Circulating adipose stromal cells (CASCs) as a potential biomarker of response to weight loss interventions in obese women at high risk for breast cancer

By

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Circulating Adipose Stromal Cells (CASCs) as a Potential Biomarker of Response to Weight Loss Interventions in Obese Women at High Risk for Breast Cancer

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Abstract

Background. Obesity is a modifiable risk factor for breast cancer in the United States. White adipose tissue is increased in obese (BMI ≥ 30 kg/m^2) women and the dysfunction resulting from adipocyte hyperplasia and hypertrophy as well as the increase in visceral and ectopic fat results in an increase in local and often systemic pro-inflammatory cytokines and bioavailable estrogen. Adipose stromal cells play a key role in releasing estrogens and pro-inflammatory cytokines, while circulating adipose stromal cells (CASCs) home to tumor sites and promote angiogenesis and vascularization. CASCs have been implicated in promoting metastases in individuals with cancer and have been correlated with BMI in both cancer and non-cancer patients. In a cross sectional study in women at high risk for development of breast cancer, we examined whether CASC frequency correlates with additional measures of adiposity and tissue measures of short term risk. In a pilot study of a 3 month weight loss intervention in obese sedentary breast cancer survivors we also assessed whether CASC frequencies changed with weight and fat loss.

Methods. 34 women at high risk for development of breast cancer were recruited primarily for random peri-areolar fine needle aspiration (RPFNA) for risk assessment and also underwent Dual-energy X-ray absorptiometry (DEXA) body composition, anthropomorphic assessment, and non-fasting venous blood collection as part of HSC4601. 10 obese sedentary breast cancer survivors recruited as part of a weight loss and exercise trial (STUDY00004575) underwent DEXA body composition, anthropomorphic measures, and phlebotomy prior to and after a 3-month intervention. Mononuclear cells were isolated from the non-fasting blood and the frequency of CASCs (characterized as $CD34^{bright}CD31^{-}CD45^{-}$ cells) was assessed by flow cytometry.

Results. In the cross sectional study in high risk women, CASC frequency ranged from 0 to 0.013% (median 0.001%) for 14 non-obese and 20 obese women. There was an association between CASC frequency and BMI (range 19 – 46 kg/m^2), as both a linear correlation (P=0.03) and when dichotomized at a BMI of 30 kg/m^2 (P=0.05). A stronger relationship was observed between BMI and CASCs when dichotomizing BMI at < 35 kg/m^2 and ≥ 35 kg/m^2 (P=0.009). CASC frequency was correlated with low mammographic breast density (P=0.018) in high risk women possibly due to high BMI in women with < 5% density. Decrease in CASC frequency in 10 obese breast cancer survivors undergoing a 3 month diet and exercise intervention was linearly correlated with decreases in weight, BMI, and visceral fat.

Conclusions. These findings suggest that evaluation of circulating adipose stromal cells could have value as a response biomarker in weight loss intervention trials of both high risk women and breast cancer survivors.

Acknowledgments

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I would also like to acknowledge Won Choi, Tanya Honderick, Beth Kerling, Farah Marhusin, and the rest of the Department of Preventive Medicine for the opportunity to participate in the Masters of Science in Clinical Research program. Their dedication to the success of my research and career development will certainly accelerate my path to becoming a clinician-scientist.

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List of Abbreviations

ASC	Adipose Stromal Cell
BMI	Body Mass Index
CASC	Circulating Adipose Stromal Cell
CT	Computerized Tomography
DEXA	Dual-Energy X-Ray Absorptiometry
HSC	Human Subjects Committee
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
MRI	Magnetic Resonance Imaging
RPFNA	Random Periareolar Fine Needle Aspiration
WAT	White Adipose Tissue

1 Background

Breast cancer is the most common cancer among women (excluding skin cancers) and the second leading cause of cancer-related mortality in women [1]. Although mortality rates have been decreasing over time due to both early detection and more effective treatment strategies [1], successful primary and secondary prevention interventions can also reduce morbidity by reducing or eliminating local and systemic treatment [2]. Obesity is associated with a 30% increase in risk of breast cancer in post-menopausal women and increased risk of recurrence and mortality once breast cancer is diagnosed [3]. The link between obesity and breast cancer risk and recurrence is likely mediated at least in part by local and systemic increases in bioavailable estrogen and testosterone and inflammatory factors [4]. Obesity is also likely to foster angiogenesis necessary both for development of invasive cancer and metastases although this link is less well studied [5, 6]. Adipose stromal cells are hypothesized to contribute to cancer risk by increasing estrogen levels in response to aromatase, releasing inflammatory cytokines, and promoting angiogenesis [7-9]. Reversible risk biomarkers are often used as surrogate markers of response in phase II prevention and survivorship interventional trials for dose finding or to inform the design of a Phase III cancer incidence or recurrence trial. They can also be used to help improve short and long term compliance with an intervention. These surrogate markers of response should be biologically plausible and associated either with the outcome of interest or other known risk factors. Increased number of circulating adipose stromal cells (CASCs) has been positively correlated in cross sectional studies with BMI in individuals with and without cancer [10-12], but relation to other measures of adiposity and risk biomarkers in women at increased risk based on family history has not been explored. Probably most important is the issue of whether CASCs are altered by successful weight and fat loss in sedentary obese breast

cancer survivors. If so serial CASC assessment could serve as a potential surrogate indicator of the effect of weight and fat mass loss on disease free survival.

1.1 Statement of Purpose

The purpose of this investigation was to explore the potential of CASCs to be used as a risk biomarker for obese women at increased estimated risk of breast cancer due to family history or other health history variables; and to explore if CASCs were associated with any other measures of adiposity besides body mass index (BMI). We also wished to evaluate the potential utility of CASCs as a response biomarker in behavioral diet and exercise trials by assessing whether successful weight and fat loss modulated CASCs in the blood of breast cancer survivors.

1.2 Research Questions

1. Is body mass index (BMI) associated with CASCs in women who are at high risk for the development of breast cancer?
2. Are other anthropomorphic measures of adiposity such as waist circumference, total fat mass or visceral fat mass more tightly correlated with CASCs than BMI?
3. Are CASCs associated with strong risk factors for breast cancer risk such mammographic breast density and Random Peri-Areolar Fine Needle Aspiration (RPFNA) atypia?
4. Can CASCs be modulated with successful weight and fat loss in a diet and exercise intervention in sedentary obese breast cancer survivors?

2 Literature Review

2.1 Breast Cancer Prevention

Breast cancer has the highest incidence rates of all cancers in women globally [13]. In the US alone, there will be an estimated 252,710 new cases of invasive breast cancer and 63,410 in situ lesions diagnosed in 2017, accounting for 30% of all incident cancers in post-menopausal women. Improved survival for women with breast cancer due to early detection and effective treatment is important progress, but only prevention can decrease incidence and thus avoid the side effects of treatment [12]. Breast cancer is the leading cause of disability-adjusted life-years for women in both developed and developing countries, and the second leading cause of loss of productivity due to cancer diagnosis in the US [13, 14]. Therefore, it is critical to focus on prevention to reduce the burden of breast cancer on women and society as a whole.

2.2 Breast Cancer Risk

Risk prediction models help clinicians stratify risk and make risk level appropriate recommendations for surveillance and risk reduction [15, 16]. Risk factors for breast cancer include non-modifiable (*e.g.* genetics, age of menarche, *etc.*) and modifiable risk factors (*e.g.* obesity, hormone replacement therapy, alcohol use, *etc*.) [17-19]. Women without a prior history of breast cancer are classified as “high risk” for developing breast cancer if they have greater than a 20% lifetime risk or ≥ 1.7% 5-year risk for developing breast cancer [20]. Although these models are generally good for classification of cohorts and are extensively used in research, short term risk prediction for an individual patient is more problematic. We are increasingly using biomarkers to help stratify risk prediction based on family history, medical and reproductive

variables. Adiposity is one such variable but if it is considered at all in these models it is only in the context of Body Mass Index (BMI) which may have limited specificity (see next section).

2.2.1 Obesity as a Risk Factor

At least 1/3 of US adult women are obese (BMI 30 kg/m^2 or higher). Obesity is associated with a 30% or greater increase in breast cancer risk in post-menopausal women [3]. The underlying problem is not necessarily increasing total mass, but rather dysfunctional fat mass which tends to increase with increasing BMI [21]. For instance, individuals with high muscle mass may have a high BMI but little adiposity or risk of metabolic abnormalities. There may be better measurements of adiposity that are more tightly correlated with risk for breast cancer and other diseases, such as waist circumference, total fat or visceral fat by Dual-energy X-ray absorptiometry (DEXA), CT, or MRI scans [22-24]. Further, there may be better measures of the consequences of excess and dysfunctional fat. White adipose tissue (WAT) is increased in obese women and acts as an active endocrine organ, releasing estrogens, pro-inflammatory cytokines, and pro-angiogenic factors locally and systemically. Visceral adipose tissue (VAT) compared to subcutaneous adipose tissue (SAT) has greater proportional responsibility metabolic abnormalities in obese women [8].

Because obesity is a very common modifiable risk factor, diet and exercise studies have been of utmost interest to reduce initial development of breast cancer, risk of breast cancer recurrence, or risk of cardiovascular disease [25]. For breast cancer survivors, maintaining a normal weight and increasing moderate to vigorous physical activity to 150-200 minutes/week can substantially decrease their risk of death, but few cancer survivors achieve this level of physical activity [26, 27]. Studies in the general population have shown that the amount of initial weight loss and the amount of exercise are the most important factors in maintaining long-term

weight loss [28, 29]. However, the amount of moderate to vigorous physical activity that older obese sedentary breast cancer survivors are capable of achieving is unknown and is the goal of the ongoing pilot study by Fabian, *et al*.

2.3 Biomarkers

Risk biomarkers help personalize risk assessment and make risk estimates more precise for individuals. The Biomarker Working Group has defined biomarkers as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [30]. Biomarkers can improve the accuracy of risk prediction models by identifying who is most likely to progress to malignancy or if they have cancer already if they are likely to develop metastases [31]. Established breast cancer risk biomarkers of short term risk include: atypical hyperplasia or LCIS by diagnostic biopsy, atypical cytomorphology by RPFNA especially when combined with measures of proliferation, high area of mammographic breast density, high levels of circulating hormones in postmenopausal women, and the ratio of IGF-1: IGFBP [32-36].

Obese postmenopausal women present special problems in the use of risk biomarkers to refine risk. They often have low yields of epithelial cells on RPFNA and low or absent proliferation by Ki-67, low area of mammographic density which becomes higher with weight loss due to reduction in breast fat, and low IGF-1:IGFBP3 ratio, which increases weight loss. Thus, for obese postmenopausal women more mechanistic and reliable risk biomarkers are needed.

2.4 Circulating Adipose Stromal Cells

Adipose stromal cells (ASCs) are the adipose tissue-derived multipotent cell population in WAT that allow for proliferative adipogenesis in obesity [37]. ASCs taken from individuals on

high fat diets have been shown to promote a pro-inflammatory state via release of pro-inflammatory cytokines such as, IL-1, IL-6, and TNF-α, and pro-inflammatory adipokines such as leptin [37, 38]. In vitro, ASCs are able to mobilize and invade, indicating their capability to coordinate tumorigenesis [39]. While the mechanism is not fully understood, ASCs appear to be capable of not only invading, but also circulating in the blood stream where they are referred to as circulating adipose stromal cells (CASCs) [10, 11, 40]. ASCs and CASCs play a key role in releasing estrogens in adipose tissue and in the circulation, and increased bioavailable estrogen is known to increase risk of breast cancer [41, 42]. CASCs have been shown to home to tumor sites and promote angiogenesis and vascularization, a key developmental component of breast cancer [8, 9, 43]. The surface antigen phenotype of CASCs is important and tells us about the origins and function of this cell population: CD34 is a hematopoietic stem cell and stromal cell marker; CD45 is a hematopoietic cell marker (excluding red blood cells); and CD31 is an endothelial cell and endothelial progenitor marker [44]. Therefore, excluding CD31 and CD45, while expressing CD34 identifies a stromal cell progenitor population (i.e., $CD34^{+}CD31^{-}CD45^{-}$ as characterized by flow cytometry).

Previous studies have shown that CASCs (expressed as the proportion of all single mononuclear cells in blood) are directly correlated with BMI in disease-free patients, cancer patients, and cancer survivors [10-12]. However, BMI alone as a marker of dysfunctional adipose predisposing to disease has its limitations [23, 45]. Total body fat and in particular visceral fat and ectopic (liver and muscle) fat, and even waist circumference may be better anthropomorphic predictors of metabolic dysfunction than BMI [46]. Visceral fat is much more metabolically active than subcutaneous fat [47] and individuals with high amounts of visceral and ectopic fat, especially those with lower muscle mass, are more prone to insulin resistance,

and higher levels of local and systemic bioavailable hormones, pro-inflammatory and pro-angiogenic factors [46]. To our knowledge, no other cross sectional studies have examined the association of CASC and other risk variables including Tyrer-Cuzick model risk estimation, area of mammographic breast density, atypical cytomorphology and Ki67. Nor have other studies examined CASC in relation to other anthropomorphic variables associated with obesity (waist circumference, DEXA total fat, lean mass). Finally, our study looks at change in CASC with weight loss in breast cancer survivors, a topic of high current interest and association of change in CASCs (%) with change in other anthropomorphic variables. Of these visceral fat is of greatest interest as higher levels are more likely to be associated with adipose dysfunction. Given the importance of CASCs in angiogenesis, CASCs could be used effectively as a risk and response biomarker in trials of calorie restriction and exercise in obese high risk women and breast cancer survivors.

3 Methods

3.1 Participants

Participants were recruited through the University of Kansas Medical Center's Breast Cancer Prevention and Survivorship Research Center. 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.

3.1.1 Cross-Sectional Study in High Risk Women

Thirty-four women at high risk for development of breast cancer were recruited primarily for random peri-areolar fine needle aspiration (RPFNA) for risk assessment. These women also underwent DEXA body composition scans (GE Lunar Prodigy), anthropomorphic assessment,

and non-fasting venous blood collection as part of HSC # 4601. This cross-sectional study was specifically investigating biomarkers in women at high risk of developing breast cancer, but who do not have a prior history of invasive breast cancer. Risk eligibility criteria for the cross-sectional study were any one or more of the following: known high penetrance germline gene mutation (e.g. BRCA1, BRCA2, p53), a first degree relative or multiple 2nd degree relatives with a diagnosis of breast cancer under age 60 (family history), prior breast biopsy, ≥25% mammographic breast density, 5 year Gail risk of ≥ 1.67% or twice the 10-year average population Tyrer-Cuzick risk. A Gail risk of 1.67% is the average risk of a 60 year old with no other risk factors. The DEXA scanner used for the cross sectional study measured total and lean mass, total fat mass, android fat mass, but did not have the software to assess visceral fat mass. Carol Fabian, MD, or Kandy Powers, NP, performed the RPFNA.

3.1.2 Diet and Exercise Intervention in Breast Cancer Survivors

Eleven obese sedentary breast cancer survivors who were recruited as part of a weight loss and exercise trial (STUDY00004575) underwent anthropomorphic measures, initial DEXA body composition, and phlebotomy before and after the intervention. DEXA used in this study was a GE Lunar iDXA which does assess visceral fat in addition to other body composition measures.

This prospective interventional study was designed to determine whether obese sedentary breast cancer survivors could reliably achieve 300 minutes per week of planned physical activity/week with the help of a trainer during a 12 week program of moderate calorie restriction. Inclusion criteria for this study were prior breast cancer and completion of any surgery, radiation or cytotoxic chemotherapy, a BMI of ≥30 kg/m^2, < 60 minutes of exercise per week, possession of a smart phone and the physical ability to exercise. Exclusion criteria were history of diabetes

or metformin use and inability to meet with an exercise trainer twice weekly at a local YMCA. One woman was African-American and all other women were Caucasian.

3.2 Setting

Both studies were conducted as outpatients at the University of Kansas Cancer Center (Westwood Campus) and at the University of Kansas Clinical and Translational Science Unit (CTSU).

3.2.1 Cross-Sectional Study in High Risk Women

Patient demographics, medical history, clinical breast exam, mammogram, RPFNA, anthropomorphic measures including dual-energy x-ray absorptiometry (DEXA). Biologic specimens were collected by clinic staff under proscribed procedures and then transferred to the Breast Cancer Prevention Laboratory for further processing.

3.2.2 Diet and Exercise Intervention in Breast Cancer Survivors

Women were generally referred for this study by their physicians although self-referral was allowed. The baseline visit included collecting patient demographics, performing a medical history and physical exam, anthropomorphic measures, a DEXA scan, and a 6 minute walk test. Baseline fitness was measured with VO_2 peak performed as part of cardio-pulmonary testing. Following two initial in person group meetings explaining the diet and exercise program and how to use their activity trackers and the MyFitnessPal application, participants were started on the program of portion-controlled meals, 5 servings of fruits and vegetables daily (1200-1400 cal/day), and an escalating exercise program. Weekly group phone sessions were conducted by research staff with study participants to discuss their progress and any challenges or issues. The 12 week off-study appointment included all of the same assessments as the baseline appointment.

3.3 Procedures

Venipuncture blood was drawn by one of two trained clinical trial staff in two blue tiger-top tubes for mononuclear cells (8 mL BD Vacutainer CPT tubes). Tubes were immediately put on wet ice and transferred to the breast cancer prevention laboratory within two hours. Identification of CASCs followed the modified protocol outlined by Duda, *et al* [48]. Cells were spun at 1,600 g at 20°C for 25 minutes with no brake. Plasma was separated from the mononuclear layer and cells were counted. Based on cell count calculations, 20 μL per 10^7 cells of Fc-receptor blocking antibody (Miltenyi Biotec) was added and incubated for 10 minutes on ice. Isotype controls and individual fluorochrome conjugated antibodies for CD31 (FITC), CD45 (eFluor), and CD34 (PE) were added to 500 μL aliquots of cells and incubated in the dark on ice for 30 minutes. Anti-CD34 PE and CD34 PE isotype control were added at 20 μL per 10^7 cells via the manufacturer's protocol (BD Pharminogen). Cells were washed twice with 9 mL of ice-cold 1X PBS and spun down at 250 g for 5 minutes with a brake. Pellets were re-suspended in 500 uL of ice-cold 1X PBS for flow cytometry.

3.4 Flow Cytometry

Flow cytometry was performed at KUMC's Flow Cytometry Core Laboratory on a Becton-Dickinson model LSR II equipped with 405, 488, 552, and 633 nm lasers for single-cell suspensions. Unstained, isotype controls, and single antibodies were run to allow for compensation of the machine. Due to the low cell count of circulating $CD34^+$ cells, manual compensation was occasionally necessary to facilitate appropriate cell gating. Cells were gated on the $CD34^{bright}$ cell population and gates were reviewed by two investigators (Appendix Figure 7). Gates were applied to cell suspensions with all three antibodies. The number of

$CD34^{bright}CD31^{-}CD45^{-}$ cells detected was expressed as a percent of all single mononuclear cells. FlowJo®, LLC (Ashland, Oregon, USA) software version 10.2 was used for all analyses.

3.5 Statistical Analysis

All statistical analyses were performed using SAS® Statistical Software version 9.4, SAS Institute Inc., Cary, North Carolina, USA.

3.5.1 Cross-Sectional Study in High Risk Women

CASC frequency was not normally distributed (Shapiro-Wilk $P<0.0001$) and therefore non-parametric analyses were used to evaluate statistical significance. Kruskal-Wallis test was used to evaluate differences in CASC levels between BMI groups ($< 30 kg/m^2$ vs. $\geq 30 kg/m^2$), and to evaluate differences in CASC and BMI levels for mammographic breast density groups. Spearman's correlation coefficient was used to evaluate linear relationships between BMI values and CASC levels.

3.5.2 Diet and Exercise Intervention in Breast Cancer Survivors

CASC frequencies at baseline and 12 weeks were not normally distributed. Paired Wilcoxon signed-rank test was used to compare baseline CASCs to 12 week CASC frequencies. Changes in CASC frequency (12 weeks – 0 weeks) were normally distributed and parametric procedures were used for all analyses comparing 12 week changes in CASCs. Correlation and linear regression were used to evaluate linear relationships between change in CASCs and other continuous variables. Non-parametric analyses or ANOVA were used to compare categorical variables with change in CASC frequencies if the CASCs of categorical groups were non-normally or normally distributed, respectively.

4 Results

For both studies, similar levels of CASCs and single mononuclear cell counts were observed for high risk women and breast cancer survivors with BMI ≥ 30 kg/m^2. For the cross-sectional study, obese high risk women had a mean CASC of 0.002% (range 0.000 – 0.013%). Median single mononuclear cell count was 590,798 cells (range 263,639 – 1,201,123 cells). For the weight loss study, obese breast cancer survivors had a mean CASC of 0.003% (range 0.000 – 0.010) at baseline. Median baseline single mononuclear cell count was 793,486 (range 542,961 – 1,514,672 cells). These consistent findings for both studies in obese women indicate reliability of our results.

4.1 Cross-Sectional Study in High risk Women

There were 14 women with BMI < 30 kg/m^2 and 20 women with BMI > 30 kg/m^2. CASC frequency was found to be nearly statistically different between dichotomized groups with BMI < 30 kg/m^2 and ≥ 30 kg/m^2 (Figure 1A, P=0.05). The mean CASC (%) for BMI < 30 kg/m^2 was 0.001 (range 0.000-0.002). The mean CASC (%) for BMI ≥ 30 kg/m^2 was 0.002 (range 0.000 – 0.013). However, when women were dichotomized into BMI groups < 35 kg/m^2 and ≥ 35 kg/m^2 there was a significant difference between groups (Figure 1B, P=0.009). A linear correlation was noted with CASCs (%) and both BMI (kg/m^2) and total body fat (kg) (Figure 2, P=0.029 and P=0.035, respectively) for the 34 high risk women. Waist circumference, percent fat, lean mass, and android fat were not found to be correlated with CASC levels with Spearman's correlation. We were unable to measure visceral fat in the cross sectional study as the GE Healthcare Lunar Prodigy DEXA scanner used for this cohort did not have the appropriate software. Other breast cancer risk variables factors investigated were age, menopause status, hormone therapy, breast density, cytomorphology, Ki67, hours of self- reported aerobic exercise per week exercise, and

Tyrer-Cuzick estimated 10 year risk. Of these variables only very low breast density (<5%) (Figure 3) was found to be significantly associated with CASC levels (Figure 4A, P=0.02). In this group, the five women with very low mammographic breast density estimates (<5%) were also all obese (Figure 4B, BMI range 32.81-42.15 kg/m^2).

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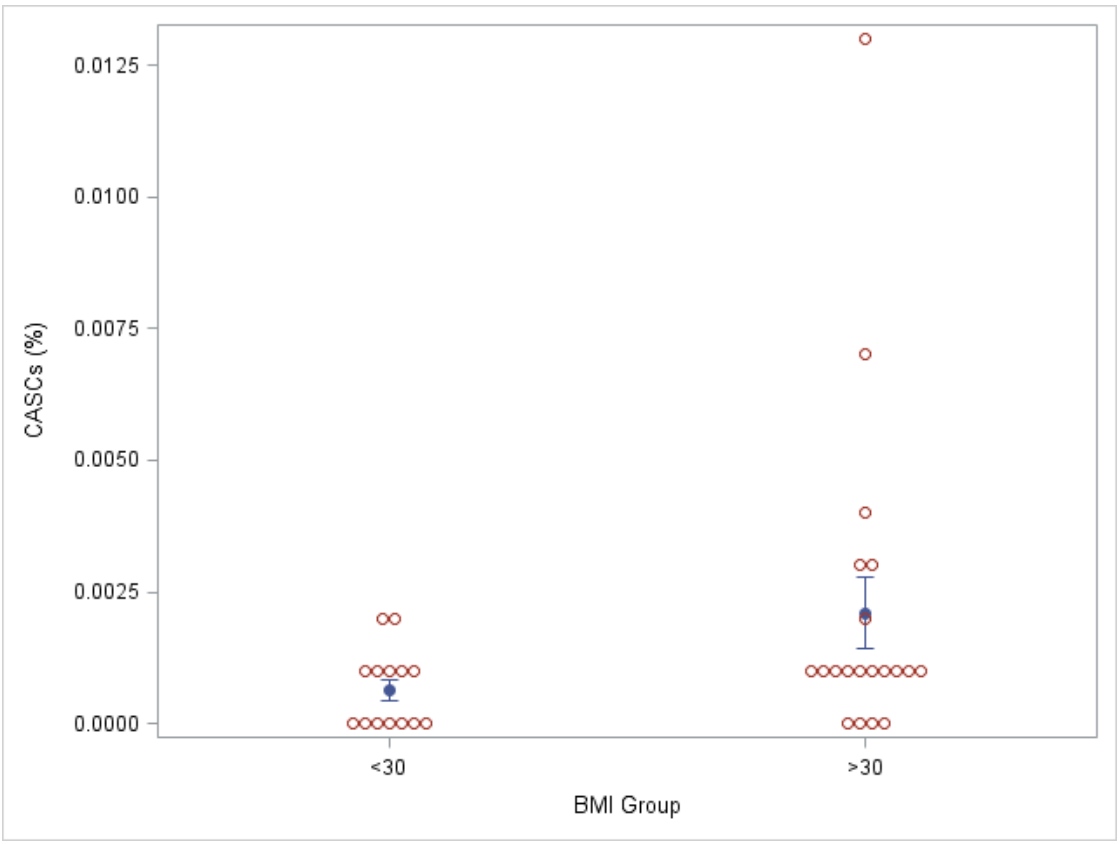

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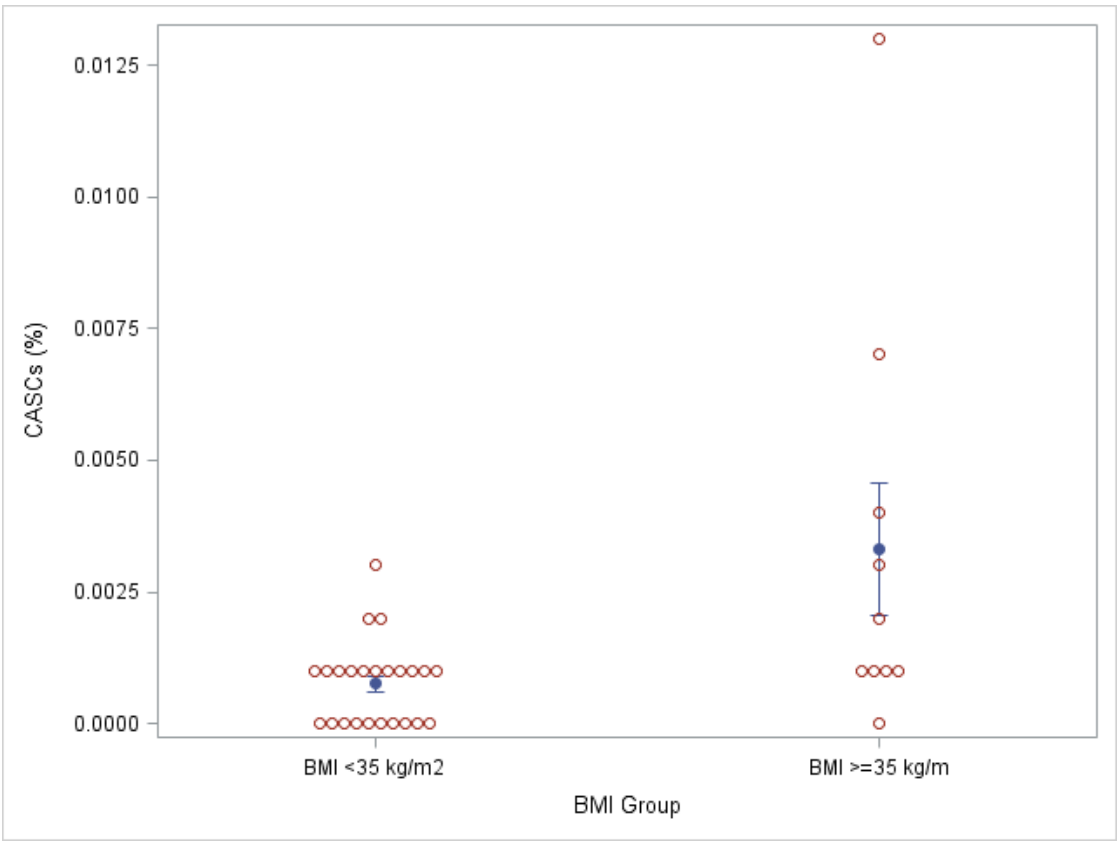

Figure 1: Distribution of CASC levels (%) by Dichotomized BMI Groups in High Risk Women

(A) BMI groups dichotomized at < 30 kg/m^2 (N=14) and ≥ 30 kg/m^2 (N=20). Kruskal-Wallis test used to evaluate significance (P=0.05). Error bars indicate mean and standard error of the mean. (B) BMI groups dichotomized at < 35 kg/m^2 (N=24) and ≥ 35 kg/m^2 (N=10). Kruskal-Wallis test used to evaluate significance (P=0.009). Error bars indicate mean and standard error of the mean.

Table 1: Comparing Characteristics of High Risk Women with BMI < 30 kg/m^2 vs. BMI ≥ 30 kg/m^2

	BMI < 30 kg/m^2 (N=14)	BMI ≥ 30 kg/m^2 (N=20)	P-Value
Age (years) (mean ± SD) Range	49.9 ±9.7 36 – 64	50.4 ± 10.5 33 – 71	0.90
Menopause Status (N, %)			
Pre-Menopause	6 (42.9)	7 (35.0)	0.39
Peri-Menopause	1 (7.1)	0 (0.0)	
Post-Menopause	7 (50.0)	13 (65.0)	
Hormone Replacement Therapy (N, %)			
Yes	5 (35.7)	7 (35.0)	0.97
No	9 (64.3)	13 (65.0)	
Mammographic Breast Density (N, %)			
<5%	0 (0.00)	5 (26.3)	0.02
5-25%	3 (21.4)	7 (36.8)	
25-50%	5 (35.7)	6 (31.6)	
50-75%	6 (42.9)	1 (5.3)	
Tyrer-Cuzick Lifetime Risk (%) (mean ± SD) Range	27.5 ± 11.7 11.4 – 54.5	29.9 ± 18.4 10.2 – 75.9	0.97
Tyrer-Cuzick 10-year Risk (%) (mean ± SD) Range	7.6 ± 6.8 1.8 – 28.5	8.2 ± 7.0 1.6 – 27.1	0.41
Waist Circumference (cm) (mean ± SD) Range	82.5 ± 18.0 32 – 108	105.9 ± 6.3 97 – 120	<0.0001
Total Fat (kg) (mean ± SD) Range	28.1 ± 11.5 12.8 – 43.3	46.6 ± 6.3 37.4 – 59.4	<0.0001
Body Fat (%) (mean ± SD) Range	39.2 ± 9.4 25.1 – 52.5	51.1 ± 3.2 44.0 – 55.6	0.0002
Lean Mass (kg) (mean ± SD) Range	40.9 ± 4.5 33.7 – 46.5	44.5 ± 5.0 36.9 – 54.2	0.087
Android Fat (kg) (mean ± SD) Range	42.3 ± 13.8 16.4 – 58.7	56.3 ± 2.8 49.8 – 60.7	0.0045
Cytomorphology Index Score* (mean ± SD) Range	14.2 ± 1.0 13 – 16	14.3 ± 1.2 12 – 16	0.72
Ki67 (%) (mean ± SD) Range	0.06 ± 0.05 0 – 0.2	0.05 ± 0.05 0 – 0.1	0.61
Weekly Exercise (hours) (mean ± SD) Range	3.9 ± 2.2 0 – 7	2.1 ± 1.9 0 – 7	0.02

*Cytomorphology Index Score Ranges: <11 = Normal; 11-14 = Hyperplasia; 15-18 = Hyperplasia with Atypia.

Kruskal-Wallis tests were performed to evaluate significance between BMI groups and all continuous variables due to lack of normality. Fisher's exact test was used to test differences between BMI groups and categorical variables (menopause status, hormone replacement therapy, mammographic breast density).

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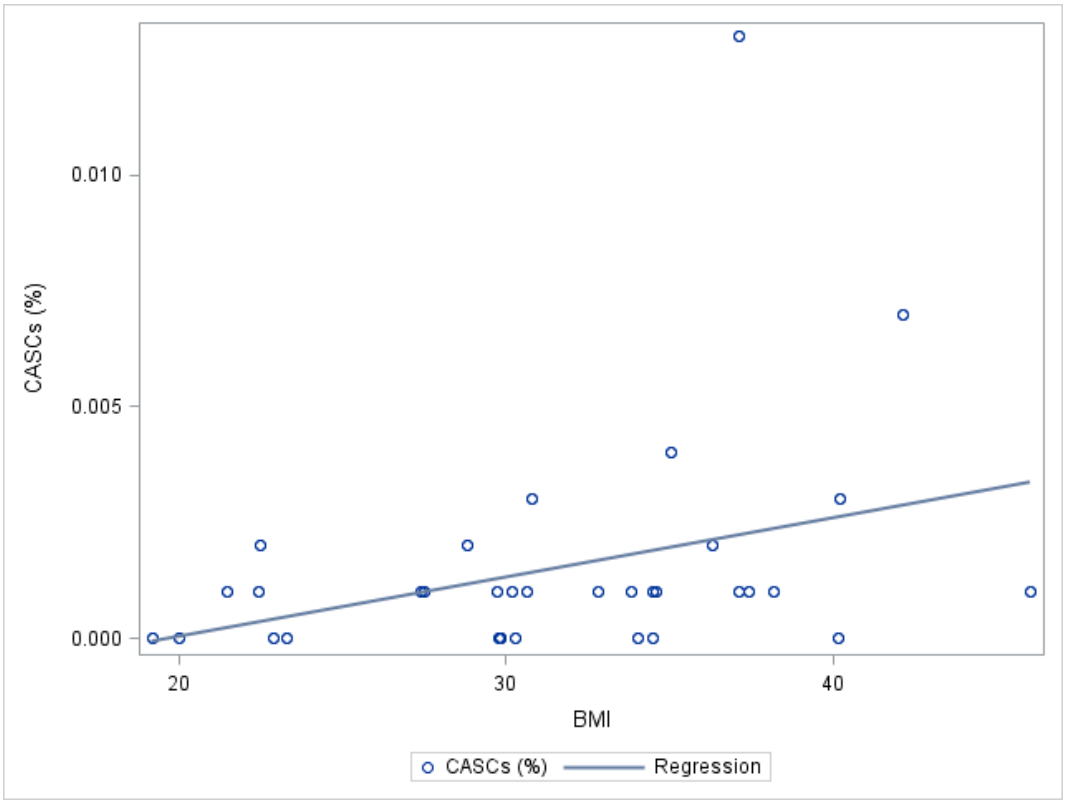

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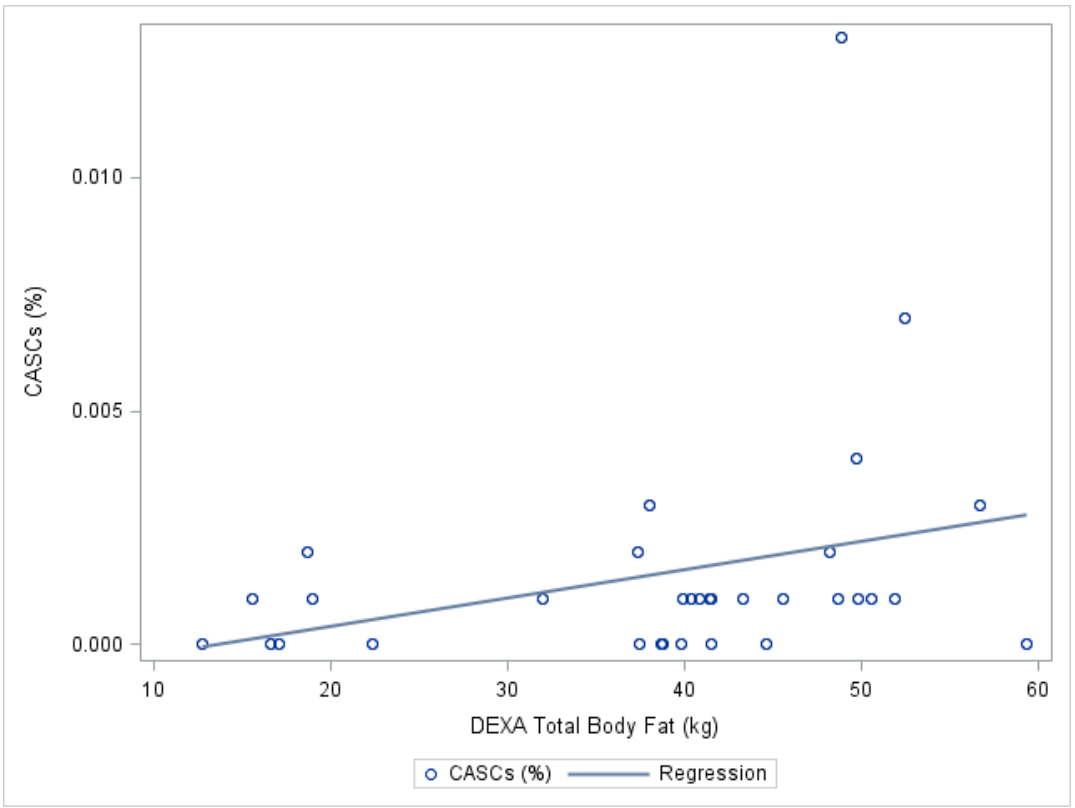

Figure 2: Linear relationship of CASCs (%) with BMI (kg/m^2) and Fat Mass (kg)

(A) Linear correlation between CASCs (%) and BMI of women at high risk for developing breast cancer. Spearman's correlation coefficient R=0.38, P=0.029. (B) Linear correlation between CASCs (%) and total body fat (kg) of women at high risk for developing breast cancer. Spearman's correlation coefficient R=0.37, P=0.035.

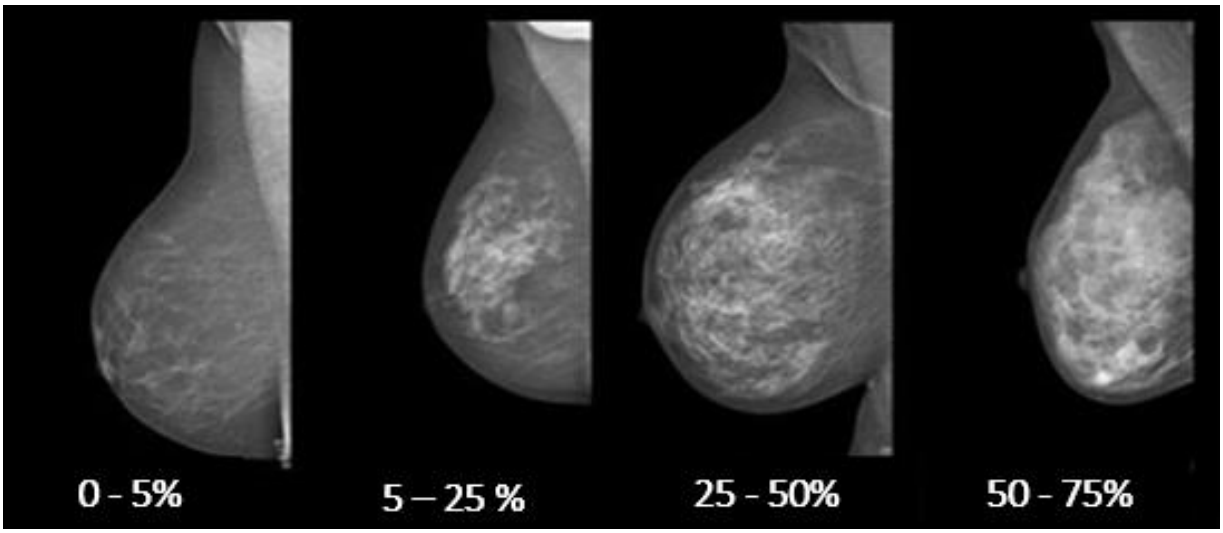

Figure 3: Depiction of Mammographically Dense Area Groups

Mammographic dense area <5% consists of mostly fatty breasts. Estimates of 5-25% generally consists of scattered densities throughout the breast. An estimate of 25-50% is considered moderately dense with > 50% at increased risk for breast cancer [49]. No women in this cohort had > 75% density.

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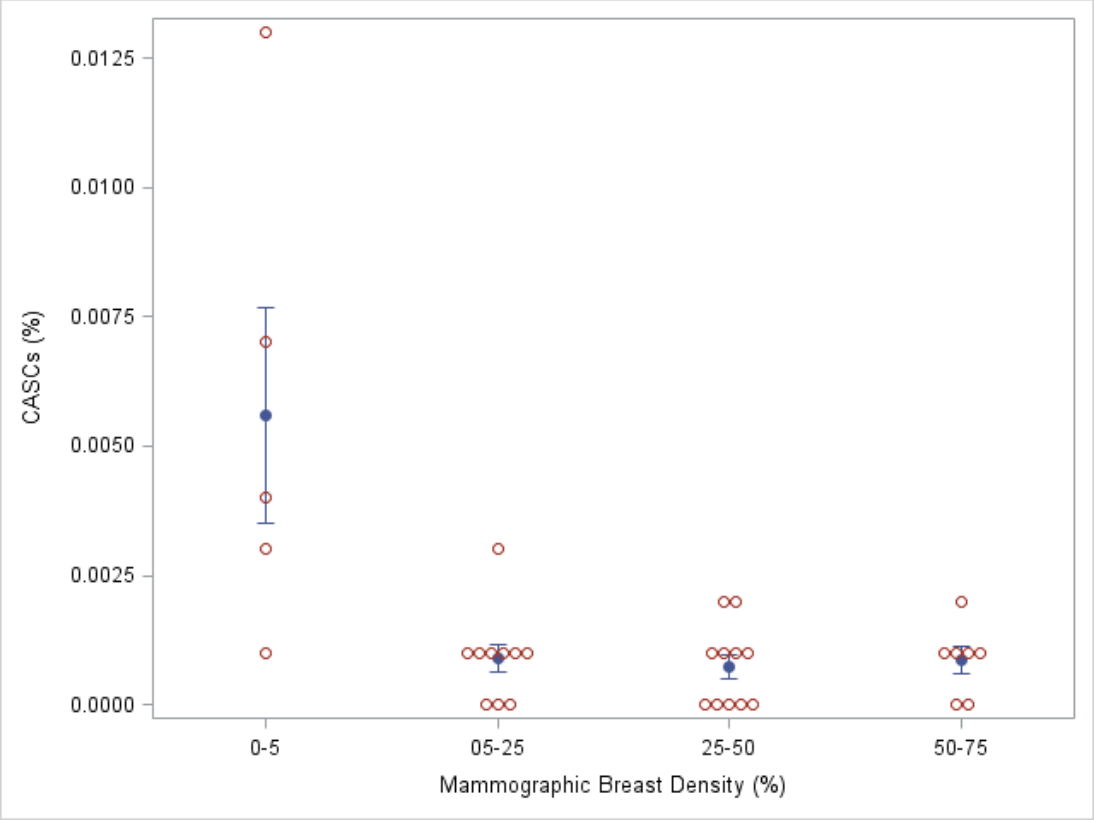

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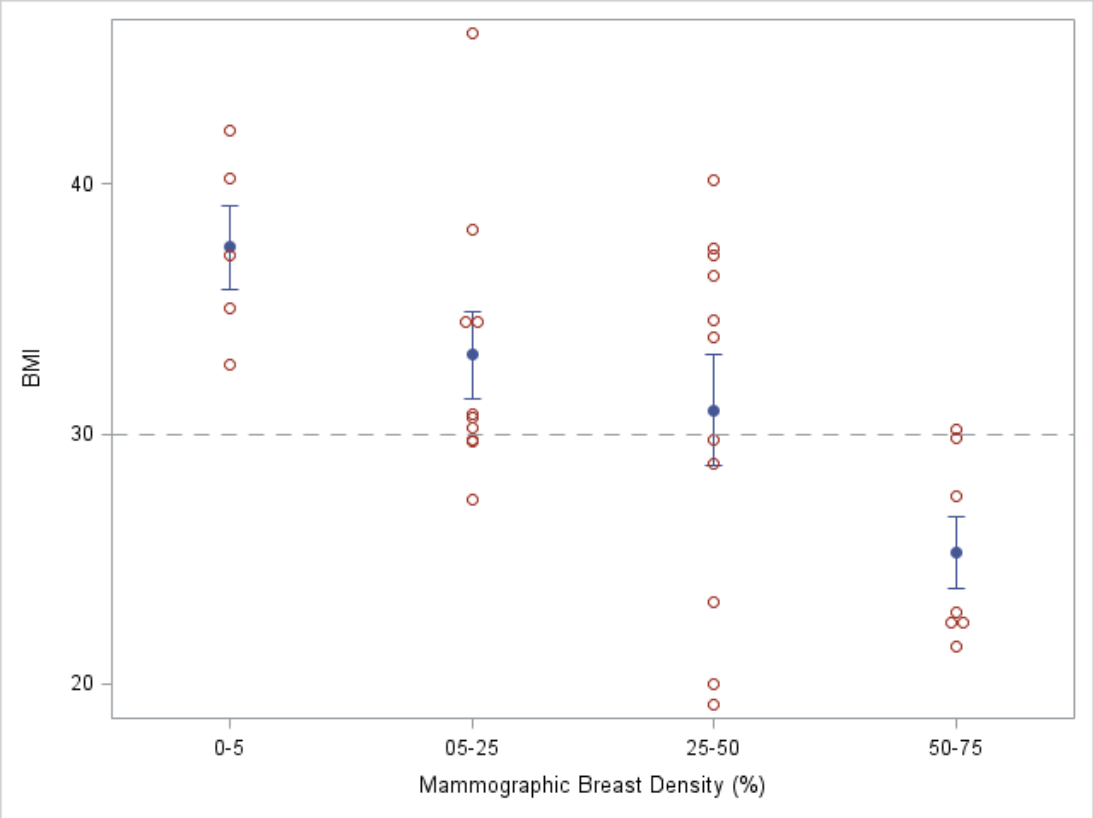

Figure 4: CASC and BMI Distributions for Mammographically Dense Area Groups

(A) Comparison of CASC levels (%) in different mammographic breast density estimate groups. Kruskal-Wallis test used to evaluate significance (P=0.018). Error bars indicate mean and standard error of the mean. (B) Comparison of BMI (kg/m^2) in different mammographic breast density estimate groups. Kruskal-Wallis test used to evaluate significance (P=0.010). Error bars indicate mean and standard error of the mean. Dotted line indicates a BMI of 30 kg/m^2 and visually divides the graph into non-obese (< 30 kg/m^2) and obese categories (≥ 30 kg/m^2).

4.2 Diet and Exercise Intervention in Breast Cancer Survivors

There were 11 women who passed all screening tests and were able to initiate the 12 week diet and exercise intervention. Only 10 of these women had blood drawn for CASC at both baseline and 12 weeks, although all 11 completed the intervention. Baseline characteristics of the 10 participants for which we have specimen for CASCs are included in Table 2. Median age was 61 and median BMI was about 36 kg/m^2. Of the 11 women who completed the weight loss intervention, 9 were able to achieve at least 200 minutes of purposeful physical activity(exercise) /week by week 8 and 6 were able to consistently perform at that level during weeks 8-12 of the interventions. Six women were able to achieve 200 or more minutes of moderate to vigorous physical activity (45-65% of heart rate reserve which we termed “Zone 3”) by week 8 and 4 of those were able to consistently perform at that level during weeks 8-12 of the intervention. Women with higher weight and fat mass loss achieved more zone 3 minutes. Absolute changes in anthropomorphic measures at 12 weeks are given in Table 3. For the weight loss intervention study we were able to use a newer model DEXA (the GE Healthcare iDXA) that uses a fully automated program to segment 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 [50, 51].

Ten women lost weight, with only one woman increasing her BMI by 0.2 kg/m^2. Individual changes in CASCs (%) did correlate with individual changes in adiposity measures. Four women had a decrease in their CASC levels (%), 4 women had no change, and 2 women had an increase in their CASC (%) levels (Figure 5). There were no patient characteristic differences or baseline adiposity variable differences in women whose CASCs decreased, increased, or had no changes (table 3). The women with the greatest proportional decreases in BMI, weight, and fat at 12 weeks exhibited the greatest changes in their CASCs (%). There were linear correlations between changes in CASCs and changes in BMI (kg/m^2), weight (kg), fat mass (kg), and visceral fat (kg). There was no correlation with absolute change in CASCs and absolute change in waist circumference or percent android fat (Table 4).

Table 2: Baseline Characteristics of Breast Cancer Survivors in Diet/Exercise Intervention

Variable	Subject Information (N=10)
Age (years) (median) Range	61 51 – 70
BMI (Mean ± SD) Range	35.76 ± 3.0 30.8 – 39. 8
Days Since Diagnosis (Mean ± SD) Range	1,099 ± 1,043.2 395 – 3,800
Chemotherapy, N (%) Yes No	 4 (40%) 6 (60%)
Anti-Hormonal Therapy, N (%) Yes No	 7 (70%) 3 (30%)

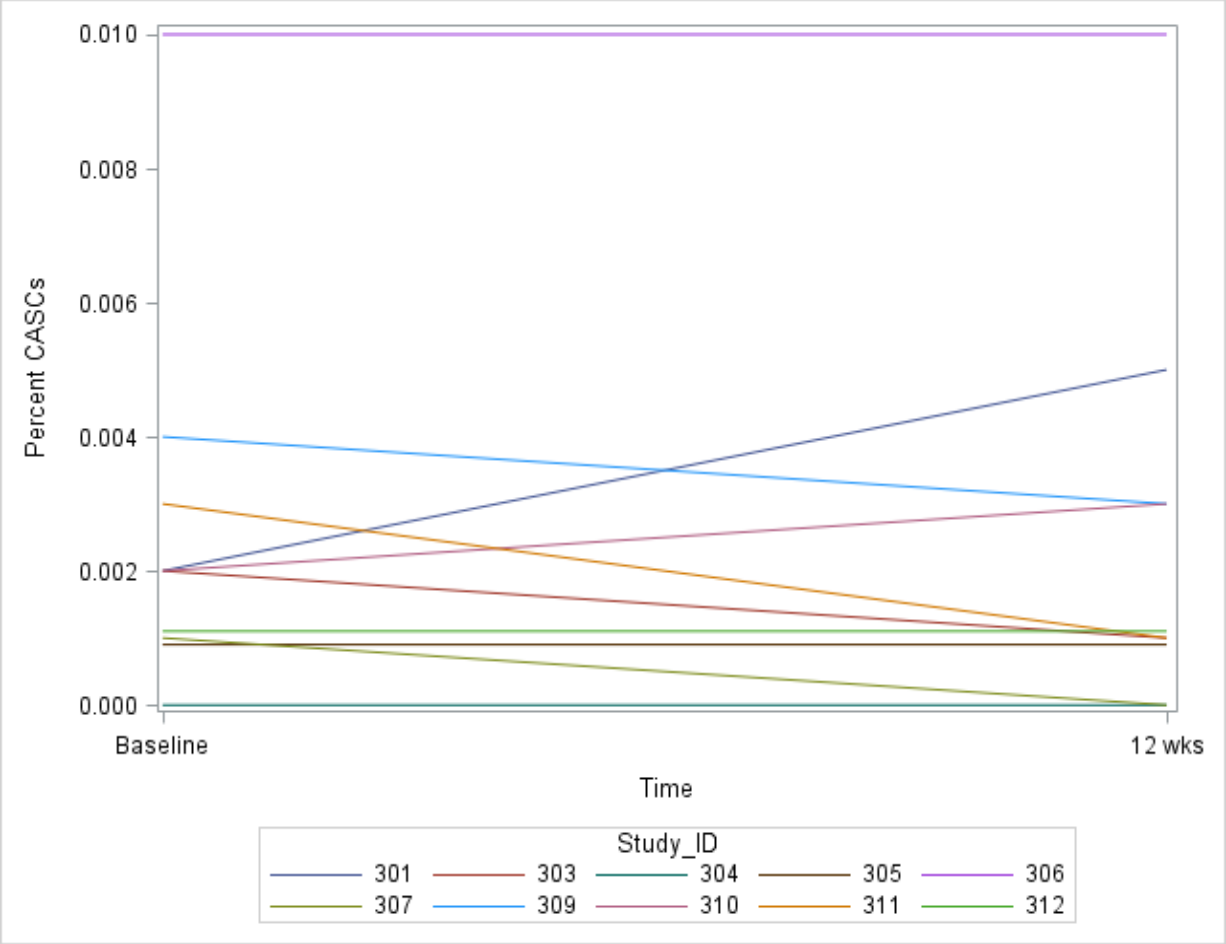

Figure 5: Individual changes in CASCs (%) from Baseline to 12 Weeks

Absolute changes of CASC levels (%) from baseline to 12 weeks are color-coded by participant study ID. Four participants decreased their CASCs. Four participants experienced no changes in their CASCs. Two participants' CASC levels increased.

Table 3: Characteristics of Participants based on Absolute Changes in CASC Levels at 3 Months

		CASC Levels Decreased (N=4)	**CASC Levels No Change (N=4)**	**CASC Levels Increased (N=2)**	**P-Value**
Patient Characteristics	Baseline CASCs Mean (Range)	0.003 (0.001-0.004)	0.003 (0.000-0.01)	0.002 (0.002-0.002)	0.54
	Age (years) Mean (Range)	57 (51 – 62)	63 (59 – 70)	64.5 (64 – 65)	0.16
	Chemotherapy N (%)	2 (50%)	1 (25%)	1 (50%)	1.00
	Anti-Hormonal Therapy N (%)	2 (50%)	4 (100%)	1 (50%)	0.33
	Days Since Diagnosis Mean (Range)	576.3 (395-820)	1,701.3 (395-3800)	940 (485-1395)	0.43
Baseline Adiposity Variables	BMI Baseline (kg/m^2) Mean (Range)	38.0 (37 – 39.8)	34.4 (30.8 – 37.7)	33.9 (31 – 36.9)	0.12
	Weight Baseline (kg) Mean (Range)	103.2 (92.5-121.6)	86.3 (75.9 – 97.7)	96.6 (89.8 – 103.4)	0.15
	Visceral Fat Baseline (kg) Mean (Range)	2.5 (1.9-3.3)	2.0 (1.5-3.1)	1.9 (1.9-2.0)	0.21
Absolute Changes in Adiposity Variables at 12 weeks	BMI Change (kg/m^2) Mean (Range)	-4.9 (-7.2, -3.2)	-2.1 (-2.9, -0.7)	-0.45 (-1.1, 0.2)	0.026
	Weight Change (kg) Mean (Range)	-13.8 (-20.5, -9.8)	-5.4 (-8.0, -1.8)	-2.0 (-4.4, 0.4)	0.026
	Body Fat Change (%) Mean (Range)	-4.6 (-8, -2.4)	-1.7 (-2.2, -0.6)	-0.95 (-1.4, -0.5)	0.026
	Visceral Fat Change (kg) Mean (Range)	-0.8 (-1.1, -0.6)	-0.5 (-0.7, -0.3)	-0.02 (-0.03, -0.01)	0.057

A Kruskal-Wallis nonparametric test was used to evaluate differences between changes in CASC levels (decrease, no change, increase) and continuous measures of patient characteristics, baseline adiposity measures, and absolute changes in adiposity variables at 12 weeks. Fisher's exact test was used to evaluate differences in CASC level changes and categorical variables (chemotherapy, anti-hormonal therapy).

BMI Change = (12 week BMI – 0 week BMI)

Weight Change (kg) = [12 week weight (kg) – 0 week weight (kg)]

Body Fat Change (%) = [12 week body fat (%) – 0 week body fat (%)]

Visceral Fat Change (kg) = [12 week visceral fat (kg) – 0 week visceral fat (kg)]

Table 4: 12 Week Outcomes of Adiposity Variables and their Linear Relationship with Change in CASCs (%)

	Univariate Statistics (N=10)					**Correlation with Change in CASCs (%)**	
	Mean	**Median**	**SD**	**Min**	**Max**	**R**	**P-value**
BMI Change (kg/m^2)	-2.9	-2.7	2.2	-7.2	0.2	0.85	0.002
Weight Change (kg)	-8.1	-7.5	6.1	-20.5	0.4	0.87	0.0009
Fat Mass Change (kg)	-6.2	-5.7	4.5	-15.6	-1.2	0.87	0.0009
Body Fat Change (%)	-2.7	-2.1	2.3	-8.0	-0.5	0.85	0.0018
Visceral Fat Change (kg)	-0.5	-0.6	0.3	-1.1	-0.01	0.73	0.016
Waist Circumference Change (cm)	-9.6	-8.0	6.6	-22.0	-3.0	0.25	0.49
Android Fat Change (%)	-3.3	-2.5	3.8	-10.5	2.1	0.73	0.17

Spearman's correlation was used to evaluate significance of linear relationships between changes in CASC (%) and changes in adiposity measures at 12 weeks.
CASC Change = [12 week CASC (%) – 0 week CASC (%)]
Fat Mass Change (kg) = [12 week fat (kg) – 0 week fat (kg)]
Waist Circumference (WC) Change = [12 week WC (cm) – 0 week WC (cm)]
Android Fat Change (%) = [12 week android fat (%) – 0 week android fat (%)]

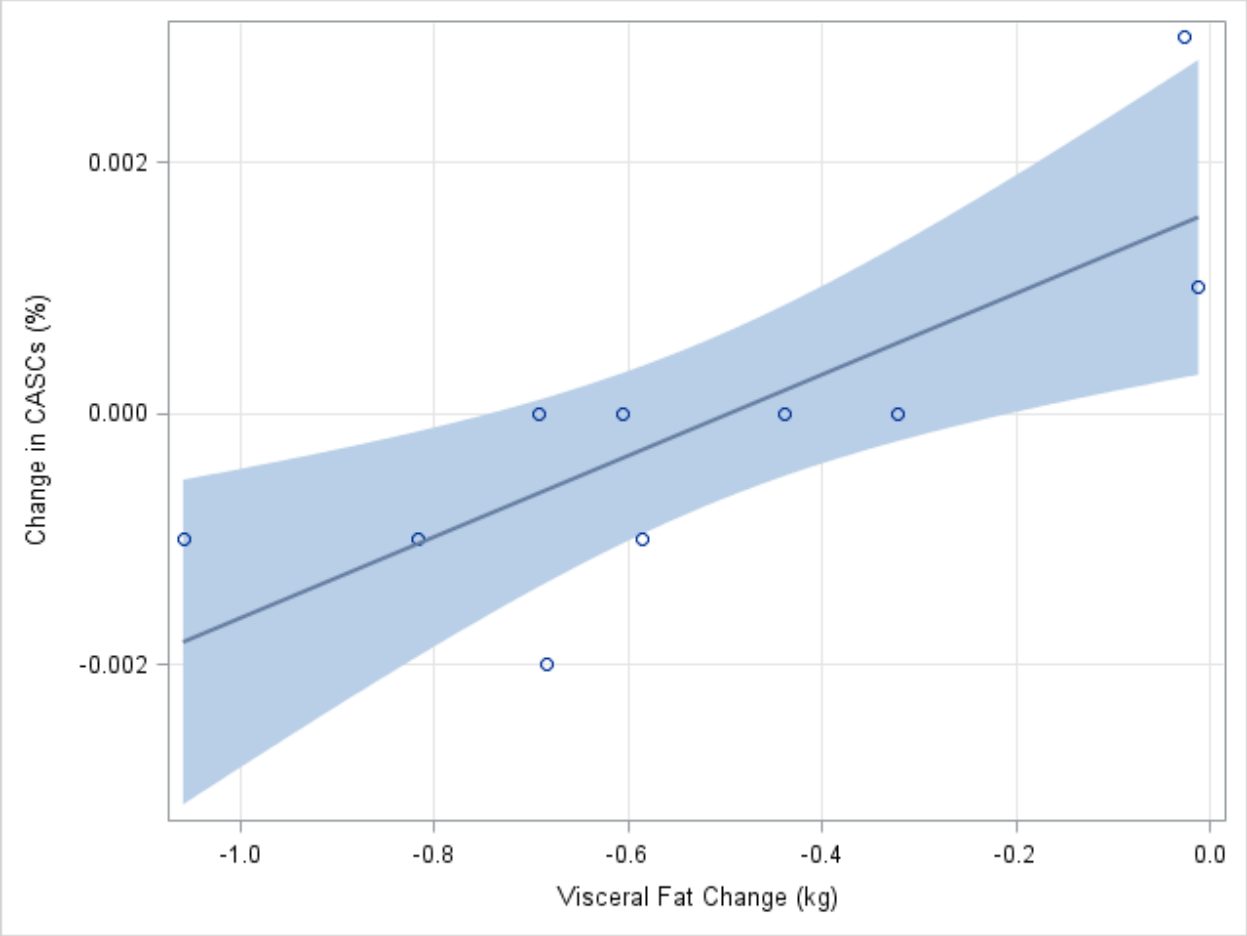

Figure 6: Linear Correlation of Changes in Visceral Fat (kg) and Changes in CASCs (%)

Linear correlation plot of changes in visceral fat [12 week visceral fat (kg) – 0 week visceral fat (kg)] with changes in CASC levels [12 week CASC (%) – 0 week CASC (%)], P=0.016. Shaded blue area represents the 95% confidence interval for the line of best-fit.

5 Discussion

Adipose stromal cells are increased in obesity, and release of these cells into the circulation from obese white adipose tissue may be due to tissue hypoxia resulting from adipocyte hypertrophy and hyperplasia in the context of an increase in extracellular matrix as well as an increase in local inflammation and tissue remodeling [52]. The observations that co-culture of human adipose stromal cells with various cell lines can result in leptin and estrogen dependent breast cancer cell proliferation, invasion and metastases [53-55] and that circulating adipose stromal cells home to sites of tumor cells via chemokine gradients [56] suggest an important role for CASCs in developing invasive cancer and metastatic disease in obese individuals. It further suggests that reduction in dysfunctional fat mass via calorie restriction and exercise in obese individuals should reduce CASCs as hypoxia and inflammation are reduced. These hypotheses led to our studies assessing CASCs across a spectrum of adiposity in high risk women and a pilot study in obese breast cancer survivors undergoing a short term diet and exercise intervention.

5.1 Cross-Sectional Study in High risk Women

Our cross-sectional study findings that there was a highly significant difference in CASCs (%) in women with BMI of < 35 kg/m^2 vs. ≥ 35 kg/m^2 (Class II obesity) (P=0.0009) and marginally with BMI < 30 kg/m^2 vs. ≥ 30 kg/m^2 (P=0.05) (Class I obesity) are consistent with the findings of others that CASCs are often not detectible in non-obese women. The increased risk of metabolic abnormalities with Class II vs. I obesity and our relatively small sample size provide a logical explanation for a stronger association of CASCs (%) with Class II obesity [52, 53].

BMI is not always an optimal measure of adiposity. Obesity can be overestimated with BMI in men with a large muscle mass and underestimated in women. We found that that total body fat (kg) is also linearly associated with CASCs (%), and this association provides a more mechanistic association than BMI. For high risk women, we did not find that percent body fat, android fat or waist circumference to have stronger associations with CASCs than BMI, although visceral fat could not be assessed with the DEXA scanner used in this cross-sectional study.

We did observe that low mammographic breast density, specifically < 5%, was inversely associated with CASC levels in obese women. This is an interesting observation because high mammographic breast density (50% or greater) is well-established as one of the strongest independent risk factors for both sporadic breast cancer and breast cancer development in high risk populations [57-60]. In fact, interventions such as tamoxifen or premenopausal oophorectomy which decrease breast density have been shown to decrease risk of breast cancer [59, 61]. Our observations do not challenge these well-documented findings, rather offer an explanation for the potential biological mechanism of increased risk despite decreased breast density in obesity. Mammographically dense area (%) is decreased in overweight and obese women due to adipose hypertrophy and hyperplasia in the breast. The increase in adipose may be associated with an increase in local and circulating adipose stromal cells [62-64]. The median BMI of high risk women with mammographic density less than 5% was 37.1 kg/m^2 (range 32.81-42.15 kg/m^2) and this group also had the highest CASCs (median 0.004%, range 0.001-0.013%). Therefore, our findings suggest that obese high-risk women with very fatty breasts may be releasing more adipose-derived CASCs into the blood stream.

From our cross-sectional study, we can conclude that in high-risk women, we are more likely to observe CASC in women with BMI above 35 kg/m^2 and that further studies are

indicated looking at correlations of CASC with Fat Mass Index (total fat mass kg divided by height in meters squared) and visceral fat, as well as adipokines and cytokines. We also found that increased proportions of breast fat (low mammographic density) in obese high-risk women appears to be associated with CASCs, but this may be a more of a representation of their BMI than mammographic density. In a random sample of high-risk women, there are many potential factors influencing CASC levels at any point in time.

5.2 Diet and Exercise Intervention in Breast Cancer Survivors

To address the question of how CASCs are affected by weight loss interventions, we quantified CASCs before and after a 3 month weight loss regimen consisting of moderate calorie restriction and high volume exercise in older obese sedentary breast cancer survivors. Ghosh *et al.* looked at the effect of a 6 month exercise program on CASCs in breast cancer survivors and found no statistically significant difference in CASCs from baseline to 6 months [39]. While they found no difference from the cohort's baseline to 6 month measures (N=13), they did observe that women whose CASCs decreased (N=6) lost about 2.79% body fat on average. The only adiposity measures they assessed were BMI and body fat (%), which they estimated through skinfold assessments. We found that women who had the largest decreases in weight, BMI, total body fat, and visceral fat had the greatest decreases in their CASCs. From Table 3, we can see that women who lost a mean of 13.8 kg (range -20.5 to -9.8 kg) of their body weight exhibited a decrease in their CASCs. Changes in weight (kg) and fat mass (kg) were found to have the strongest linear correlation (P=0.0009). Changes in BMI (kg/m^2), body fat (%), and visceral fat (kg) also expressed significant linear relationships with changes in CASCs (%) and therefore justify further investigation in larger cohort studies. These findings provide promising potential for the ability of change in CASCs to be an indicator of the type of fat loss (visceral and ectopic

fat) associated with the greatest metabolic dysfunction and greatest breast cancer risk. These results warrant further investigation into this relationship with larger sample size.

Our studies were both limited by small sample sizes as well as the low number of CASCs in the mononuclear cell population. Next steps include assessment of change in measures of insulin resistance and pro-inflammatory adipokines and cytokines including leptin as a function of change in CASCs. We also intent to expand our weight loss intervention data in breast cancer survivors and high risk participants where change in CASCs, fat mass index, visceral fat, bioavailable hormones and blood adipokines and cytokines are measured in all participants.

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7 Appendix

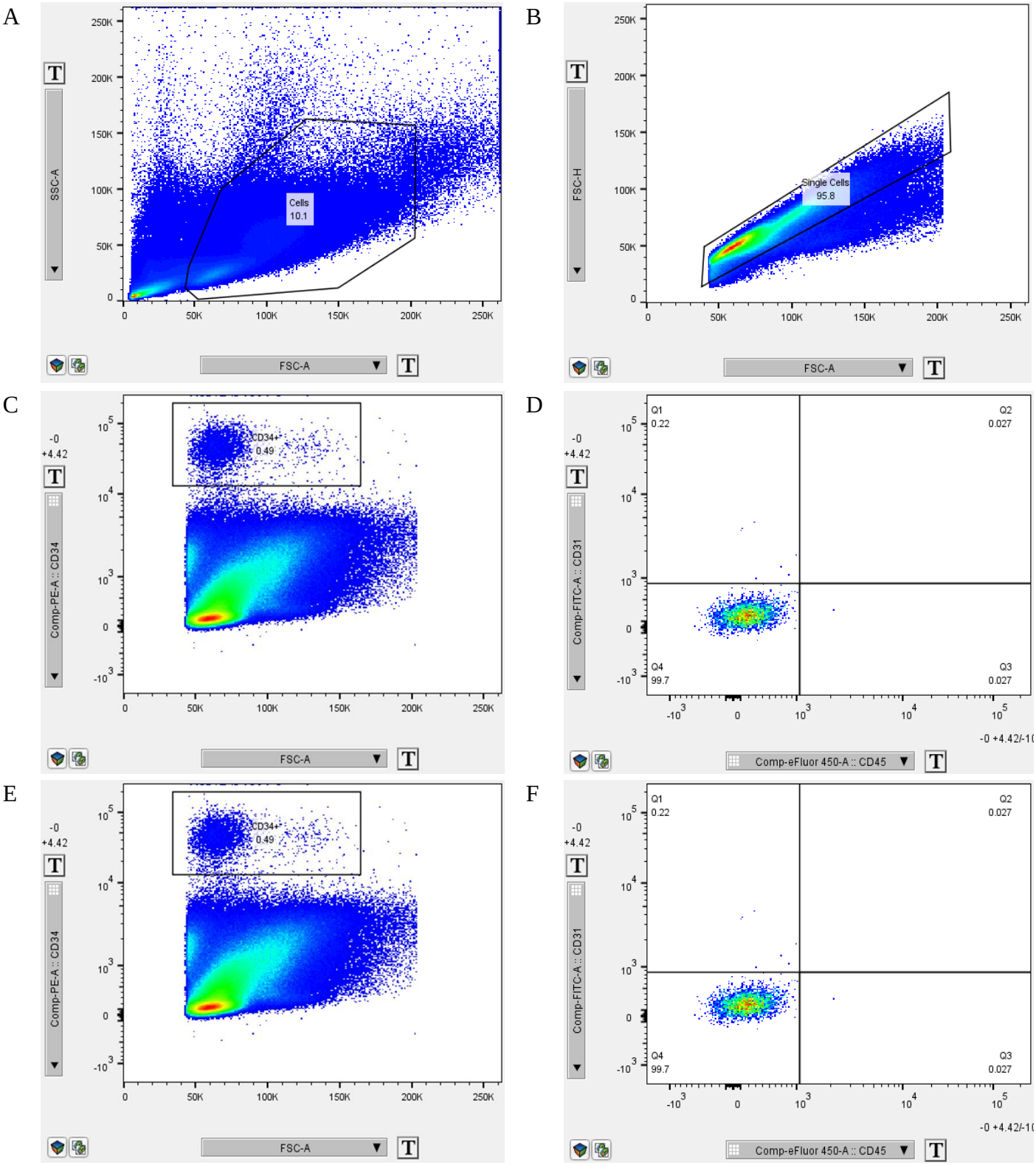

Figure 7: Flow Cytometry Gating Protocol with FlowJo Software

(A) FSC-A/ SSC-A: Excluding cell debris and gating for mononuclear cells. (B) FSC-A/FSC-H: Excluding cell clumps and selecting only single cells. (C) FSC-A/CD34: From single cells, select discrete $CD34^{bright}$ population from CD34 single antibody tube. (D) CD45/CD31: From $CD34^{bright}$ population, set gate around population to define CD31 and CD45 negatives. Apply this gate to sample with all 3 antibodies. (E) Sample with all three antibodies from high risk woman of BMI < 30 kg/m^2. Cells in Q4 taken as a percentage of all single mononuclear cells. (F) Sample with all three antibodies from high risk woman of BMI ≥ 30 kg/m^2. Cells in Q4 taken as a percentage of all single mononuclear cells.

Table 5: Raw Data of CASCs from Cross-Sectional Study in High Risk Women

Participant Number	Single Mononuclear Cell Counts (N)	CASC Number	CASC (%)
9	749,319	8	0.001
15	577,209	1	0.000
16	656,479	0	0.000
17	698,373	13	0.002
18	1,020,415	22	0.002
19	1,055,266	138	0.013
20	948,013	25	0.003
22	988,324	5	0.001
24	582,524	23	0.004
26	590,541	4	0.001
27	376,338	0	0.000
28	291,304	0	0.000
30	395,522	0	0.000
32	384,372	1	0.000
34	369,819	0	0.000
35	263,639	1	0.000
36	646,893	3	0.000
37	711,825	10	0.001
38	550,291	3	0.001
39	417,255	0	0.000
40	558,892	6	0.001
41	525,537	5	0.001
42	651,430	17	0.003
43	815,972	8	0.001
44	520,096	3	0.001
45	591,055	8	0.001
46	996,813	11	0.001
47	509,598	11	0.002
48	731,888	9	0.001
49	552,416	4	0.001
50	438,538	5	0.001
51	767,462	3	0.000
52	798,913	58	0.007
65	1,201,123	16	0.001
Median	590,798	5	0.001
Min	263,639	0	0.000
Max	1,201,123	138	0.013

Table 6. Raw Data of CASCs from Baseline and 3 months of Cohort Study in Obese Sedentary Breast Cancer Survivors

Study ID	Baseline			3 Months		
	Single Mononuclear Cell Counts (N)	CASC Number	CASCs (%)	Single Mononuclear Cell Counts (N)	CASC Number	CASCs (%)
301	809,521	13	0.002	2,222,210	103	0.005
303	905,363	20	0.002	2,026,995	28	0.001
304	616,091	3	0.000	1,979,686	9	0.000
305	542,961	5	0.001	1,817,123	27	0.001
306	928,044	91	0.010	1,421,737	141	0.010
307	736,938	5	0.001	866,011	3	0.000
309	648,726	29	0.004	694,747	18	0.003
310	777,451	36	0.002	888,584	31	0.003
311	1,099,218	30	0.003	1,103,567	7	0.001
312	1,514,672	21	0.001	1,574,821	19	0.001
Median	793,486	20.5	0.002	1,498,279	23	0.001
Min	542,961	3	0.000	694,747	3	0.000
Max	1,514,672	91	0.010	2,222,210	141	0.010