 5 h of purposeful physical activity per week during calorie restriction. Eligibility criteria for the weight loss intervention included completion of any surgery, radiation or cytotoxic chemotherapy for breast cancer at least 3 months prior to registration, a BMI of ≥ 30 kg/m^2, and < 60 min of exercise per week. Women were excluded if they were taking metformin or insulin. At baseline and again after the 3-month weight loss intervention, 10 participants had assessment of CASC in addition to body composition. Body

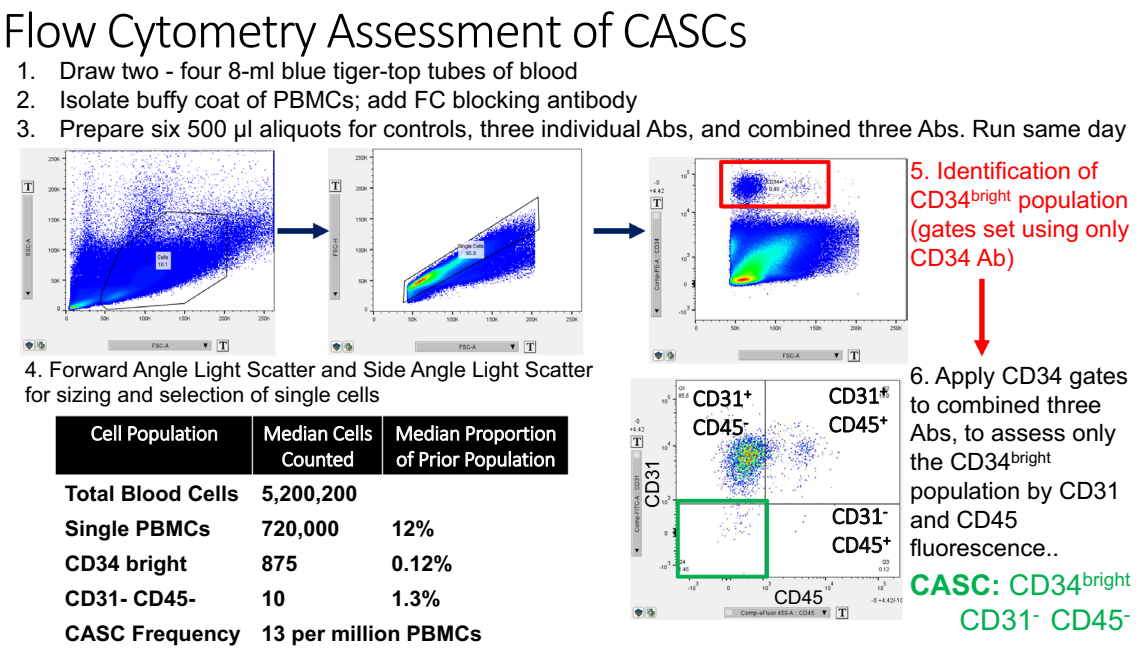
composition was measured by a GE Lunar iDXA™ unit, which in addition to traditional adiposity assessments measured visceral adipose automatically via a software program that segments android fat into subcutaneous fat and visceral fat. Visceral fat measured by this technique has been found to have a strong correlation with visceral fat assessed by MRI and CT with an R^2 with MRI of 0.948 [28, 29]. Participants were started on a reduced calorie diet and a partially supervised program of moderate to vigorous physical activity that was escalated over time in both volume and intensity.

Specimen processing for CASC

Identification of CASCs followed the modified protocol outlined by Duda et al. [30] and utilized by Ghosh et al. in their study of breast cancer survivors [18]. Complete methodological details are provided in the Supplementary File; a brief description is provided here. Typically, blood was collected in at least two 8-ml cell preparation tubes with anticoagulant and a gel separator (Becton–Dickinson, catalog# SKU 362761) and transferred on ice to the laboratory. Blood was centrifuged at 20 °C and the buffy coat located just above the gel barrier was removed for analysis of peripheral blood mononuclear cells (PBMCs). Viable cell number was determined by trypan blue exclusion. After Fc-receptor blocking, six replicate aliquots of 500 µL each were processed as follows: one unstained; one each for the three individual antibodies FITC-anti-human CD31, eFluor® 450-anti-human CD45, PE-anti-human CD34; one for the combined three antibodies; and one for the three matched isotype controls. After incubation in the dark on ice for 30 min, aliquots were rinsed twice and resuspended in a final volume of 500 µL of phosphate buffered saline. See Fig. 1 for a general schematic of the procedure.

Flow cytometry

Flow cytometry was performed on the day of collection at the University of Kansas Medical Center Flow Cytometry Core Laboratory on a Becton–Dickinson model LSR II equipped with 405, 488, 552, and 633 nm lasers. Unstained, isotype controls, and single antibodies were run to allow compensation for fluorescence overlap between fluorophores. Non-mononuclear cells, cell clumps, and debris were excluded by gating on forward and side scatter assessment. All PBMCs in a 500 µL aliquot were counted. Automatic fluorescence compensation was found to be inaccurate for samples with low numbers of $CD34^+$ cells. Therefore, manual compensation was performed on all samples to ensure appropriate cell gating. The gate for the $CD34^{bright}$ cell population was intentionally set high to assure selection of circulating hematopoietic progenitor cells, even though this reduced the



Cell Population	Median Cells Counted	Median Proportion of Prior Population
Total Blood Cells	**5,200,200**	
Single PBMCs	**720,000**	**12%**
CD34 bright	**875**	**0.12%**
CD31- CD45-	**10**	**1.3%**
CASC Frequency	**13 per million PBMCs**	

Fig. 1 Flow cytometry assessment of circulating adipose stem cells from peripheral blood. This illustrates the processing of blood specimens to obtain peripheral blood mononuclear cells (PBMCs) followed by interrogation by flow cytometry. First, debris is gated out and then single cells are identified by size and shape using forward and side angle light scatter parameters. Next, cells with very bright fluorescence for CD34 are selected. Finally, $CD34^{bright}$ are further characterized by CD31 and CD45 fluorescence (four populations). The $CD34^{bright}$ $CD31^-$ $CD45^-$ cells are classified as CASCs. The inset table indicates the successive application of selection criteria, resulting in the identification of a very rare population of cells in peripheral blood

number of cells identified as CASC. CASC frequency was expressed as the number of $CD34^{bright}CD31^{-}$ $CD45^{-}$ cells per million mononuclear cells assessed. FlowJo®, LLC (Ashland, Oregon) software version 10.2 was used for all analyses.

Statistical analysis

All statistical analyses were performed using SPSS version 24 (IBM). Due to most variables not being normally distributed and overall small sample sizes, non-parametric methods were used to evaluate statistical significance (defined as $P < 0.05$); e.g., Mann–Whitney test was used to evaluate differences in CASC frequencies for categorical variables, Spearman's correlation coefficient for continuous variables, and paired Wilcoxon signed-rank test for within-subject changes over time. Two-sided tests were used throughout, with no adjustment for multiple comparisons.

Results

Characteristics of the high-risk cohort

Women in the high-risk cohort had Tyrer–Cuzick risk predictions, anthropomorphic assessments, DXA body composition, and blood obtained for CASC evaluation between February and December of 2016. Median age was 52 and median residual lifetime risk calculated by the Tyrer-Cuzick model was 27%. Median BMI was 30.7 kg/m^2 (range 19.2–46.1 kg/m^2) and fat mass index 15.2 kg/m^2 (range 4.8–23.4 kg/m^2). Note that a fat mass index of 13 kg/m^2 by DXA is considered as obese [22].

An average of 3 ml of buffy coat was obtained for the high-risk group for CASC assessments. However, given the need for multiple single antibody and control tube aliquots, material prepared from one buffy coat aliquot of 500 µL was generally all that was available for CASC assessments for high-risk women. Using this 500 µL aliquot, a median of 0.59 million PBMCs (range 0.26–1.20) were examined by flow cytometry with a median frequency of 9.7 CASC per million PBMCs (range 0–138). Table 1 gives the age, menopause status, BMI, fat mass index, PBMC number, CASC number, and CASC frequency for each of the 33 high-risk individuals. Summary demographic and anthropomorphic characteristics and breast cancer risk variables are shown in Supplementary Table 1. The datasets analyzed are available from the corresponding author on reasonable request.

There were no statistically significant correlations of CASC frequency with age, waist circumference, or Tyrer-Cuzick risk estimates. CASC frequency trended positively with BMI ($p = 0.066$; Spearman correlation), Fat Mass Index ($p = 0.062$), and percent android fat ($p = 0.077$). For BMI dichotomized at < 35 versus > 35 kg/m^2, i.e., less than severely versus severely obese, CASC frequency was higher in the ten women in the severely obese group than the 23 women in the less than severely obese group (medians of 16.3 vs. 8.8 CASC per million PMBCs) ($p = 0.018$; Mann–Whitney test) (Supplementary Fig. 1). Because there were seven specimens where less than 0.5 million PBMCs were assessed (and CASC were undetectable in four of these), we increased the amount of blood collected in the subsequent breast cancer survivor cohort so as to ensure examination of > 0.5 million PBMC in all cases.

Characteristics of the breast cancer survivor cohort

After completion of CASC assessments in the high-risk cohort, CASC frequency was incorporated into a pilot clinical trial initiated to test a 3-month diet and physical activity intervention in obese sedentary breast cancer survivors. Ten survivors had blood drawn for CASC frequency at enrollment (November 2016) and again immediately following the 3-month intervention (February–March 2017). Median age was 61 years (range 50–70 years), all were post-menopausal, nine were Caucasian, and seven were currently taking aromatase inhibitors. Median time since breast cancer diagnosis was 25 months. All had a BMI > 30 kg/m^2 (median 37 kg/m^2) and self-reported < 60 min of purposeful exercise per week. All had a fat mass index > 13 kg/m^2 (median 17 kg/m^2). Median visceral fat was 1.7 kg (range 1.4–3.0); with values in excess of 1.18 kg by DXA considered to confer an increased risk for development of metabolic syndrome [21]. Baseline median CASC frequency for survivors was 16.4 per million PBMCs (similar to that observed for the ten severely obese high-risk women). Individual demographic, treatment, and baseline anthropomorphic data are shown in Supplementary Table 2. No statistically significant correlations were detected between these variables and baseline CASC frequency. While there were, as expected, correlations between the iDXA derived and weight-related (weight, BMI, waist circumference, FMI) baseline values shown in Supplementary Table 3, only a few correlations were detected with the variables of race, age, prior chemotherapy, current aromatase inhibitor use, and interval from diagnosis (Supplementary Table 2). Associations of greater android fat (percent of region) and visceral fat mass with prior chemotherapy use were marginal ($p = 0.033$ and $p = 0.019$) and did not survive correction for multiple comparisons.

Change in CASC following a diet and exercise intervention

Median weight change was -6.4 kg ($+0.4$ to -20.5 kg) after the 3-month intervention with loss occurring in 9/10 breast cancer survivors. Supplementary Table 3 also

Table 1 Individual data from flow cytometric assessment of CASC frequency for 33 high-risk women (arranged in order of increasing PBMCs assessed)

Age years	Menopause status	Hormone replacement therapy	BMI (kg/m^2)	Fat Mass Index	Single PBMC count	CASC count	CASC frequency (per 10^6 PBMCs)
37	Pre	No	22.9	7.6	263,639	1	3.8
58	Post	Yes	32.8	17.0	342,390	5	14.6
56	Post	No	19.2	4.8	369,819	0	0.0
49	Post	Yes	23.3	6.5	376,338	0	0.0
50	Pre	No	20.0	6.6	384,372	1	2.6
64	Post	Yes	29.8	15.6	395,522	0	0.0
56	Post	No	30.3	15.3	417,255	0	0.0
44	Pre	No	28.8	13.3	509,598	11	21.6
56	Post	No	33.9	18.4	520,091	3	5.8
59	Post	No	34.5	18.0	525,537	5	9.5
47	Post	No	37.1	20.6	550,288	3	5.5
58	Post	No	38.2	19.7	552,416	4	7.2
44	Pre	No	30.2	14.4	558,892	6	10.7
54	Post	Yes	29.8	13.8	577,209	1	3.5
35	Pre	No	35.0	18.1	582,524	23	39.5
57	Post	No	34.6	17.7	590,541	4	6.8
49	Pre	No	27.5	13.3	591,055	8	13.5
41	Post	Yes	40.2	21.2	646,872	3	4.6
40	Pre	No	40.2	22.2	651,383	17	26.1
33	Pre	No	34.0	16.0	656,479	0	0.0
61	Post	No	22.5	7.8	698,373	13	18.6
64	Post	Yes	22.4	7.4	711,825	10	14.0
56	Post	Yes	29.7	15.1	731,883	9	12.3
35	Pre	Yes	46.1		749,319	8	10.7
36	Pre	No	29.8	14.4	767,462	3	3.9
54	Pre	No	42.2	23.4	798,913	58	72.6
59	Post	Yes	30.7	14.3	815,892	8	9.8
40	Post	Yes	30.8	13.6	948,013	25	26.4
38	Pre	No	27.4	11.1	988,324	5	5.1
47	Pre	No	37.4	17.9	996,813	11	11
59	Post	Yes	36.3	18.2	1,020,415	22	21.6
71	Post	Yes	37.1	19.9	1,055,266	138	130.8
41	Pre	No	21.5	6.2	1,201,123	16	13.3
50	Median		30.7	15.2	590,541	5	9.8
33	Minimum		19.2	4.8	263,639	0	0.0
71	Maximum		49.1	23.4	1,201,123	138	130.8

provides change over time values for the various body composition parameters. Again, there were correlations between change and baseline values for most body composition parameters, as well as correlations for change values between the parameters. However, with the exception of a marginal correlation observed between change in waist circumference and prior chemotherapy use ($p = 0.019$), the personal history variables in Supplementary Table 2 had no impact on weight or fat loss.

Table 2 provides individual CASC values at baseline and 3 months, as well as total mass change and visceral fat lost after the 3-month intervention. Median CASC frequency at baseline was 16.4 cells per million PBMCs and at 3 months was 14.3 cells per million PBMCs. CASC frequency reduction was observed in 6/10 survivors. CASC frequency decreased in all survivors with at least a 5 kg total mass loss or a 0.7 kg visceral fat loss.

Table 2 Individual data for CASC assessment at baseline and after 3 months of weight loss intervention for ten breast cancer survivors

Study ID	Baseline			3 months			Change w/intervention	
	Single PBMC count	CASC count	CASC frequency (per 10^6 PBMCs)	Single PBMC count	CASC count	CASC Freq.	Weight change, kg	Visceral fat loss, kg
301	809,521	13	16.1	2,222,210 ↑	103	46.4 ↑	+0.4	.023
303	905,363	20	22.1	2,026,995 ↑	28	13.8 ↓	−14.0	.737
304	616,091	3	4.9	1,979,686 ↑	9	4.5 ↓	−7.0	.290
305	542,961	5	9.2	1,817,123 ↑	27	14.9 ↑	−4.7	.395
306	928,044	91	98.1	1,421,737 ↑	141	99.2	−1.8	.623
307	736,938	5	6.8	866,011 ↑	3	3.5 ↓	−9.8	.528
309	648,726	29	44.7	694,747 ↑	18	25.9 ↓	−20.5	.954
310	777,451	13	16.7	888,584 ↑	31	34.9 ↑	−4.4	.010
311	1,099,218	30	27.3	1,103,567 ↑	7	6.3 ↓	−10.8	.616
312	1,514,672	21	13.9	1,574,821 ↑	19	12.1 ↓	−8.0	.545
Median	793,486	16.5	16.4	1,498,279	23	14.3	6.4	.537
Minimum	542,961	3	4.9	694,747	3	3.5	−20.5	.010
Maximum	1,514,672	91	98.1	2,222,210	141	99.2	+0.4	.954

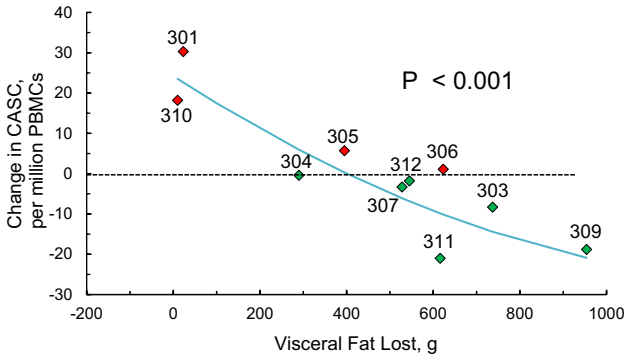


Fig. 2 Correlation of change in CASC frequency (per million PBMCs) with amount of visceral fat lost (in grams) for ten breast cancer survivors who completed a 3-month diet and exercise weight loss intervention. Numbers indicate subject study ID numbers, as per Table 2

In univariate analyses, change in CASC frequency was significantly associated with change in waist circumference, BMI, and iDXA-measured total mass, lean mass, fat mass, fat mass index, percent total body fat and percent fat in gynoid and android regions, android region, and visceral fat mass ($p < 0.01$ for all; Spearman correlation). None of the clinical history characteristics in Supplementary Table 2 had an effect on change in CASC frequency. Change in visceral adipose had the strongest association ($p < 0.00078$) and was the only significant independent predictor of change in CASC frequency in a multivariate analysis. Figure 2 shows the change in CASC frequency as a function of the amount of visceral fat lost.

Discussion

Our pilot study is the first to examine CASC frequency in high-risk women and breast cancer survivors as a function of DXA measures of adiposity. In our high-risk cohort a positive trend was observed between CASC frequency, fat mass index and percent android fat in addition to BMI. Visceral fat could not be measured in the high-risk cohort given the type of machine and software program used but visceral fat is a subset of android fat.

When only the 33 high-risk women are considered, those with severe obesity (BMI > 35 kg/m^2) had a significantly higher CASC frequency than women with a BMI < 35 kg/m^2 (16.3 vs. 6.8 CASC per million PBMCs). CASC were undetectable in four of seven specimens where < 0.5 million PBMCs were assessed. However, these seven women were not obese, which may have introduced bias. Optimally, subsequent studies should ensure a minimum of 0.5 million PBMCs be assessed (which may require more than 16 mL of blood being collected). This would provide greater confidence for any observed low or zero values.

Our results suggesting an association with CASC and BMI are in general agreement with those of Bellows et al. and Ghosh et al., despite our ~ 10-fold lower CASC frequency both in high-risk women and cancer survivors [17, 18]. The lower CASC frequency is likely due to conservative gating on our part for CD34^{bright} cells and thus fewer cells detected as CASC. At such low event rates, we found that compensation for auto fluorescence was important in defining appropriate gating parameters. It is also possible that the specific monoclonal antibody clones may be important given

considerations of specificity and background. We used the same CD31 antibody clone (WM59) that was used by Bellows [17] and Ghosh [18]. We used the same antibody clones for CD34 (563) and CD45 (2D1) as Ghosh [18]; however, these were different than those used by Bellows (HI30 and 8G12, respectively) [17].

Our study is also the first to correlate change in CASC frequency with change in multiple DXA-determined adiposity measures after a diet and exercise intervention aimed at weight reduction in obese breast cancer survivors. On univariate analyses, there were highly significant associations between change in CASC frequency and change in multiple adiposity measures as measured by iDXA. On multivariate analysis, change in visceral adipose mass by itself explains 75% of the variance in change in CASC frequency, as might be expected from preclinical studies [12]. Visceral adipose amount in obese women is strongly correlated with development of insulin resistance and metabolic syndrome, both of which are implicated in risk of recurrence [25]. Given the high VAT values of our cohort at baseline, we could predict that approximately half of these women were at increased risk to develop metabolic syndrome [21]. Limitations to the interventional portion of the study in cancer survivors include treatment heterogeneity (some of the patients were on aromatase inhibitors) and small sample size. Nonetheless, our findings would support those of Ghosh et al. who found no overall change in CASC with an exercise intervention but reduction in CASC for those with a decrease in skin fold thickness (a surrogate for fat loss) [18]. These investigators are currently exploring CASC along with pro-inflammatory cytokines as response indicators in breast cancer survivors randomized to an anti-inflammatory nutrition intervention vs control [31]. Our findings require duplication in a much larger group of survivors in which similar numbers of PBMCs are examined before and after a diet and exercise intervention.

Finally, there are several limitations to the assay itself, including the need to perform the assay the same day as the blood draw to avoid fixation or freezing artefacts, the subjective nature of the gating procedure, and the very small numbers of CASCs.

For women at increased risk for development of breast cancer, CASC frequency may be higher for those with BMI-defined severe or worse obesity (Class II, III obesity); although studies should be repeated with a larger sample size. Given the strong correlation between changes in visceral adipose and change in CASC frequency in breast cancer survivors with significant mass and fat loss, CASC assessment should be further explored as a bio-indicator of obesity-related risk of recurrence and response in Phase II calorie restriction and physical activity trials.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The conduct of the trial complies with the current laws of the United States of America.

Research involving human participants and/or animals All procedures and protocols were approved by the Human Subjects Committee (Institutional Review Board) of the University of Kansas Medical Center. All studies have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent Written informed consent was obtained from all participants.

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