T.C.
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

RACIAL DISPARITY IN TUMOR MICROENVIRONMENT AND OUTCOMES IN RESIDUAL BREAST CANCER AFTER NEOADJUVANT CHEMOTHERAPY

Burcu KARADAL-FERRENA, MD/PhD

Tumor Biology and Immunology
DOCTOR OF PHILOSOPHY THESIS

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This thesis study has been approved and accepted as a PhD dissertation in "Tumor Biology and Immunology Program" by the assessment committee, whose members are listed below, on 07.06.2023.

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisors (Gurcan GUNAYDIN, MD/PhD and Maja H. OKTAY, MD/PhD) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

(Signature)

Burcu KARADAL-FERRENA, MD/PhD

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ABSTRACT

Karadal-Ferrena, B., Racial Disparity in Tumor Microenvironment and Outcomes in Residual Breast Cancer after Neoadjuvant Chemotherapy, Hacettepe University Graduate School of Health Sciences Basic Oncology Department of Tumor Biology and Immunology Doctor of Philosophy Thesis, Ankara, 2023. Black patients with residual estrogen receptor-positive (ER+) breast cancer after neoadjuvant chemotherapy (NAC) have inferior survival compared to white women resulting racial disparity in breast cancer survival. Differences in the tumor microenvironment (TME) might be one of the mechanisms behind the racial disparity in outcome. The hypothesis of this thesis study is "Racial disparity in Distant Recurrence-Free Survival (DRFS) in patients with residual ER+/ Human Epidermal Growth Factor Receptor 2 negative (HER2-) disease is due to enhanced pro-metastatic components (macrophage, microvasculature, cancer stem cell, and Tumor Microenvironment of Metastasis (TMEM) doorway density) in the tumor microenvironment post-NAC". We stained 183 invasive ductal carcinoma tissue samples (96 Black women, 87 white women) for TMEM doorways (Pan-Mena expressing tumor cell, CD68 macrophages, and CD31 endothelial cells) and SOX9 expressing cancer stem cells (CSCs). TMEM doorway score and macrophage density were more in Black patients in the entire cohort and in the ER+/HER2- disease. TMEM doorway was an independent prognostic factor overall. There was no racial disparity in microvascular density and CSCs. In conclusion, high-TMEM doorway score was an independent prognostic factor of worse survival in patients with residual cancer post-NAC. Racial disparity in outcome might be due to an increased pro-metastatic response to chemotherapy in Black relative to white patients with residual ER+/HER2- disease.

Keywords: Breast Cancer, Metastasis, Neoadjuvant Chemotherapy, Cancer Recurrence, Tumor Microenvironment

Supporting Institutions: Albert Einstein College of Medicine

ÖZET

Karadal-Ferrena, B., Neoadjuvan Kemoterapi Sonrası Rezidü Meme Kanserinde Tümör Mikroçevresi Ve Nükssüz Sağ Kalımındaki Irksal Farklılılar, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Temel Onkoloji Anabilim Dalı Tümör Biyolojisi ve İmmünolojisi Doktora Tezi, Ankara, 2023. Neoadjuvan kemoterapiden (NAK) sonra rezidü östrojen reseptörü pozitif (*Estrogen Receptor,*ER+) meme kanseri olan Siyahi kadınlar, Beyaz kadınlara kıyasla daha düşük sağkalıma sahiptir ve meme kanseri sonuçlarında ırksal eşitsizlik görülmektedir. Irksal sağ kalım eşitsizliğini açıklayabilecek faktörlerden biri tümör mikroçevresindeki değişiklikler olabilmektedir. Bu tez çalışmasının hipotezi, "Rezidü ER+/ İnsan Epidermal Büyüme Faktörü Reseptör 2 negatif (*Human Epidermal Growth Factor Receptor 2 negative*, HER2-) meme kanseri olan hastalarda nükssüz sağ kalımdaki (*Distant Recurrence-Free Survival*, DRFS) ırksal eşitsizlik, neoadjuvan kemoterapi sonrası tümör mikroçevresindeki artmış pro-metastatik bileşenlerden (makrofaj, mikrovaskülerite, kanser kök hücresi ve tümör mikroçevresi metastaz (*Tumor Microenvironment of Metastasis,* TMEM) kapısından yoğunluğu) kaynaklanmaktadır". TMEM kapıları (Pan-Mena eksprese eden tümör hücresi, CD68 makrofajlar ve CD31 endotel hücreleri) ve SOX9 eksprese eden kanser kök hücreleri (KKH'ler) için 183 invaziv duktal karsinom örneğini (96 Siyahi kadın, 87 Beyaz kadın) boyadık. Tüm kohortta ve ER+/HER2- alt tipinde Siyahi hastalarda TMEM kapı skoru ve makrofaj yoğunluğu daha yüksekti. TMEM kapı skoru genel olarak bağımsız bir prognostik faktördü. Mikrovasküler yoğunluk ve KKH'lerde ırksal bir farklılık yoktu. Sonuç olarak, NAK sonrası rezidüel kanserli hastalarda yüksek TMEM kapı skoru düşük sağkalım için bağımsız bir prognostik risk faktörüdür. Irksal farklılık, rezidü ER+/HER2- meme kanseri olan Beyaz hastalara kıyasla Siyahi hastalarda kemoterapiye daha belirgin bir pro-metastatik yanıttan kaynaklanıyor olabilir.

Anahtar Kelimeler: Meme Kanseri, Metastaz, Neoadjuvan Kemoterapi, Kanser Nüksetmesi, Tümör Mikroçevresi

Destekleyen Kurumlar: Albert Einstein Tıp Fakültesi

CONTENTS

ABBREVIATIONS

Ang1	Angiopoietin-1
Ang2	Angiopoietin-2
BP	Black Patients
CI	Confidence Interval
CSC	Cancer Stem Cell
CSF-1	Colony-Stimulating Factor
CSF-1R	CSF1-Receptor
DRFS	Distant Recurrence Free Survival
EMT	Epithelial-to-Mesenchymal Transition
ER	Estrogen Receptor
ER+/HER2-	Estrogen Receptor Positive – Human Epidermal Growth Factor Receptor 2 Negative
FFPE	Formalin Fixed Paraffin Embedded
G-CSF	Granulocyte Colony Stimulating Factor
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hazard Ratio
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile Range
MDSC	Myeloid-Derived Suppressor Cell
Mena	Mammalian-enabled, Actin Regulatory Protein
MenaINV	Invasive isoform of Mena protein
NAC	Neoadjuvant Chemotherapy
NF-κB	Nuclear Factor Kappa B
NSABP	National Surgical Adjuvant Breast and Bowel Project
NYPOG	**N**ew York Pathology Oncology Group

ROI	Region of Interest
SD	Standard Deviation
SOX9	SRY-Box 9
TAILORx	The Trial Assigning Individualized Options for Treatment
TGF-β	Transforming Growth Factor-β
TIE Domains	Tyrosine Kinase with Immunoglobin and EGF Homology
TILs	Tumor-Infiltrating Lymphocytes
TME	Tumor Microenvironment
TMEM	Tumor Microenvironment of Metastasis
TMEM-MRI	TMEM-Magnetic Resonance Imaging
TN	Triple Negative
TNBC	Triple Negative Breast Cancer
Tregs	Regulatory T cells
VEGF-A	Vascular Endothelial Growth Factor-A
WP	White Patients
yPN	Lymph Node Status after Neoadjuvant Chemotherapy
yPT	Tumor Stage after Neoadjuvant Chemotherapy

FIGURES

TABLES

1. INTRODUCTION

Breast cancer is responsible for a significant proportion of cancer-related deaths, with the majority of deaths caused by distant metastases (1,2). Black patients have higher death rates by 40% than white patients (3). Mortality rates declined by 40% in the past thirty years, however the gap between Black patients and white patients remained similar (3,4). Randomized clinical trials showed that Black women have higher recurrence risk in patients who treated with adjuvant chemotherapy, especially in estrogen receptor positive/human epidermal growth factor receptor 2 negative (ER+/HER2-) disease (5–9). Additionally, Black patients have worse survival in residual ER+ breast cancer after neoadjuvant chemotherapy (NAC) (10).

Racial disparity in outcome might be related to differences in the tumor microenvironment (TME). Specifically, black patients found to have higher levels of regulatory T cells (Tregs), macrophages, microvascular density, adipocytes, and inflammatory cytokines in their TME when compared to white patients (11). Understanding the underlying factors that contribute to racial disparities in TME could provide important insights into how to overcome these disparities and improve outcomes for all patients with breast cancer. Such insights help scientists for the development of targeted treatments or personalized treatment approaches that take into account the unique characteristics of each patient's TME.

NAC, which is administered prior to surgery of the cancer, has been found to induce a reparative response in the TME, resulting in angiogenic TIE2+ (TIE: tyrosine kinase with immunoglobin and EGF homology domains) monocyte migration into the tumor (12,13). NAC increases the formation and density of portals for cancer cell intravasation called “Tumor Microenvironment of Metastasis (TMEM) doorways” These doorways provide a direct route for cancer cells to enter the bloodstream and spread to other organs, leading to metastasis (14,15).

TMEM doorways are complex structures consisting of direct and tight interaction of three different cell types: Mena (mammalian-enabled, actin regulatory protein) positive cancer cell, a vascular-adjacent TIE2 high macrophage, and an endothelial cell (16–21). These structures provide a direct route for cancer cells to enter

the bloodstream, then initiate metastatic spread. According to three case-control studies, the TMEM doorway score has been found to be a reliable predictor of the increased metastasis risk in patients with ER+/HER2- disease (16,17,22). Further characterization of the TME has revealed that TMEM doorways serve as microanatomical niches for cancer stem cells (CSCs). Interaction between cancer cells and macrophages induces CSC phenotype around TMEM doorways (23). Increased density of CSCs is important because these cells have the potential to drive both primary tumor and metastatic growth (24). Understanding the complex interactions between TMEM doorways, CSCs, and the TME is crucial for developing effective treatment strategies for breast carcinoma patients.

The focus of this study is to examine the potential racial disparities that exist in breast carcinoma outcomes among patients with residual ER+/HER2- disease. The study hypothesis posits that Black patients may experience a more enhanced pro-metastatic response compared to white patients, which may contribute to the observed racial disparities. In order to test this hypothesis, a retrospective, multi-institutional case-control study was conducted, 96 tissue samples from Black patients and 87 tissue samples from white patients were analyzed with the investigation of several pro-metastatic markers such as TMEM doorway score, macrophage density, microvascular density, and CSCs in residual ER+/HER2- breast carcinoma.

The research objectives of the study are twofold. Firstly, the study aimed to determine whether pro-metastatic changes observed in residual cancer post-NAC were associated with inferior outcomes. Secondly, the study aimed to investigate whether racial disparities exist in the pro-metastatic changes observed in residual cancer post-NAC. The study investigated various markers associated with pro-metastatic response in order to identify potential underlying contributors of racial disparities in breast carcinoma outcomes. By conducting a rigorous investigation into the potential racial disparities that exist in breast cancer outcomes, this study contributes valuable insights into the underlying factors that contribute to such disparities. Ultimately, the results of this study could inform the development of more efficient and equitable strategies for personalized treatments for patients from different racial backgrounds.

1. BACKGROUND

2.1. Breast Cancer Epidemiology

Breast carcinoma has the highest incidence rate among women globally (25). The risk of breast carcinoma diagnosis for each women is one in eight in their lifetime (2,3). Age of 50 and above is the majority age group for both diagnosis with average eighty percent and mortality with average of ninety percent (3). Breast carcinoma accounts for a significant number of new cases diagnosed per year, with a variety of clinical presentations. With the advent of more commonly used screening with mammography, more than half of breast carcinoma are diagnosed on screening mammogram, and average one-third are diagnosed with palpable breast mass (26). However, it is vital to note that breast carcinoma can also present with other symptoms such as a breast asymmetry, nipple inversion, nipple discharge, palpable axillary mass, breast skin thickening (also known as peau d'orange), and breast skin erythema (27). These presentations are less common but should not be overlooked, as they can be indicative of breast cancer. Breast carcinoma diagnosis often involves multiple different imaging studies, including ultrasound, mammography, and magnetic resonance imaging, as well as histopathological examination of breast tissue obtained by biopsy. Early detection of breast carcinoma is critical, as the survival rate is much higher when the cancer is detected and treated at an early stage (2,3).

The estimated breast cancer incidence in 2023 is 300,590 for both genders, and 297,790 for female breast carcinoma in the United States, and the estimated breast carcinoma related deaths is 43,700 deaths for both genders, and 43,170 deaths for female breast cancer (2). In the past decade, the incidence rates have been on the rise, and this trend is likely due to a combination of multiple factors. One significant contributor to the increased incidence rates is the widespread and advanced use of cancer screening methods, particularly mammography. These screening methods have enabled doctors to detect breast cancer earlier and more accurately, leading to an increased number of diagnoses (2,3). However, while screening has a crucial role in early diagnosis, this is not the only factor driving the rise in breast cancer incidence rates. In the ER+ subset of breast carcinoma patients, several other contributors may be at play. For instance, studies have shown that the age of pregnancy has been

increasing in the past few decades, and this may be contributing to the rise in breast cancer rates. Finally, increased rates of obesity may also have a role in increasing incidence rates. Obesity is a known risk factor for breast cancer. While rates of obesity increasing worldwide, so too do the rates of breast cancer (3,11). These multiple factors together have likely effecting the rising number of breast carcinoma diagnoses observed in recent years, highlighting the need for continued research into breast cancer prevention, diagnosis, and treatment.

From 1989 to 2017, the United States mortality decreased by 40% driven by improvements in screening and treatment (3). Metastatic disease causes 90% of breast cancer related deaths (2,3). Five-year survival of localized breast cancer is 99%, whereas survival of metastatic (distant) disease is only 30% (2) (**Figure 2.1A**). There are 150,000 patients with metastatic breast cancer reported in 2019 (3). Studying and targeting metastatic disease in breast cancer is crucial when considering incidence rates and deaths due to metastasis. Triple Negative Breast Cancer (TNBC) has the worse outcome among breast cancer subtypes with an average of 77% survival rate (3) (**Figure 2.1B**). This is due to diagnosis at late stages, early recurrence with metastases, and dearth of effective targeted therapies (28–30).

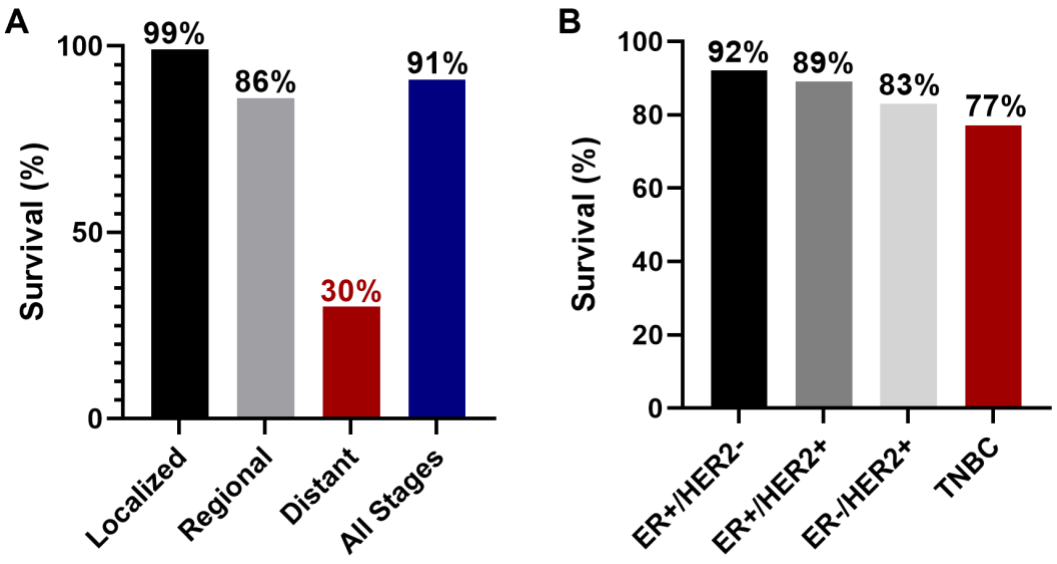


Figure 2.1. A) Five-year relative survival of women with breast cancer, United States 2012-2018. This figure is adapted from Siegel et al. CA Cancer J Clin, 2023 (2). **B)** Five-year relative survival of women with breast cancer by subtype, United States 2010-2015. This figure is adapted from DeSantis et al. CA Cancer J Clin, 2019 (3).

2.2. Breast Cancer Subsets

Breast cancer, a prevalent malignancy among women, is a multifaceted disease characterized by significant heterogeneity. This complexity stems from the fact that it encompasses diverse subsets that differ in their molecular and histological characteristics, leading to distinct clinical behaviors and responses to treatment. In clinical practice, three receptors – ER, HER2, and progesterone receptor (PR) - are commonly used to classify breast cancer subtypes. These biomarkers are identified through immunohistochemical staining and help in determining the appropriate treatment strategy for each subtype (2,3,27).

Among subsets, the most common subtype is the hormone receptor-positive subset, accounting for an average of eighty percent of all cases. This subset is characterized with expression of ER and/or PR and the absence of HER2 overexpression, making it a less aggressive subtype (3,31).

In contrast, HER2+ breast carcinoma is characterized by the overexpression of the HER2 protein. This subset is associated with an high cell growth and proliferation rate. This subtype is approximately twenty percent of all cases and typically more aggressive than the hormone receptor-positive subset (3,31).

Another important subtype is TNBC, which lacks the expression of ER, PR, and HER2 receptors. This feature makes this subset the most difficult subtype to treat, as it lacks targetable receptors (27). TNBC accounts for an average of ten to fifteen percent of all breast carcinomas (3,31).

2.3. Breast Cancer Treatment

Breast carcinoma is treated through a combination of various therapies, including surgery, radiotherapy, chemotherapy, hormone therapy, and targeted treatment. Chemotherapy can occur either before or after surgery, depending on the timing of administration. Pre-surgical chemotherapy, also known as neoadjuvant chemotherapy (NAC), aims to downsize the tumor, enabling less aggresive surgery and potentially increasing the chance of breast-conserving surgery. On the other hand, adjuvant chemotherapy is given after surgery for eliminating any remaining tumor cells and reduce the recurrence risk (3). The selection and sequence of these treatments

are determined based on several factors, including the stage at diagnosis. This is due to the fact that treatment goals and the likelihood of achieving them differ based on the stage. For example, early-stage breast carcinoma may be treated with surgery alone, whereas advanced stages may require a combination of several therapies (27).

Typically, early-stage breast carcinoma patients receive primary surgery, which may include mastectomy or breast conserving surgery (lumpectomy) of the breast and resection of local lymph nodes (3,27). Following the completion of surgery, additional systemic treatment or radiotherapy can be recommended depending on various tumor features such as grade, size, lymph node involvement, and the ER, PR, and HER2 expression (3,27). When a patient has higher grade, tumor bigger than two centimeters, and positive local lymph node, adjuvant chemotherapy is added into the treatment regimen. Patients with TNBC, even if the tumor is small (more than 0.5 centimeter), are usually treated with adjuvant chemotherapy (3,27,32–34). Patients with HER2+ tumor that is more than one centimeter are treated with targeted HER2 inhibitors (35).

Locally advanced breast carcinoma is defined as tumor size is typically more than 5 centimeters or two-three lymph node positivity are found without any distant organ metastases (36,37). This stage is commonly managed with both surgery and chemotherapy, which is similar to high-risk early-stage carcinoma. NAC is typically preferred over adjuvant chemotherapy for early-stage TNBC, HER2+ disease, and locally advanced breast carcinoma. The main aim in NAC is to downsize the tumor and early recurrence risk (38–40).

In contrast, the treatment approach for metastatic disease is mainly based on endocrine and chemotherapy. For hormone receptor positive cancers endocrine therapy combination with targeted therapy is preferred. Metastatic TNBC is treated with chemotherapy (41–44). Most widely used endocrine therapy agents are ER modulator, tamoxifen; aromatase inhibitors letrozole, anastrozole, exemestane which inhibit conversion of androgens to estrogen (27). The most common chemotherapy agents are: 1) Taxane groups: docetaxel, paclitaxel which inhibits microtubule function and disrupts mitosis, 2) Cyclophosphamide: an alkylating agent, inhibits

DNA replication, 3) Doxorubicin: topoisomerase-two inhibitor that interferes with DNA replication (27).

In addition to chemotherapy and endocrine therapy, targeted therapies are available for specific mutations in breast cancer. Notably, targeted agents have been developed for “breast cancer susceptibility gene 1 or 2 (BRCA1/2)” mutations and the immune checkpoint molecule “programmed cell death ligand 1 (PD-L1)”. These targeted therapies aim to specifically address the underlying molecular alterations associated with these mutations (3,27). For individuals with BRCA1/2 mutations, targeted agents such as “poly (ADP-ribose) polymerase (PARP)” inhibitors have shown efficacy in inhibiting the DNA repair process specifically in cancer cells carrying these mutations (27). This targeted approach can lead to enhanced treatment response and improved outcomes. Similarly, targeted therapies that target PD-L1, such as immune checkpoint inhibitors, harness body's immune system and enhance anti-tumor responses (27).

In conclusion, the management of breast carcinoma is a multidisciplinary approach that requires a comprehensive evaluation of several factors, such as the stage, primary tumor characteristics, and individual patient factors. Decisions are made based on a combination of different options, such as surgery, chemo-, radio-, hormone-therapy, and targeted therapy, aimed at improving the patient's survival and quality of life.

2.4. Racial Disparity in Breast Cancer

Breast cancer related deaths declined approximately by 40% in the last three decades, however death rates decline less in Black race. Black patients have worse survival and increased distant recurrence compared to white patients (3,45–47).

Black patients are diagnosed younger than white patients (average 60 versus 63 years). Furthermore, these patients are dying younger due to breast cancer (average 63 years versus 70 years) (3). Five-year relative survival of distant disease is 32% for white patients whereas it is only 21% for Black patients (**Figure 2.2**). The main factors contributing to this are: (i) advanced stage at presentation, (ii) limitations in access to care, (iii) higher TNBC incidence, (iv) dropout from endocrine and chemotherapy, and

(v) more common comorbidities (48–52). Black patients tend to have higher grade cancer more often than lower grade cancer compared to other race groups, as well as bigger size tumors (more than 5 centimeter) (3).

ER+/HER2- breast cancer is more common in white patients, whereas TNBC is more common in Black patients. Especially, higher percentage of TNBC in black women younger than 50 years might be contributing the racial disparity (3,53).

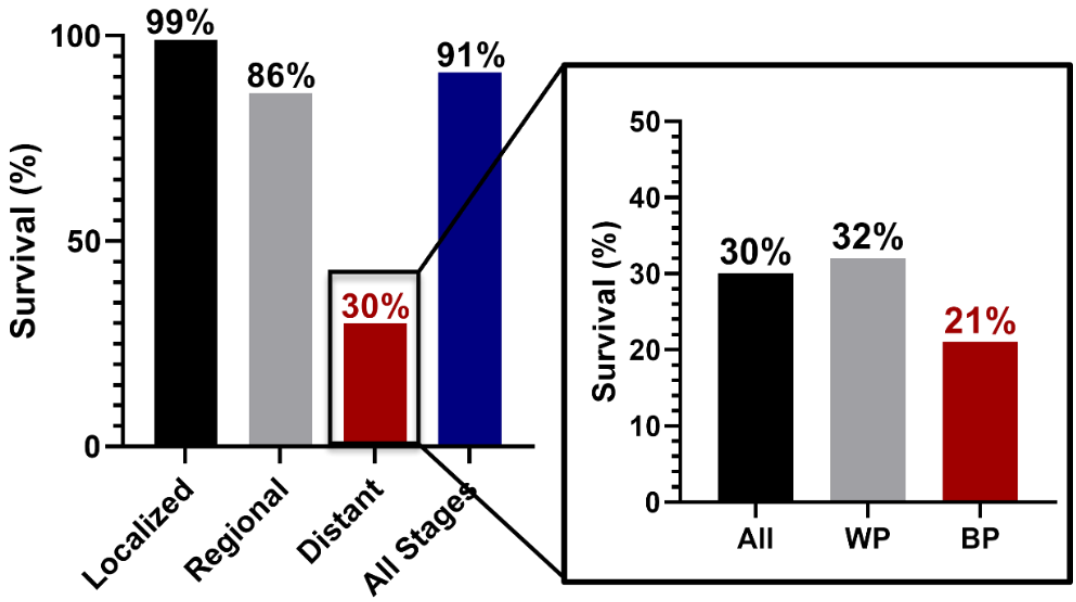


Figure 2.2. Five-year relative survival of women with distant metastatic breast cancer, United States 2012-2018. This figure is adapted from Siegel et al. CA Cancer J Clin, 2023 (2). WP: White Patients, BP: Black Patients.

Randomized clinical trials have shown that mortality gap remains even after adjusting for social and demographic factors, especially in ER+/HER2- breast cancer (5,7,8). There are three randomized adjuvant treatment breast cancer trials that reported that Black race is related with increased recurrence risk ranging from 1.5 to 1.84 fold (5,7,8) (**Table 2.1**).

Table 2.1. Randomized adjuvant breast cancer trials

Study/Cohort	Number	Black	Stage	Black race and risk of recurrence
E1199 (NCT00004125) *Sparano et al. J Natl Cancer Inst., 2012. (5)*	4,817	405 (8.4%)	II-III	↑**1.58-fold** (p=0.002) in ER+/HER2- disease
E5103 (NCT00433511) *Schneider et al. JCO Precision Oncol., 2017. (7)*	2,859	386 (13.5%)	II-III	↑**1.5-fold** (p=0.027) in ER+/HER2- disease
Montefiore-Einstein cohort *Kabat et al. J Racial Ethn Health Disparities, 2017. (8)*	3,890	1,394 (35.8%)	I-III	↑**1.84-fold** (p<0.05) in ER+/HER2- disease

↑ Increased. This table is adapted from *Kim et al. Cancer 2022* (10). No.: total number of patients.

In addition to the adjuvant setting, a recent study including eight National Surgical Adjuvant Breast and Bowel Project (NSABP) trials showed that there are also disparities in residual ER+/HER2- breast cancer in neoadjuvant setting (10). These findings indicate that disparity might be related to other factors, such as the biology of the host, the biology of cancer, host-tumor interaction, and/or the TME.

2.5. Racial Disparity in the Tumor Microenvironment

Racial disparity seen in breast carcinoma even in randomized clinical trials that controlled for social factors and access to healthcare suggests that other unknown factors may be contributing to this disparity. Potential causes may lie with parameters associated with the biology of the host and/or tumor, including the TME (11). Dynamic interaction of cancer cells and non-cancer cells can shape the growth, progression, and dissemination of the tumor (54).

Tumor associated macrophages can harbor two different roles: pro-tumorigenic roles or anti-tumorigenic roles (55,56). One study showed that Black patients have overall higher macrophage density in the TME (57), while other showed specifically high pro-tumorigenic subtype of macrophages in Black patients (58).

Similar to macrophages, neutrophils can harbor pro-tumorigenic or anti-tumorigenic roles (59). Even though it has been shown that Black race can have high neutrophils in healthy conditions (60), there is no difference in neutrophil levels among Black relative to white breast cancer patients (61).

Another cell from myeloid lineage besides macrophages and neutrophils is myeloid-derived suppressor cells (MDSCs). MDSCs have immunosuppressive properties and have two subtypes: monocytic and granulocytic (62). One study showed

that circulating granulocytic-MDSCs in blood increases after adjuvant chemotherapy and this increase is even more pronounced in Black patients than Caucasian patients (63).

Tregs cause immunosuppression and inferior survival in breast cancer (64,65). Percentage of Tregs in TME is higher in Black relative to white patients (61). Although tumor-infiltrating lymphocytes (TILs) correlate with improved survival and chemotherapy response (66,67), there is no racial differences reported in TILs between Black relative to white patients (61).

Vasculature in the tumor is important for growth, progression as well as dissemination of the tumor cells (68–70). Microvascular density is also associated with breast cancer outcome (71). Black patients with breast cancer have higher microvascular density in their TME relative to white patients (58).

Additionally, adipocytes, most common cell type in breast tissue, are also associated with inferior survival (72,73). Obesity is seen more in Black than white women (74). Obesity can induce chronic-inflammation and increased chemo-attractants in the TME (75). Black women have higher adipocyte-associated structure also known as crown-like structure relative to Caucasian women with breast cancer. Crown-like structure is composed of dying adipocytes surrounded by macrophages and correlated with inferior survival in breast cancer (57).

The racial differences in the immune cell composition in turn affects cytokine profiles in the TME. Healthy Black women have higher interleukin-8 (IL-8), interleukin-6 (IL-6), granulocyte stimulating factor (G-CSF), and TNF-alpha levels at the baseline (76,77). Black patients with breast cancer have higher IFN-gamma and resistin levels compared to white patients (58,78).

Overall, these findings show that Black women have more pro-tumorigenic and pro-metastatic TME relative to white women. This could be one of the main contributors in racial disparity among Black and white race. Understanding the mechanism and the impact of differences in the TME in Black and white race will guide further studies design new treatment strategies to overcome racial disparity in clinic.

2.6. Tumor Microenvironment of Metastasis (TMEM) Doorways

Higher macrophages and microvascular density seen in Black women are important because these are the components of portals for tumor cell dissemination called “TMEM doorways”. They are made of three different cells: a tumor cell, an endothelial cell, and a macrophage (**Figure 2.3**).

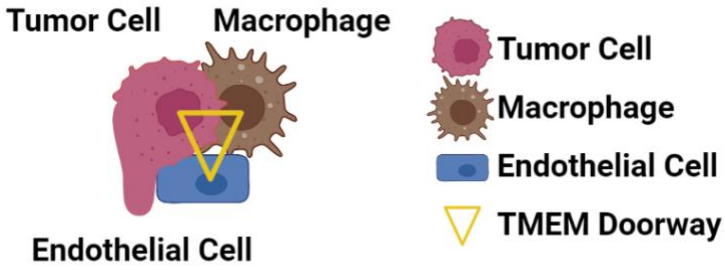


Figure 2.3. Tumor MicroEnvironment of Metastasis (TMEM) Doorway. Three different cells in direct and stable physical contact form TMEM doorway: Tumor cell, Perivascular Macrophage, and Endothelial Cell. BioRender.com was used to make this figure.

The details of TMEM doorway function have been established using pre-clinical *in vivo* and *in vitro* models, as well as *in vivo* imaging (15,18,20,79). There is a stable, tight, and direct contact between the three TMEM doorway cells. The interaction of the cells results in transient vascular opening events around TMEM doorways which cancer cells intravasate and metastasize (**Figure 2.4**). The mechanism and the pathways are activated from this interaction are well studied in *in vivo* mouse mammary tumor models.

One of the components of TMEM doorway is macrophages that are TIE2+. TIE2 is a receptor tyrosine kinase, and is expressed in macrophages, endothelial cells, and cancer cells among others. Through its receptors, Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2), TIE2 regulates angiogenesis, cell migration, and survival (80). In context of TMEM doorway, Ang2 secreted by endothelial cell stimulates TIE2 receptors on the TMEM doorway macrophage, which increases vascular endothelial growth factor-A (VEGF-A) production (**Figure 2.4A**). Another pathway that increases VEGF-A secretion around TMEM doorways is CSF-1 (colony-stimulating factor) – CSF-1R (CSF1-receptor) pathway. CSF-1 is secreted by cancer cells and binds CSF1-

R on macrophages inducing VEGF-A expression in TIE2+ macrophages at TMEM doorways (81) (**Figure 2.4B**). VEGF-A released from the TMEM doorway-bound macrophage induces dissociation of endothelial cell-cell junctions, leading to localized vascular permeability around TMEM doorways and cancer cell intravasation at TMEM doorways (**Figure 2.4C**).

Cancer cells with high invasive isoform of Mena (Mena-invasive, MenaINV) disseminate from the primary tumor via TMEM doorways. Mena is an actin regulatory protein. Mena inhibits capping proteins of actin and causes sustained actin polymerization and directional movement of the cell which helps tumor cells to migrate and intravasate. Mena protein has multiple splicing variants, including Mena11a and MenaINV (82). Mena11a is related to epithelial phenotype and associated with anti-metastatic character (82). On the other hand, MenaINV is related to epithelial-to-mesenchymal transition (EMT). It is an invasive and pro-metastatic marker (83). It has been shown in several studies that Mena11a-low, MenaINV-high tumor cells migrate towards and intravasate through TMEM doorways (84–86) (**Figure 2.4**). Further, MenaINV levels correlate with TMEM doorway score in human mammary carcinoma (82).

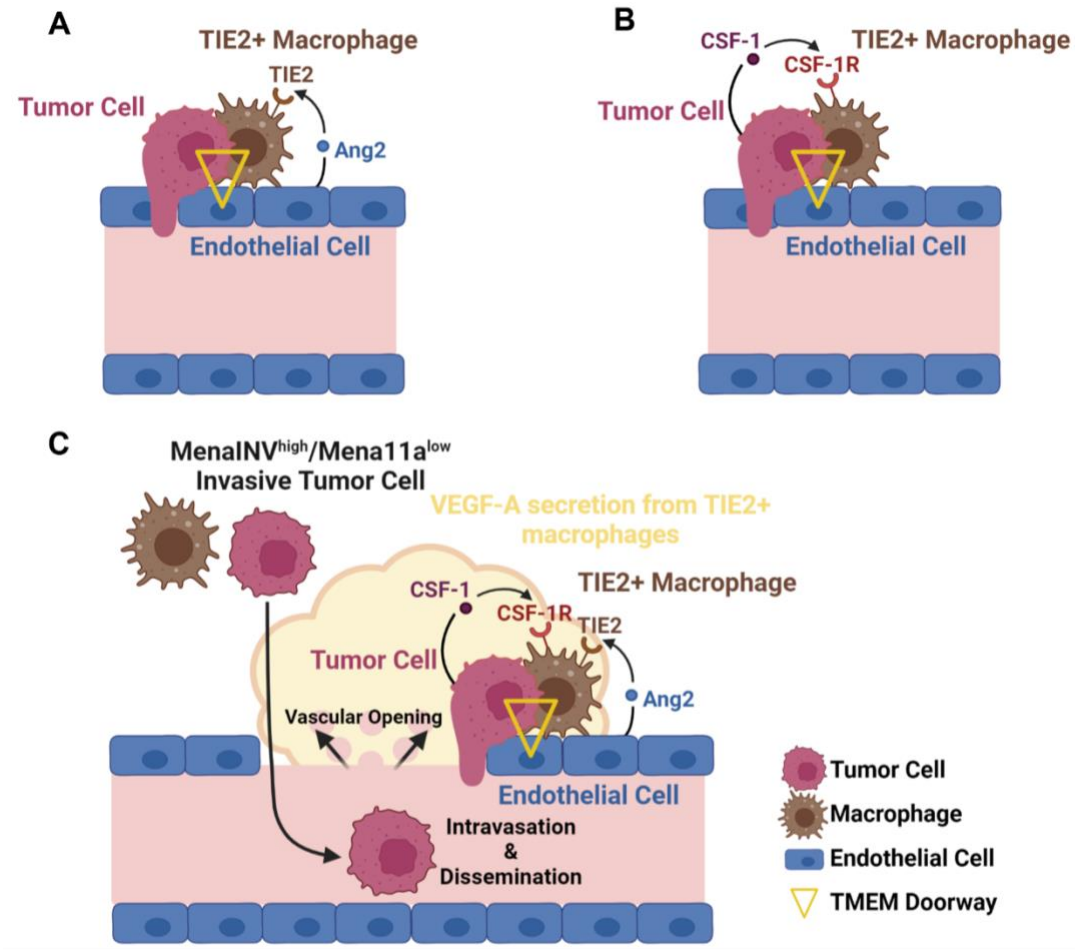


Figure 2.4. Proposed Model of TMEM Doorway Function. **A**) Endothelial cells secrete Angiopoietin 2 (Ang2) which in turn activates the tyrosine kinase receptor TIE2 in perivascular macrophages. **B**) Colony stimulating factor-1 (CSF-1) secreted by tumor cell at TMEM doorway binds to its receptor (CSF-1R) on macrophages. **C**) These interactions increase Vascular Endothelial Growth Factor-A (VEGF-A) secretion, thereby stimulating transient and local vascular opening event at the TMEM doorway. Invasive cancer cells with high MenaINV and low Mena11a expression disseminate from the primary tumor via transient vascular opening events around TMEM doorways. BioRender.com was used to make this figure.

The density of TMEM doorways is an independent prognostic indicator of distant recurrence in ER+/HER2- disease (**Table 2.2**). Metastatic breast cancer had higher TMEM doorway score than non-metastatic breast cancer patients (16). Likewise, in a largerstudy including 518 cases found that TMEM doorway score was correlated with metastasis in ER+/HER2- breast carcinoma (17). Moreover, another analysis of trial E2197 (NCT00003519) reported that there was a strong association between early recurrence in ER+/HER2 disease and TMEM doorway score (22).

Table 2.2. TMEM doorway case-control studies

Study	Number of patients	Conclusion of the Study
Robinson et al. Clinical Can. Res., 2009. (16)	60 cases (30 case-control pairs)	Patients with metastatic breast cancer have higher TMEM doorway score than patient with non-metastatic disease.
Rohan et al. JNCI, 2014. (17)	518 cases (259 case-control pairs)	TMEM doorway score was associated with increased risk of metastasis in ER+/HER2- disease.
Sparano et al. NPJ Breast Cancer, 2017. (22)	600 cases (E2197 cohort)	There was a positive association between TMEM doorway score and early recurrence in ER+/HER2- disease.

Importantly, TMEM doorway function can be inhibited by an orally available, highly specific, small molecule TIE2 inhibitor (Rebastinib) (87) which was tested in clinical trials for breast and gynecologic cancers (NCT03717415, NCT03601897) at Montefiore-Einstein and other sites. In mouse mammary tumor model, Rebastinib treated mice, compared to the control group, has decreased primary tumor growth, lower number of metastatic nodules in the lung, less TMEM doorway activity, less circulating cancer cells, and longer survival (87).

Moreover activity of TMEM-doorways can be monitored via TMEM-Magnetic Resonance Imaging (TMEM-MRI) (88). TMEM-MRI is based on first-pass measurement of contrast reagent, gadolinium, and detects TMEM doorway related vascular opening events. Pilot cohort from the study has shown association between TMEM-MRI and TMEM doorway score (88). Even though larger cohort studies are needed, this technique has the potential for real-time assessment of TMEM doorways in the future.

In the setting of chemotherapy, the number of TMEM doorways, cancer cell intravasation and metastasis were enhanced in murine mammary carcinoma models treated with NAC. Moreover, patients with ER+/HER2- disease had increased TMEM doorway numbers post-NAC (15). Although there is evidence of NAC-induced TMEM doorway mediated metastasis, the impact of NAC on TMEM doorways in the context of racial disparity is unknown.

2.7. Cancer Stem Cells in Breast Cancer

CSCs are rare stem-like cancer cells that are known to exhibit properties of self-renewal, differentiation which can initiate cancer and tumor progression (24). With these features CSCs can sustain the primary tumor, initiate metastases, and resist chemotherapy (24,89).

High circulating CSCs are associated with inferior treatment response, survival, and progression-free survival (90). Similarly, a meta-analysis showed correlation of CSCs and inferior survival in breast cancer (91). Some studies have found association between CSCs and outcome (91–94), but other studies did not (95,96).

CSC phenotype can be modulated by TME. Tumor cell-macrophage interaction around TMEM doorways induces CSCs phenotype in mouse mammary carcinoma model (23). TMEM doorway score was positively associated with CSCs (23). Although the importance and tumor initiation capacity of CSCs are well studied, CSC density in different racial backgrounds are unknown.

Transcription factor SOX9 (SRY-box 9) is one of the markers for CSCs. SOX9 mediates development of many tissues via regulating multiple downstream signaling pathways e.g., nuclear factor κB (NF-κB), Notch, Wnt-Beta catenin, Transforming Growth Factor-β (TGF-β) (97–101). SOX9 positive tumor cells can promote tumorigenesis and metastasis (101,102). SOX9 is correlated with worse survival in breast carcinoma as well as hepatocellular carcinoma, ovarian cancer, lung cancer, and gastric cancer (102,103). Furthermore, SOX9 levels are positively correlated with TMEM doorway score in human breast cancer samples (23).

In summary, studies extensively suggests that CSCs have a crucial role in cancer initiation as well as metastasis initiation. They might have a significant impact on patient survival. Therefore, it is crucial to delve deeper into the study of as a pro-metastatic tumor marker, particularly in the context of racial disparity.

3. MATERIALS AND METHODS

3.1 Patient Selection and Study Design

The study is a prospective-retrospective, multi-institutional case-control study. Patient samples are collected from New York Pathology Oncology Group (NYPOG) institutions (Montefiore Medical Center; New York University Langone Health; Memorial Sloan Kettering Cancer Center; New York-Presbyterian/Weill Cornell Medical Center, https://einsteinmed.edu/research/groups/ny-pathology-oncology/). NYPOG is a group of physicians from pathology and oncology specialties, also basic research scientist. The collaborative work of this group focuses on the TME role in cancer initiation, progression, metastasis, and dormancy in patients of all racial background in the New York, US.

This study was approved by Institutional Review Board of each institution and performed in accordance with REMARK guidelines (104,105).

A total of 183 patient samples were collected, including 96 tissue samples from Black patients and 87 tissue samples from white patients (**Table 4.1**). The inclusion and exclusion criteria are shown in **Figure 4.1**. Inclusion criteria are unilateral invasive ductal breast cancer, age older than eighteen, residual disease after NAC minimum five millimeter. Exclusion criteria are history of previous cancer, metastatic disease, and male patients. The race of each patient was self-identified as either Black or white.

For each patient, a Formalin Fixed Paraffin Embedded (FFPE) block was obtained. Blocks were cut into 5 µm thickness – five sequential slides for further staining. These slides were used to investigate various tumor markers and their relationship to outcome, with the aim of improving our understanding of the mechanisms that underlie racial disparity.

The NAC treatment details were categorized into anthracycline, cyclophosphamide, taxane drug combinations, or NAC combined with endocrine therapy, HER2 inhibition, radiation therapy. Treatment regimens were similar between patients, as outlined in **Tables 4.2 and 4.3**.

3.2 Immunohistochemistry (IHC) and Immunofluorescence (IF) Staining

One of the sections was stained for TMEM doorway triple IHC staining, as previously described (17,22). Antibodies used for TMEM doorway triple IHC staining as follows:

- pan-Mena antibody (P/N: 610692, BD Biosciencesthat stain all isoforms of Mena with Fast Red chromogen (Bond Polymer Refine Red Detection, Leica Biosystems) for cancer cells,
- CD-68 antibody (clone PG-M1; 1:300 dilution; DAKO) with antigen retrieval using Bond Epitope Retrieval Solution 1 and 3,3'-Diaminobenzidine (DAB) chromogen for macrophages,
- CD-31 antibody (clone JC70A; 1:800 dilution; DAKO) with Bond Epitope Retrieval Solution 2 and Vector Blue chromogen for endothelial cells.

One of the serial sections for each patient was stained with SOX9 for CSCs SOX9 (anti-rabbit Millipore 3205915, 1:100 dilution) and DAPI (1:1000 dilution for nuclei) were used for IF staining of CSCs. Alexa Fluor-546 goat anti-rabbit (H+L) (Thermofisher, Cat# A11035, 1:200 dilution) was used as secondary antibody for SOX9. 3D Histech P250 High-Capacity Slide Scanner was used for scanning the slides.

Details of SOX9 staining protocol is:

<u>First Day:</u>

1. Dewaxing the slides
    a. Leave the slides in 60 degree Celsius incubator for an hour.
    b. Place the slides in a rack, and dewax the slides two times with xylene, 10 minutes for each time.
    c. Perform following washes:
        1. 100% ethanol, 2 minutes
        2. 95% ethanol, 2 minutes
        3. 70% ethanol, 2 minutes
        4. 50% ethanol, 2 minutes
        5. Last wash in water
        6. Keep the slides in water

2. Perform antigen retrieval
    a. Prepare 1X pH 9, antigen unmasking solution, tris-based (100X, vector labs, catalog number H-3301)
    b. Place antigen retrieval buffer in a glass jar and microwave to boiling without the slides.
    c. Place the slides into the retrieval solution and put the jar in the steamer with lid for 20 minutes.
    d. Remove the slide from the steamer and cool it down for 30 minutes at room temperature.
3. Wash the slides in phosphate-buffered saline, 5 minutes for three times.
4. Use a hydrophobic pap pen to outline tissue area.
5. Prepare blocking buffer, 10 mL:
    i. 8.8 mL tris buffered saline-tween 20 (0.05% tween)
    ii. 0.1 gram bovine serum albumin, fraction V
    iii. 1 mL normal goat serum, cell signaling catalog number 5425S
6. Block the slides at room temperature for one hour.
7. Add primary antibody (SOX9 anti-rabbit Millipore 3205915, 1:100 dilution in blocking buffer) and incubate the slides at 4 degree solution over-night.

<u>Second day:</u>

1. Wash the slides in tris buffered saline-tween 20 (0.05% tween) for 5 minutes, three times.

(From second step, protect the slides from the light)

2. Add Alexa Fluor-546 goat anti-rabbit (H+L) as secondary antibody, Thermofisher, Cat# A11035, 1:200 dilution in blocking buffer) and incubate the slides for one hour at room temperature.
3. Wash the slides in tris buffered saline-tween 20 (0.05% tween) for 5 minutes, three times.
4. Stain the slides with DAPI for 5 minutes, (1:1000 dilution, diluted in tris buffered saline-tween 20).

5. Wash the slides in tris buffered saline-tween 20 (0.05% tween) for 5 minutes, three times.
6. Place the slides in phosphate-buffered saline.
7. Mount the slides with Vectashield hardset antifade mounting medium (Vector labs catalog number H-1400) and place the cover glass on top.
8. When the slides are dried, store the slides in the dark 4 degree Celsius fridge.
9. Scan the slides as soon as possible with 3D Histech P250 High-Capacity Slide Scanner.

3.3 Automated Analysis and Quantification of TMEM Doorways

Ten 20X (660x880 μm^2) region of interests (ROIs) were drawn in scanned slides in Visiopharm image analysis software (Hørsholm, Denmark). The areas of tissue fold, necrosis, and inflammation were avoided. Automated analysis of TMEM doorways were done in all 20X ROIs as previously described (106). The published analysis were done in 40X ROIs, which is 4 times of 20X ROIs (22,106,107). To normalize the data, the sum of the results was divided by 4. High versus mid/low TMEM doorway scores were separated by highest tertile and lowest 2/3 tertiles, respectively.

3.4 Macrophage Density and Microvascular Density Analysis

In addition to its primary function of detecting TMEM doorways in tissue samples, the algorithm used in this study is also capable of identifying areas of CD31 staining (blue) for endothelial cells, CD68 staining (brown) for macrophages within the same TMEM doorway slides (106).

To perform macrophage and microvascular density analysis, the same ROIs from TMEM doorway analysis were utilized. The algorithm identified blue areas within ROIs as the microvascular component and brown areas as the macrophage component. The areas of macrophage or microvascular staining were divided to area of each ROI to calculate macrophage density and microvascular density.

3.5 Analysis of Cancer Stem Cells

The same ROIs from TMEM doorway analysis were also used to analyze CSCs – specifically, nuclear SOX9 expressing cells in Visiopharm software. To do this, the ROIs were saved as BMP (bitmap) format and opened via Fiji software (108). To separate the red channel for SOX9 (CSCs) and blue channel for DAPI (nucleus) the Trainable Weka Segmentation plugin algorithm on Fiji was used (109). The intensity of each nuclear SOX9 was measured with an algorithm via separating the red areas on the image from the surrounding area.

The cut-off value for high nuclear SOX9 cells was determined based on the value capturing top 5% of SOX9 expressing cells in control slides (three untreated ER+/HER2- white patient samples). The top 5% high expressing cells was decided to be a cutoff point for CSC determination based on previous data showing that the percentage of CSCs in mouse mammary tumor does not exceed 5% of the cancer cell population (23). The average cut-off value from control slides was set up as high SOX9 expression threshold and applied to all ROIs. For each patient, average of high-nuclear SOX9 expressing cell percentage in each ROIs were averaged.

3.6 Data Analysis

GraphPad-Prism v9.1 (Dotmatics, Boston, MA) and SAS 9.4 (SAS Institute Inc., Cary, NC 2014) used for analyses.

The Fisher's exact or Chi-squared test for categorical variables, Wilcoxon test for continuous variables, were used to compare demographic variables in different groups (Black versus white patients, ER+/HER2- versus TNBC).

The Spearman correlation test used for correlation in TMEM doorway score, macrophage density, CSCs, microvascular density. DRFS analysis was performed with Kaplan-Meier curve and log-rank tests.

Cox proportional hazard model was used for multivariate analysis.

The power of the study was designed to have two-sided type-I error rate of not more than 5% with 80% power to detect difference of 0.61 standard deviation in each marker. Based on these criteria N=50 no-distant recurrence, N=25 distant-recurrence

for each race was enough to match power analysis. Patient numbers N=49 no-distant recurrence, N=47 distant recurrence in Black patients, N=57 no-distant recurrence, N=30 distant recurrence in white patients fulfilled this requirement, except 1 patient missing in no-distant recurrence group in Black patients.

P value <0.05 was statistically significant. P values reported in the analyses are two-sided.

4. RESULTS

4.1 Patient Demographics and Characteristics

Study design, inclusion criteria, and exclusion criteria are shown in **Figure 4.1**.

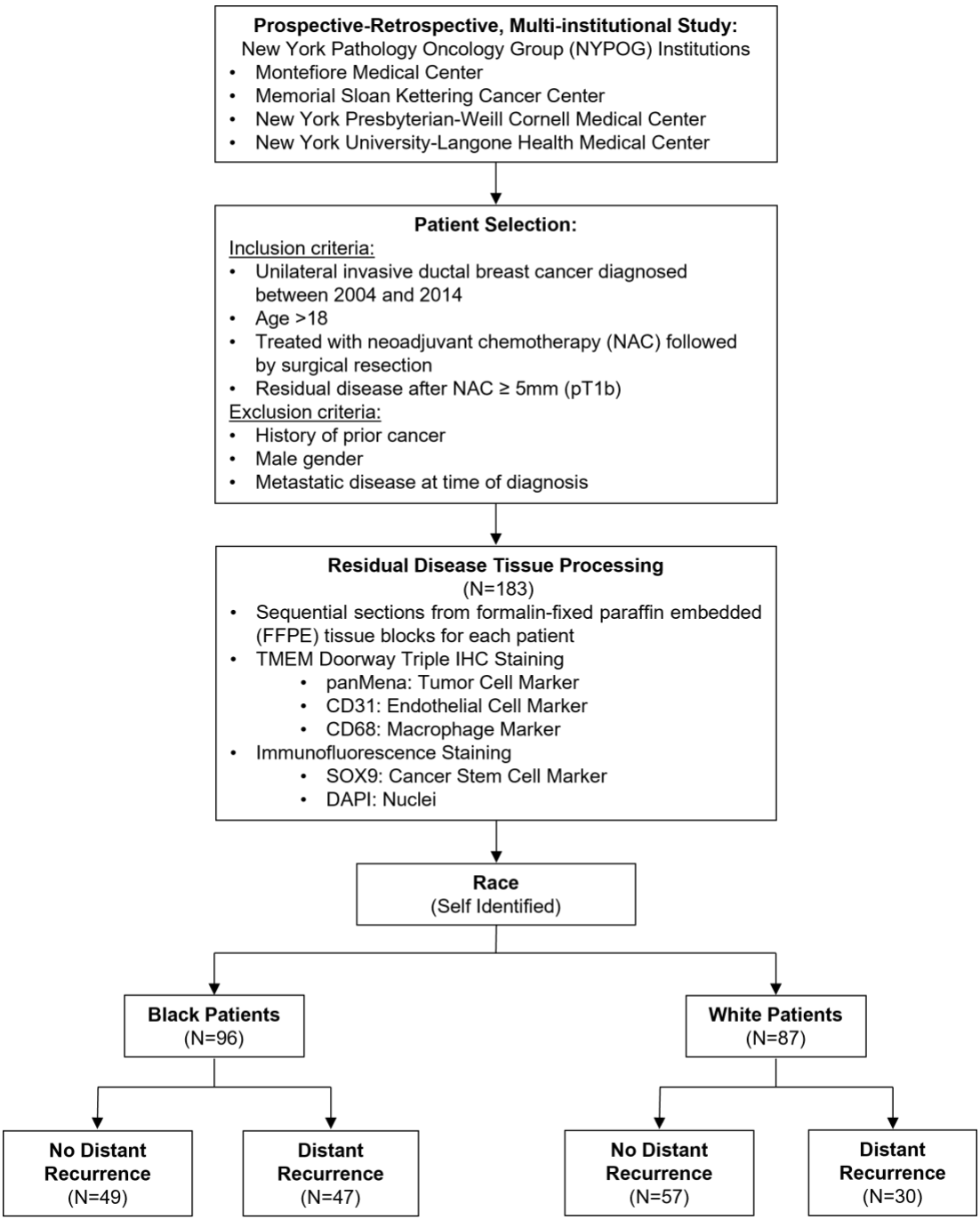


Figure 4.1. Study Design

We collected residual invasive ductal breast cancer tissue samples from 96 Black patients and 87 white patients (52.5% and 47.5%, respectively) from NYPOG institutions (**Figure 4.1, Table 4.1**). Black patients had higher distant recurrence (p=0.07, 49% in Black relative to 34.5% in white patients), mastectomy over breast conserving therapy (p=0.04, 69.8% in Black relative to 54% in white patients) (**Table 4.1**). Tumor grade was higher in Black versus white patients (p=0.002) with 76% Black versus 52.9% white patients in Grade 3, 22.9% Black versus 32.2 white in Grade 2, 0% Black versus 6.9 white in Grade 1 (**Table 4.1**). Although grade was statistically different, stage was not different between two (p=0.84, 46.9% Black versus 42.5% white patients in T1, 37.5% Black versus 40.2% white patients in T2, 15.6% Black versus 17.2% white patients in T3) (**Table 4.1**).

Age was not different (p=0.69, range [45.8-58.2], mean 51.6 in Black patients versus range [43.5-61], mean 52.3 in white patients. Time to distant recurrence (p=0.62, range [26.6-94.3], mean 63.9 in Black versus range [25.8-89.5], mean 59.8 in white patients) were similar in two groups (**Table 4.1**).

Lymph node status (p=0.67, 72.9% positive, 27.1% negative in Black patients; 69% positive, 31% negative in white patients) or subtype (p=0.1, 42.7% Black versus 57.5% white patients in ER+/HER2- breast cancer; 38.5% Black versus 25.3% white patients in TNBC; 18.8% Black versus 17.2% white patients in other subtypes) were also not statistically significant in the entire cohort (**Table 4.1**).

Table 4.1. Patient demographics and characteristics, entire cohort.

	Number of Patients (%)			
	All Patients N=183 (100)	Black Patients N=96 (52.5)	White Patients N=87 (47.5)	P Value
Distant Recurrence				0.07
Yes	77 (42.1)	47 (49)	30 (34.5)	
No	106 (57.9)	49 (51)	57 (65.5)	
Age				0.69
Mean [SD]	51.9 [11.5]	51.6 [10.3]	52.3 [12.7]	
Median [IQR]	52 [44, 60]	51 [45.8, 58.2]	52 [43.5, 61]	
Range	28 - 95	29 - 78	28 - 95	
Time to Distant Recurrence, Months				0.62
Mean [SD]	61.9 [40.4]	63.9 [42.9]	59.8 [37.7]	
Median [IQR]	62.5 [26.1, 92.9]	60.7 [26.6, 94.3]	64 [25.8, 89.5]	
Range	1 – 160.7	9.4 – 159.8	1 – 160.7	
Surgery				0.04
Mastectomy	114 (62.3)	67 (69.8)	47 (54)	
Breast Conserving Therapy	69 (37.7)	29 (30.2)	40 (46)	
Tumor Stage (ypT)				0.84
T1 (<2cm)	82 (44.8)	45 (46.9)	37 (42.5)	
T2 (2-5 cm)	71 (38.8)	36 (37.5)	35 (40.2)	
T3 (>5 cm)	30 (16.4)	15 (15.6)	15 (17.2)	
Lymph Node Status (ypN)				0.67
Positive	130 (71)	70 (72.9)	60 (69)	
Negative	53 (29)	26 (27.1)	27 (31)	
Grade				0.002
1	6 (3.3)	0 (0)	6 (6.9)	
2	50 (27.3)	22 (22.9)	28 (32.2)	
3	119 (65)	73 (76)	46 (52.9)	
Unknown	8 (4.4)	1 (1)	7 (8)	
Subtype				0.1
ER+/HER2-	91 (49.7)	41 (42.7)	50 (57.5)	
TNBC	59 (32.2)	37 (38.5)	22 (25.3)	
Other	33 (18)	18 (18.8)	15 (17.2)	

SD: standard deviation, IQR: interquartile range, yPT: tumor stage after neoadjuvant chemotherapy, yPN: lymph node status after neoadjuvant chemotherapy, ER+: estrogen receptor positive, TNBC: triple negative breast cancer.

Distant recurrence in ER+/HER2- disease was 46.3% in Black and 28% in white patients, however it was not significant (p=0.11) (**Table 4.2**). Black patients were treated with mastectomy over breast conserving therapy (82.9% Black versus 52% white patients in mastectomy, 17.1% Black versus 48% white patients in breast conserving therapy, p=0.004) (**Table 4.2**). Black women also had more positive lymph nodes (90.2% versus 66%, p=0.01) in ER+/HER2- breast cancer. Additionally, they had higher grade (p=0.01, 63.4% versus 32% in Grade 3, 36.6% versus 50% in Grade 2, 0% versus 8% in Grade 1) in ER+/HER2- subtype. However, stage was not statistically significant (p=0.91, 43.9% Black versus 40% white patients in T1, 41.5%

Black versus 46% white patients in T2, 14.6% Black versus 14% white patients in T3) (**Table 4.2**). There was no racial difference between in time to distant recurrence (p=0.78, range [33-93.4], mean 66.5 in Black versus range [42.2-95.3], mean 67.7 in white patients) in ER+/HER2- disease (**Table 4.2**).

Table 4.2. Patient demographics and characteristics in ER+/HER2- breast cancer.

	ER+/HER2- Number of Patients (%)			
	All Patients N=91 (100)	Black Patients N=41 (45.1)	White Patients N=50 (54.9)	P Value
Distant Recurrence				0.11
Yes	33 (36.3)	19 (46.3)	14 (28)	
No	58 (63.7)	22 (53.7)	36 (72)	
Age				0.13
Mean [SD]	52 [12.4]	49.6 [11.3]	54 [12.9]	
Median [IQR]	50 [43.5,61]	49 [42,56]	52 [44,62]	
Range	29 – 95	29 – 78	31 – 95	
Time to Distant Recurrence, Months				0.78
Mean [SD]				
Median [IQR]	67.1 [36.9]	66.5 [38.2]	67.7 [36.1]	
Range	71.5 [35.7,94.7]	68.6 [33,93.4]	72.7[42.2,95.3]	
	1 – 160.7	12 – 151	1 – 160.7	
Surgery				0.004
Mastectomy	60 (65.9)	34 (82.9)	26 (52)	
BCT	31 (34.1)	7 (17.1)	24 (48)	
Tumor Stage (ypT)				0.91
T1 (<2cm)	38 (41.8)	18 (43.9)	20 (40)	
T2 (2-5 cm)	40 (44)	17 (41.5)	23 (46)	
T3 (>5 cm)	13 (14.3)	6 (14.6)	7 (14)	
Lymph Node Status (ypN)				0.01
Positive				
Negative	70 (76.9)	37 (90.2)	33 (66)	
	21 (23.1)	4 (9.8)	17 (34)	
Grade				0.01
1	4 (4.4)	0 (0)	4 (8)	
2	40 (44)	15 (36.6)	25 (50)	
3	42 (46.2)	26 (63.4)	16 (32)	
Unknown	5 (5.5)	0 (0)	5 (10)	

SD: standard deviation, IQR: interquartile range, yPT: tumor stage after neoadjuvant chemotherapy, yPN: lymph node status after neoadjuvant chemotherapy, ER+: estrogen receptor positive, TNBC: triple negative breast cancer.

In TNBC, distant recurrence status between Black and white patient was similar (p=1, 54.1% Black versus 54.5% white patient distant recurrence) (**Table 4.3**). Time to distant recurrence in Black patient was [16.3-99.7] range, 62.5 mean; in white patients was [13-63.5] range, 43.6 mean. However, this was not significant (**Table 4.3**). Age was similar between Black and white patients (53.4 versus 50.9 mean, p=0.44). 56.8% of Black patient were received mastectomy, 43.2% were received breast conserving therapy. 68.2% white patients were received mastectomy, 31.8% were

received breast conserving therapy. Grade and stage were not different between two patient groups (p=0.4 and p=0.55, respectively) (**Table 4.3**). Lymph node positivity was similar between patients (p=0.52, 51.4% positive and 48.6% negative in Black patients, 63.6% positive and 36.4% negative in white patients) (**Table 4.3**).

Table 4.3. Patient demographics and characteristics in TNBC.

	TNBC Number of Patients (%)			
	All Patients N=59 (100)	Black Patients N=37 (62.7)	White Patients N=22 (37.3)	P Value
Distant Recurrence				1
Yes	32 (54.2)	20 (54.1)	12 (54.5)	
No	27 (45.8)	17 (45.9)	10 (45.5)	
Age				0.44
Mean [SD]	52.5 [9.7]	53.4 [8.3]	50.9 [11.8]	
Median [IQR]	52 [47,58]	52 [48,58]	53.5[42.5,57]	
Range	30 - 75	33 - 70	30 - 75	
Time to Distant Recurrence, Months				0.14
Mean [SD]	55.4 [44.5]	62.3 [47.9]	43.6 [36.1]	
Median [IQR]	36.6[15.1,95.4]	37.1[16.3,99.7]	31.3[13,63.5]	
Range	1.2 – 158.2	10.6 – 158.2	1.2 – 113.5	
Surgery				0.55
Mastectomy	36 (61)	21 (56.8)	15 (68.2)	
BCT	23 (39)	16 (43.2)	7 (31.8)	
Tumor Stage (ypT)				0.49
T1 (<2cm)	20 (33.9)	14 (37.8)	6 (27.3)	
T2 (2-5 cm)	25 (42.4)	16 (43.2)	9 (40.9)	
T3 (>5 cm)	14 (23.7)	7 (18.9)	7 (31.8)	
Lymph Node Status (ypN)				0.52
Positive	33 (55.9)	19 (51.4)	14 (63.6)	
Negative	26 (44.1)	18 (48.6)	8 (36.4)	
Grade				0.4
1	0 (0)	0 (0)	0 (0)	
2	6 (10.2)	5 (13.5)	1 (4.5)	
3	51 (86.4)	31 (83.8)	20 (90.9)	
Unknown	2 (3.4)	1 (2.7)	1 (4.5)	

SD: standard deviation, IQR: interquartile range, yPT: tumor stage after neoadjuvant chemotherapy, yPN: lymph node status after neoadjuvant chemotherapy, ER+: estrogen receptor positive, TNBC: triple negative breast cancer.

Treatments between patients in all cohort, ER+/HER2- breast carcinoma and TNBC were compared to avoid treatment effect on DRFS (**Tables 4.4, 4.5, and 4.6**). Treatments were categorized as NAC treatment regimen details based on the agent used and NAC combination with other treatments “called as All Treatments”, such as endocrine treatment, radiation, and HER2 inhibition.

Both NAC and all treatment categories were similar between Black and white patient in the entire cohort (p=0.33 and p=0.51, respectively) (**Table 4.4**). Additionally, NAC and all treatment categories did not show any statistical differences in ER+/HER2- subset (**Table 4.5**) and TNBC (**Table 4.6**).

Table 4.4. Neoadjuvant chemotherapy treatment details, entire cohort.

	Number of Patients (%)			
	All Patients N=183 (100)	Black Patients N=96 (52.5)	White Patients N=87 (47.5)	P Value
Neoadjuvant Chemotherapy (NAC)				0.33
Taxane-containing	157 (85.8)	80 (83.3)	77 (88.5)	
ACT	132 (84.1)	70 (87.5)	62 (80.5)	0.12
AT	3 (1.9)	2 (2.5)	1 (1.3)	
CT	5 (3.2)	0 (0)	5 (6.5)	
T	17 (10.8)	8 (10)	9 (11.7)	
No taxane	12 (6.6)	6 (6.3)	6 (6.9)	
Unknown[a]	14 (7.7)	10 (10.4)	4 (4.6)	
All Treatments				0.51
NAC	140 (76.5)	72 (75)	68 (78.2)	
NAC + Endocrine	2 (1.1)	1 (1.0)	1 (1.1)	
NAC + HER2 inhibitor	23 (12.6)	12 (12.5)	11 (12.6)	
NAC + Radiotherapy	4 (2.2)	1 (1.0)	3 (3.4)	
Unknown[a]	14 (7.7)	10 (10.4)	4 (4.6)	

[a]Detailed neoadjuvant chemotherapy information is not available. A: anthracycline, C: cyclophosphamide, T: taxane

Table 4.5. Neoadjuvant chemotherapy treatment details in ER+/HER2 breast cancer.

	ER+/HER2- Number of Patients (%)			
	All Patients N=91 (100)	Black Patients N=41 (45.1)	White Patients N=50 (54.9)	P Value
NAC				0.15
Taxane-containing	78 (85.7)	32 (78.1)	46 (92)	
ACT	66 (84.6)	27 (84.4)	39 (84.8)	0.63
AT	2 (2.6)	1 (3.1)	1 (2.2)	
CT	2 (2.6)	0 (0)	2 (4.3)	
T	8 (10.3)	4 (12.5)	4 (8.7)	
No taxane	5 (5.5)	3 (7.3)	2 (4)	
Unknown[a]	8 (8.8)	6 (14.6)	2 (4)	
All Treatments				0.29
NAC	77 (84.6)	33 (80.5)	44 (88)	
NAC + Endocrine	2 (2.2)	1 (2.4)	1 (2)	
NAC + HER2 inhibitor	0 (0)	0 (0)	0 (0)	
NAC + Radiotherapy	4 (4.4)	1 (2.4)	3 (6)	
Unknown[a]	8 (8.8)	6 (14.6)	2 (4)	

[a]Detailed neoadjuvant chemotherapy information is not available. A: anthracycline, C: cyclophosphamide, T: taxane

Table 4.6. Neoadjuvant chemotherapy treatment details in TNBC.

	TNBC Number of Patients (%)			
	All Patients N=59 (100)	Black Patients N=37 (62.7)	White Patients N=22 (37.3)	P Value
NAC				0.38
Taxane-containing	53 (89.8)	32 (86.5)	21 (95.5)	
ACT	50 (94.3)	32 (100)	18 (85.7)	0.09
AT	0 (0)	0 (0)	0 (0)	
CT	2 (3.8)	0 (0)	2 (9.5)	
T	1 (1.9)	0 (0)	1 (4.8)	
No taxane	3 (5.1)	2 (5.4)	1 (4.5)	
Unknown[a]	3 (5.1)	3 (8.1)	0 (0)	
All Treatments				0.17
NAC	56 (94.9)	34 (91.9)	22 (100)	
NAC + Endocrine	0 (0)	0 (0)	0 (0)	
NAC + HER2 inhibitor	0 (0)	0 (0)	0 (0)	
NAC + Radiotherapy	0 (0)	0 (0)	0 (0)	
Unknown[a]	3 (5.1)	3 (8.1)	0 (0)	

[a]Detailed neoadjuvant chemotherapy information is not available. A: anthracycline, C: cyclophosphamide, T: taxane

4.2 Racial Disparity in Pro-metastatic TME Markers

The study aimed to explore the potential differences in TMEM doorway score and the individual components of the TMEM doorway, including macrophage and microvascular density, after NAC. In addition, CSCs within the TME were also

investigated, as recent evidence have suggested that interactions between macrophages and TMEM doorway-related tumor cells can lead to increased density of CSCs (23).

The figures presented in 4.2 illustrates representative images of TMEM doorway-high versus TMEM doorway-mid/low, as well as nuclear SOX9-high versus nuclear SOX9-low (**Figures 4.2**).

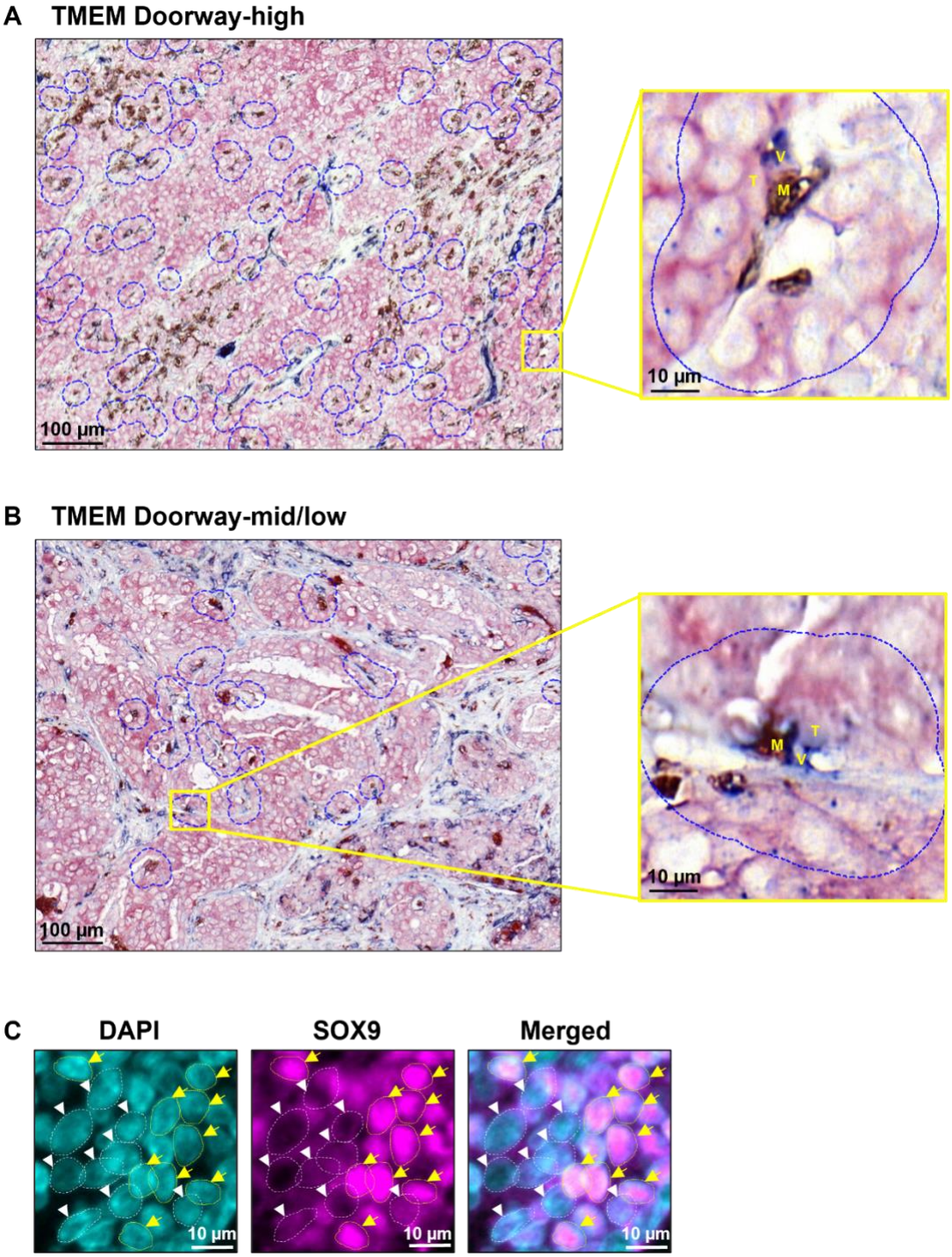


Figure 4.2. Representative images of TMEM Doorway triple IHC and SOX9 IF staining. **A**) TMEM Doorway-high and **B**) TMEM Doorway-mid/low score representative images. Tumor cells (panMena) are pink, macrophages (CD68) are brown, and endothelial cells (CD31, vasculature) are blue. TMEM doorways in automated analysis are seen as blue-dashed circles. **C**) Representative images show SOX9-high cells with yellow arrows and SOX9-low cells with white arrowheads. M: Macrophage, T: Tumor Cell, V: Vasculature.

First, we investigated differences of TMEM doorway, and related pro-metastatic tumor markers (CSCs, macrophage, and microvascular density) in most common breast cancer subtypes: ER+/HER2- and TN breast cancer.

TNBC had higher TMEM doorway score (p=0.004) (**Figure 4.3A**), macrophage density (p=0.0002) (**Figure 4.3B**), and CSCs density (nuclear SOX9^{high} cancer cells, p=0.0002) (**Figure 4.3C**) than ER+/HER2- disease. Microvascular density was similar between the two subtypes (p=0.44) (**Figure 4.3D**).

The TMEM doorway and it's individual components play crucial role in facilitating cancer cell entry into vessels and subsequent migration to distant organs, and thus may be implicated in the early recurrence of TNBC.

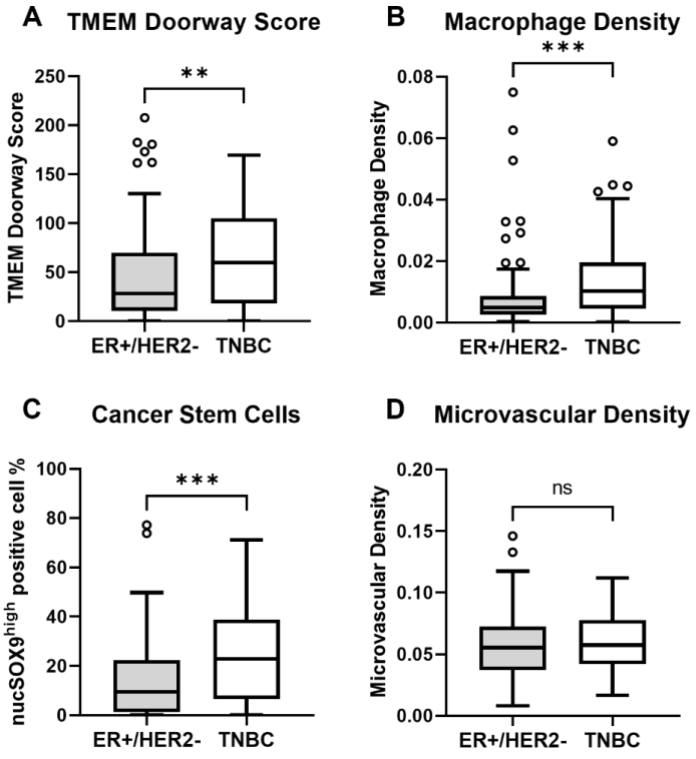


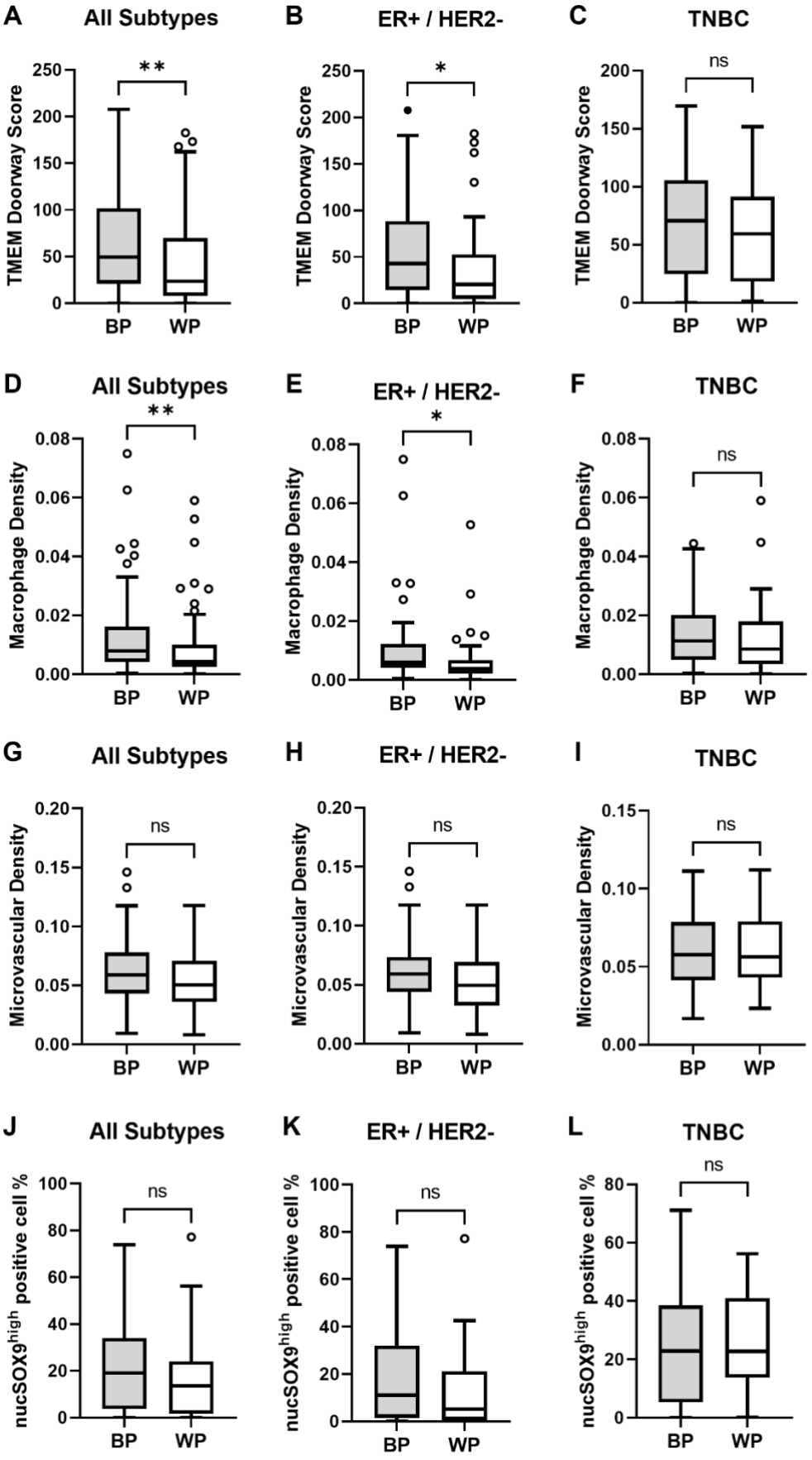
Figure 4.3. Pro-metastatic tumor markers, except microvascular density, are higher in TNBC than ER+/HER2- disease. **A**) TMEM doorway score (p=0.004); **B**) macrophage density (p=0.0002); **C**) cancer stem cells (p=0.0002); and **D**) microvascular density (p=0.44). N=91 in ER+/HER2- disease, N=59 in TNBC. ER+: estrogen receptor positive, TNBC: triple negative breast cancer. ns: not statistically significant, p>0.05, *: p≤ 0.05, **: p≤ 0.01, ***: p≤ 0.001.

Following the pro-metastatic tumor markers in breast cancer subtypes, we focused on racial disparity in same pro-metastatic tumor markers. Black patients had higher TMEM doorway score when all subtypes combined (p=0.002) (**Figure 4.4A**), and in ER+/HER2- disease (p=0.02) (**Figure 4.4B**), but not in TNBC (p=0.74) (**Figure 4.4C**). Like TMEM doorway score, macrophage density was higher in Black, compared to white patients, in all subtypes (p=0.002) (**Figure 4.4D**), and in ER+/HER2- disease (p=0.02) (**Figure 4.4E**), but not in TNBC (p=0.31) (**Figure 4.4F**).

Microvascular density was not different between patients in the entire cohort (p=0.06), ER+/HER2- disease (p=0.09), and TNBC (p=0.97) (**Figure 4.4G-I**). Similarly, CSCs density was not different between patients in the entire cohort (p=0.09), ER+/HER2- disease (p=0.09), and TNBC (p=0.73) (**Figure 4.4J-L**)

In summary, these data indicate that black patients with ER+/HER2- subset have higher TMEM doorway score and macrophage density relative to white patients. These findings mat offer an explanation for the racial differences in ER+/HER2- subset survival.

Next, to answer the question of effect of TMEM doorway and other markers on survival, we analyzed the correlation between these markers and distant recurrence free survival (DRFS).


A
All Subtypes
TMEM Doorway Score
**
BP
WP
B
ER+ / HER2-
TMEM Doorway Score
*
BP
WP
C
TNBC
TMEM Doorway Score
ns
BP
WP
D
All Subtypes
Macrophage Density
**
BP
WP
E
ER+ / HER2-
Macrophage Density
*
BP
WP
F
TNBC
Macrophage Density
ns
BP
WP
G
All Subtypes
Microvascular Density
ns
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H
ER+ / HER2-
Microvascular Density
ns
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WP
I
TNBC
Microvascular Density
ns
BP
WP
J
All Subtypes
nucSOX9high positive cell %
ns
BP
WP
K
ER+ / HER2-
nucSOX9high positive cell %
ns
BP
WP
L
TNBC
nucSOX9high positive cell %
ns
BP
WP

Figure 4.4. Racial disparity is observed in TMEM doorways and macrophages overall, and in ER+/HER2- disease, but not in TNBC. **A-C**) TMEM doorway score by race in all subtypes (A, p=0.002), in ER+/HER2- disease (B, p=0.02), and TNBC (C, p=0.74). **D-F**) Macrophage density by race in all subtypes (D, p=0.002), in ER+/HER2- disease (E, p=0.02), and TNBC (F, p=0.31). **G-I**) Microvascular density by race in all subtypes (G, p=0.06), in ER+/HER2- disease (H, p=0.09), and TNBC (I, p=0.97). **J-L**) Cancer stem cell percentage in all subtypes (J, p=0.09), in ER+/HER2- disease (K, p=0.09), and TNBC (L, p=0.73). Patient numbers: BP (N=96), WP (N=87) in all subtypes; BP (N=41), WP (N=50) in ER+/HER2- disease; BP (N=37), WP (N=22) in TNBC. BP: Black Patients, WP: White Patients, ER+: estrogen receptor positive, TNBC: triple negative breast cancer. ns: not statistically significant, $p>0.05$, *: $p\leq 0.05$, **: $p \leq 0.01$.

4.3 Association between Pro-metastatic TME Parameters and DRFS

Several previous studies have demonstrated that a high TMEM doorway score is strongly associated worse survival and increased metastasis in adjuvant treatment setting. In light of this, we sought to extend this knowledge and examine whether a high TMEM doorway score, in conjunction with other pro-metastatic marker, is also linked to inferior DRFS in NAC setting. Our objective was to gain a more understanding of the TMEM doorway and related pro-metastatic factors' role in NAC-induced tumor progression, and whether they might serve as viable prognostic markers in racial disparity context.

First, we looked at the correlation between TMEM doorway score and pro-metastatic tumor markers via Spearman correlation analysis. We found that only TMEM doorway score and macrophage density are positively correlated among other markers (Spearman Correlation Coefficient: 0.668) (**Figure 4.5**). Other combinations were:

1. TMEM doorway score and CSC (Spearman Correlation Coefficient: 0.112).
2. TMEM doorway score and microvascular density (Spearman Correlation Coefficient: -0.233). TMEM doorway score and microvascular density showed negative correlation in this analysis. To our knowledge, this finding was unexpected and need further investigation.

3. Macrophage density and microvascular density (Spearman Correlation Coefficient: 0.081).
4. Macrophage density and CSCs (Spearman Correlation Coefficient: 0.0.103).
5. Microvascular density and CSCs (Spearman Correlation Coefficient: 0.0.103).

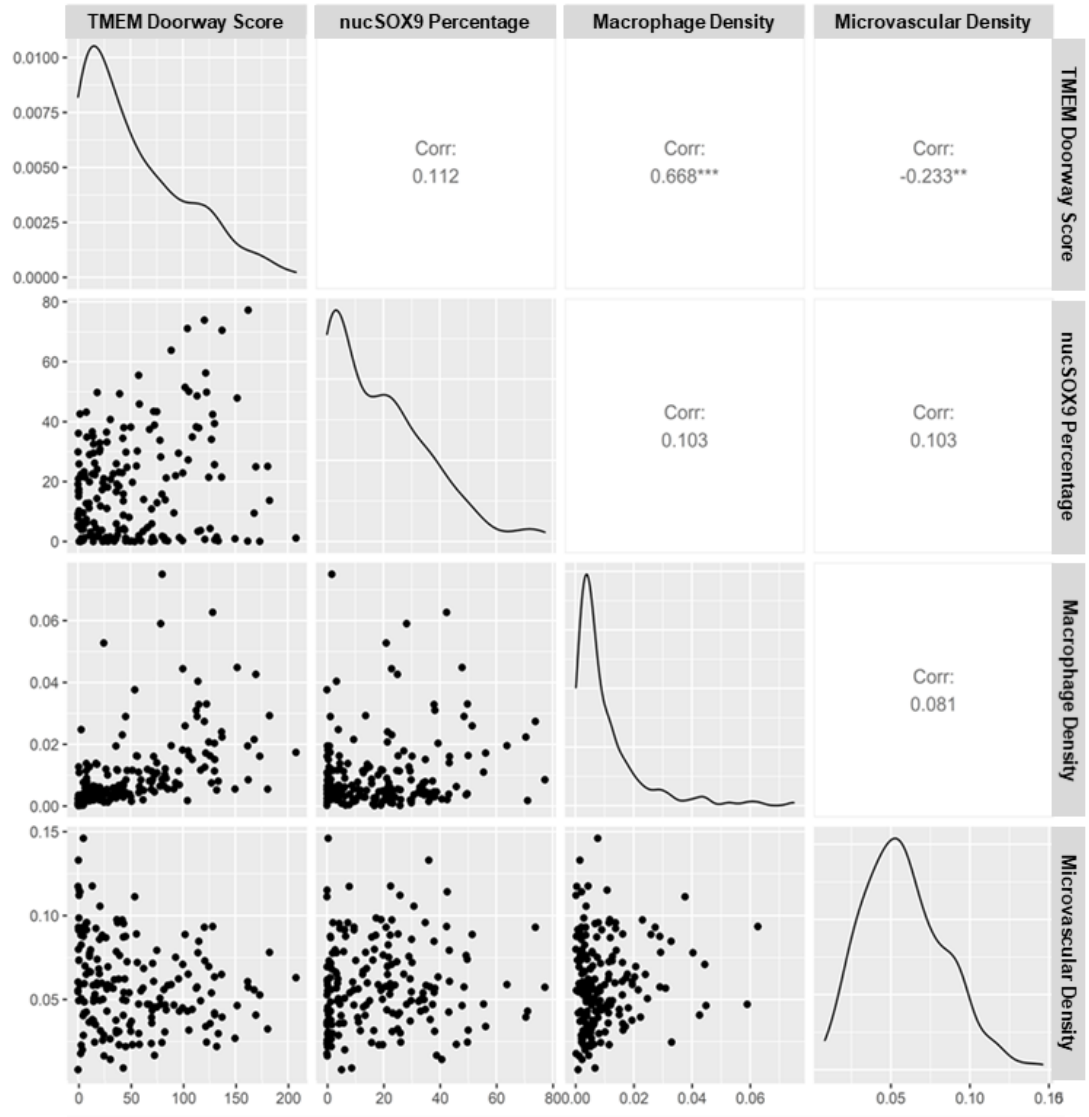


Figure 4.5. Correlation among pro-metastatic tumor markets in the entire cohort. The distribution of each marker can be seen in diagonally placed curve graphs.

Next, we investigated DRFS and racial disparity. Even though, there is a separation, DRFS in Black versus white patients did not show any statistical difference (p=0.21) (**Figure 4.6A**). In ER+/HER2- subset, the separation between two race was

more obvious, although it was not significant (p=0.15) (**Figure 4.6B**). DRFS in TNBC was similar between two race (p=0.6) (**Figure 4.6A-C**).

Following DRFS and racial disparity, we looked at the association between pro-metastatic tumor markers and DRFS. Patients with high TMEM doorway score in comparison with mid/low TMEM doorway score had inferior DRFS in all cohort (p=0.008) (**Figure 4.6D**). We also observed the separation between high- and mid/low-TMEM doorway score in ER+/HER2- subtype (p=0.08) (**Figure 4.6E**). There was no difference in TNBC (p=0.77) (**Figure 4.6F**). We asked if treatment status were different between TMEM doorway-high and TMEM-doorway-mid/low groups that might be affecting the survival. There was no difference in treatment between high vs mid/low groups (p=073 in NAC treatment and p=0.83 in all treatments) (**Table 4.7**).

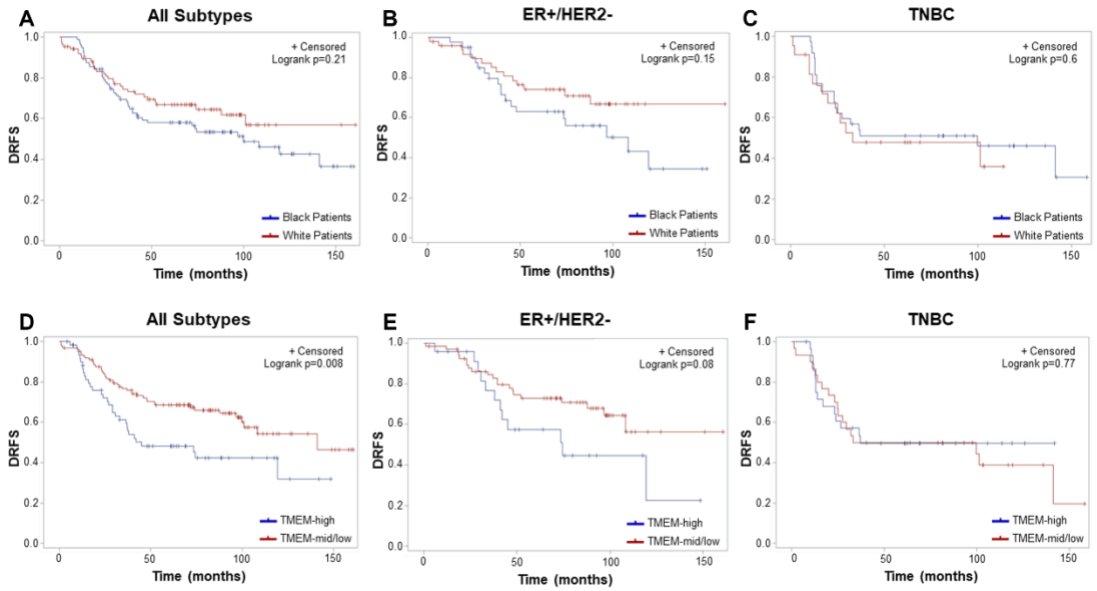


Figure 4.6. Patients with high-TMEM doorway score have inferior DRFS in the entire cohort and trended towards in ER+/HER2- disease. **A-C**) DRFS in Black vs white patients in all subtypes (A, p=0.21), ER+/HER2- subtype (B, p=0.15), and TN subtype (C, p=0.6). **D-F**) Patients with high- vs mid/low-TMEM doorway score and in all subtypes (D, p=0.008), ER+/HER2- subtype (E, p=0.08), and TN subtype (F, p=0.77).

Table 4.7. Neoadjuvant chemotherapy treatment details, by TMEM doorway score.

	Number of Patients (%)			
	All Patients N=183 (100)	TMEM Doorway-high N=61 (33.7)	TMEM doorway-mid/low N=122 (66.7)	P Value
Neoadjuvant Chemotherapy (NAC)				0.73
Taxane-containing	157 (85.8)	51 (83.6)	106 (86.9)	
ACT	132 (84.1)	42 (82.4)	90 (84.9)	0.61
AT	3 (1.9)	2 (3.9)	1 (0.9)	
CT	5 (3.2)	2 (3.9)	3 (2.8)	
T	17 (10.8)	5 (9.8)	12 (11.3)	
No taxane	12 (6.6)	4 (6.6)	8 (6.6)	
Unknown[a]	14 (7.7)	6 (9.8)	8 (6.6)	
All Treatments				0.83
NAC	140 (76.5)	47 (77.1)	93 (76.2)	
NAC + Endocrine	2 (1.1)	1 (1.6)	1 (0.8)	
NAC + HER2 inhibitor	23 (12.6)	6 (9.8)	17 (13.9)	
NAC + Radiotherapy	4 (2.2)	1 (1.6)	3 (2.5)	
Unknown[a]	14 (7.7)	6 (9.8)	8 (6.6)	

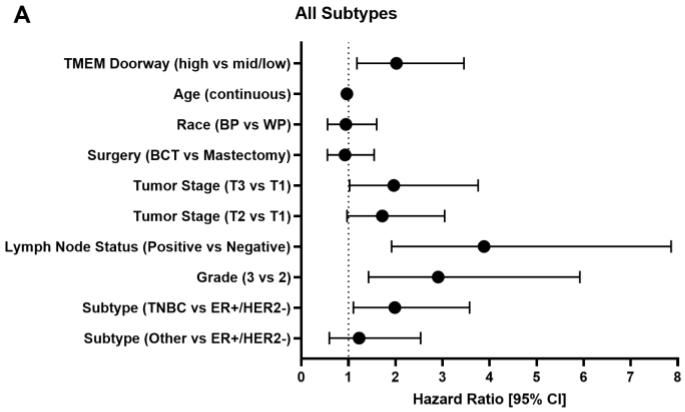
[a]Detailed neoadjuvant chemotherapy information is not available. A: anthracycline, C: cyclophosphamide, T: taxane

To avoid confounding effect on DRFS, we ran multivariate Cox regression analysis for the following variates in entire cohort and in ER+/HER2- breast cancer: TMEM doorway (high versus mid/low), age, race, surgery type (breast conserving surgery versus mastectomy), tumor stage, lymph node status, grade, and subtype.

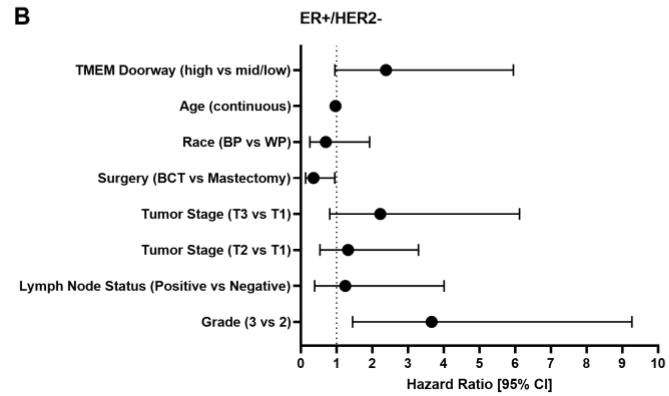
Multivariate Cox regression analysis showed that high-TMEM doorway score was an independent prognostic risk factor (HR 2.02 [95% CI 1.18-3.46], p=0.01). Other prognostic risk factors were stage (T3 versus T1) (HR 1.97 [95% CI 1.03-3.76], p=0.04), lymph node status (positive versus negative) (HR 3.88 [95% CI 1.92-7.86], p=0.0002), grade (3 versus 2) (HR 2.91 [95% CI 1.43-5.92], p=0.003), and subtype (TNBC versus ER+/HER2-) (HR 1.99 [95% CI 1.1-3.58], p=0.02) (**Figure 4.7A**). Race (HR 0.94 [95% CI 0.56-1.6], p=0.83), surgery type (HR 0.93 [95% CI 0.55-1.55], p=0.77), tumor stage for T2 versus T1 (HR 1.72 [95% CI 0.97-3.05], p=0.06), and subtype for other versus ER+/HER2- (HR 1.23 [95% CI 0.6-2.53], p=0.58) did not show any significance. Even though age p value was below 0.05 (HR 0.97 [95% CI 0.94-0.99], p= 0.01), we accepted this result not clinically important with confidence interval 0.94-0.99 (**Figure 4.7A**).

Further, high-TMEM score trended towards being an independent prognostic risk factor in ER+/HER2- subtype (HR 2.38 [95% CI 0.96-5.95], p=0.06) (**Figure**

4.7B). Only variates as prognostic risk factor in ER+/HER2- group was grade (3 versus 2) (HR 3.66 [95% CI 1.45-9.27], p=0.006) (**Figure 4.7B**). Other variates were not significant.



HR [95% CI]	P Value
2.02 [1.18 - 3.46]	0.01
0.97 [0.94 - 0.99]	0.01
0.94 [0.56 - 1.6]	0.83
0.93 [0.55 - 1.55]	0.77
1.97 [1.03 - 3.76]	0.04
1.72 [0.97 - 3.05]	0.06
3.88 [1.92 - 7.86]	0.0002
2.91 [1.43 – 5.92]	0.003
1.99 [1.1 - 3.58]	0.02
1.23 [0.6 - 2.53]	0.58



HR [95% CI]	P Value
2.38 [0.96 – 5.95]	0.06
0.97 [0.93 – 1.01]	0.12
0.70 [0.25 – 1.93]	0.49
0.35 [0.13 – 0.95]	0.04
2.22 [0.81 – 6.12]	0.12
1.32 [0.53 – 3.29]	0.55
1.24 [0.39 – 4.01]	0.72
3.66 [1.45 – 9.27]	0.006

Figure 4.7. Multivariate Cox regression model in all subtypes (**A**) and in ER+/HER2- subtype (**B**). N=175 in all subtypes and N=86 in ER+/HER2- disease, patients with unknown status are excluded. BP: Black Patients, WP: White Patients, BCT: breast conserving therapy, ER+: estrogen receptor positive, TNBC: triple negative breast cancer, HR: Hazard Ratio, CI: Confidence Interval.

Even though, high-TMEM doorway score was associated with inferior DRFS; we did not observe any association between individual components of the TMEM doorway (macrophage and microvascular density) and DRFS (**Figure 4.8A-F**). DRFS in macrophage density-high versus macrophage density mid/low groups were similar in all subsets (p=0.23), in ER+/HER2- subset (p=0.42), and in TNBC (p=0.37) (**Figure 4.8A-C**). DRFS in microvascular density-high versus microvascular density mid/low groups were similar in all subsets (p=0.82), in ER+/HER2- subset (p=0.26) (**Figure 4.8D-E**). There was a trend in high-microvascular density and DRFS TN subtype (p=0.06) (**Figure 4.8F**). Additionally, there was no association between CSC density

and DRFS in tall subsets (p=0.83), in ER+/HER2- subset (p=0.25), and in TNBC (p=0.47) (**Figure 4.8G-I**).

In summary, these results suggest that direct and stable interaction between three cells, namely tumor cells, endothelial cells, and macrophages, at the TMEM doorways is of greater importance for survival than the individual components of the doorway. It is evident from this finding along with other that TMEM doorways could be significant determinant of prognosis in clinical settings. However, due to the relatively smaller number of patients when divided into racial groups, subtypes, and TMEM doorway-high versus TMEM doorway mid/low status, it was not possible to run DRFS analysis for each racial group.

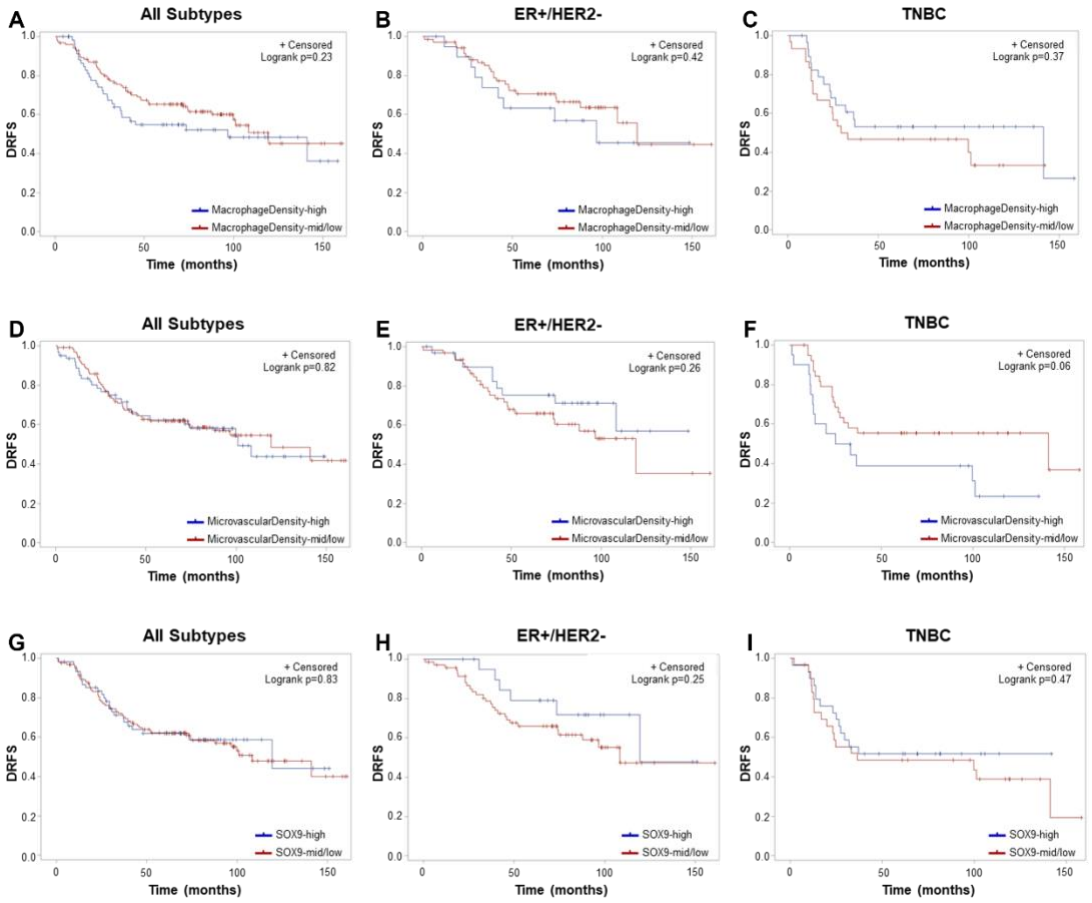


Figure 4.8. Pro-metastatic tumor markers and DRFS were not associated in all subtypes, ER+/HER2- subtype, and TN subtype. **A-C**) DRFS in high- vs mid/low-macrophage density in all subtypes (A, p=0.23), ER+/HER2- subtype (B, p=0.42), and TN subtype (C, p=0.37). **D-F**) DRFS in high- vs mid/low-microvascular density in all subtypes (D, p=0.82), ER+/HER2- subtype (E, p=0.26), and TN subtype (F, p=0.06). **G-I**) DRFS in high- vs mid/low- SOX9

density in all subtypes (G, p=0.83), ER+/HER2- subtype (H, p=0.25), and TN subtype (I, p=0.47). DRFS: Distant Recurrence Free Survival, ER+: Estrogen Receptor positive, TN: Triple Negative.

5. DISCUSSION

In this thesis study, first we explored the differences in pro-metastatic tumor markers in ER+/HER2- and TN breast cancer. TMEM doorways, macrophages, and CSCs were higher in TNBC compared to ER+/HER2- breast carcinoma. Then, we investigated the racial differences in pro-metastatic tumor markers in residual breast cancer after NAC.

Black patients had higher TMEM doorway score and macrophages compared to white patients in entire cohort and ER+/HER2- breast cancer, but not in TNBC. Only high-TMEM doorway density was correlated with inferior survival overall, not the other components of the TMEM doorway. This doorway is an important prognostic factor for distant recurrence, not individual components of the pathway (macrophage density, microvascular density). This study shows that race and breast cancer subtype are important biologic factors affecting the response to chemotherapy, to a greater degree in Black patients with residual ER+/HER2- breast carcinoma after NAC (**Figure 5.1**).

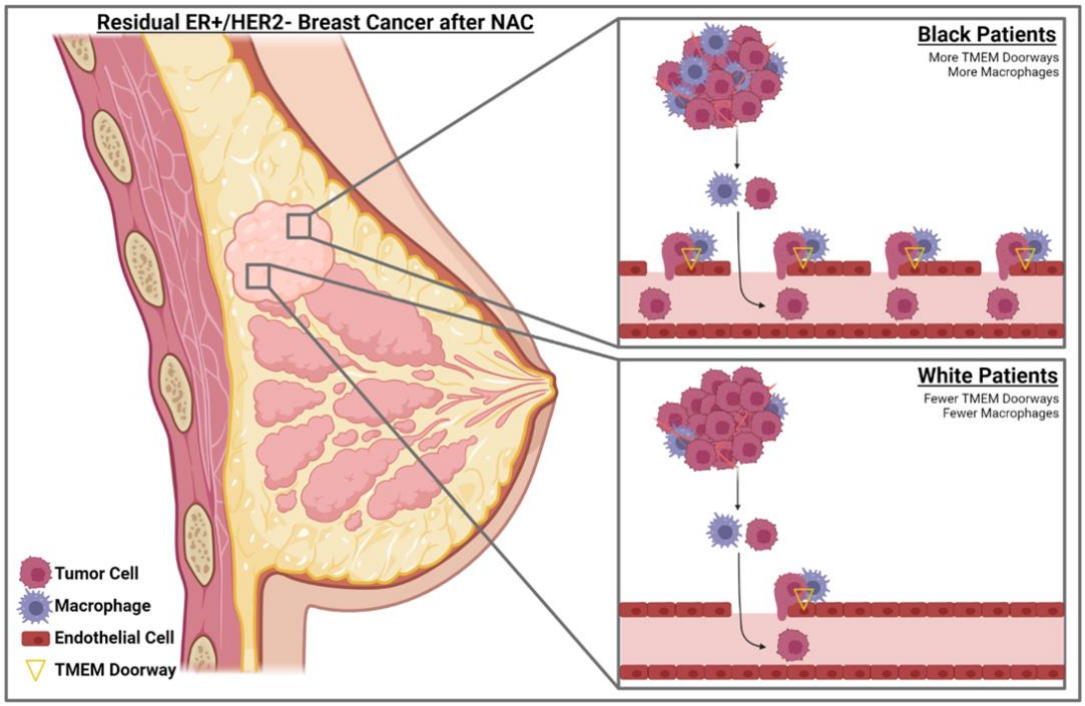


Figure 5.1. Black patients have higher TMEM doorway score and macrophage density compared to white patients in residual ER+/HER2- breast cancer after NAC. Racial disparity in outcome may be due to a more pronounced pro-metastatic

tumor microenvironment (increased TMEM doorway density) in Black, compared to white, patients with residual ER+/HER2- breast cancer. BioRender.com was used to make this figure.

TN breast cancer has the worse survival when compared to other subtypes because of lack of targeted therapies, early recurrence, and high relapse rates (3,28–30). We have found that TN, compared to ER+/HER2- breast cancer, has higher levels of TMEM doorways, macrophages, and CSCs which may explain early recurrence and high relapse nature of the disease. However, there was no racial disparity of pro-metastatic tumor markers or DRFS in this subtype. These findings might be due to the aggressive nature of TNBC masking biological race differences in TME.

Studies have been found that repair signals in tumor is induced following NAC which in turn recruits TIE2+ macrophages, one of the components of the TMEM doorway, into the TME (12,13) resulting in increased TMEM doorway numbers (14,15). TIE2+ macrophages in TMEM doorways secrets VEGF-A and opens the vasculature transiently around TMEM doorways (18). Then, MenaINV expressing invasive tumor cells can intravasate using this transient vascular openings (19,82,110) and metastasize. Increased TMEM doorway density and MenaINV tumor cell dissemination might be one the mechanisms for resistance to NAC (111). This potential resistance mechanism carries clinical importance since TMEM doorway related vascular opening can be monitored in real time via novel TMEM-MRI technology (88) and can be targeted via TIE2 inhibitor, Rebastinib (87).

Macrophage density has been shown to be correlated with inferior outcome in breast carcinoma (112–115). A meta-analysis of 8496 patients with breast carcinoma indicated that tumor associated macrophages are correlated with inferior outcome (112). Similarly, high microvascular density was correlated with metastasis and worse survival in invasive breast carcinoma (116). One study has reported that both macrophage and microvascular density were higher in Black versus white patients with breast cancer (58). Even though we have found higher macrophage density in Black patients, there was no racial disparity in microvascular density and no association between DRFS for both markers.

There are contrasting reports regarding CSCs and breast carcinoma outcomes. Some studies showed the correlation between CSCs and inferior outcome (90–94),

whereas other studies have not found any correlation (95,96). We have not found any racial disparity in CSCs or correlation between DRFS in this cohort. Additionally, recent study found induction of CSCs around TMEM doorway mouse mammary cancer *in vivo* (23). Further, same study found correlation between CSCs and TMEM doorway score (23). However, there was no correlation between CSCs and TMEM doorway score in this study. This discrepancy between studies might be related to the analysis method of CSCs in patient samples. CSCs were analyzed by flow cytometry and in-situ hybridization in fine-needle aspirate samples in Sharma et al. (23), whereas we used IF to analyze CSC density. The fine-needle aspirate samples collected from patients might have been enriched by invasive cancer cells with high CSC marker expression because these cells are discohesive and compared to more epithelial cohesive cells preferentially get collected. This might be one of the reasons for the discrepancy and needs further investigation.

Several studies showed that the racial disparity is most prominent in patients with ER+/HER2- disease who received adjuvant chemotherapy, but not in ER- disease (5,7,8,10). A study including a cohort of 3,890 cases with invasive breast carcinoma showed that Black women with ER+/HER2- disease had 2 times higher distant recurrence (8). Similarly, National Cancer Institute sponsored the Trial Assigning Individualized Options for Treatment (TAILORx) trial which includes only ER+/HER2- breast cancer found that Black race was associated with inferior survival, also 1.6 fold increase in distant recurrence (9). Furthermore, Black patients with ER+/HER2- disease was strongly associated with inferior disease-free survival in ECOG-ACRIN-5103 clinical trial with 4,994 patient cohort (7). Likewise, in a randomized study with 4,817 patients showed that Black women with ER+/HER2- disease had inferior survival (5). Besides breast cancer, Albain et al. showed that racial disparity in outcome also seen in other hormone-dependent cancers (117). In a study combined 8 NSABP trials, our group found that in addition to adjuvant chemotherapy, racial disparity also exists in neoadjuvant setting in residual ER+/HER2-, but not TN breast cancer (10). In this work, there was a trend towards inferior DRFS in ER+/HER2- disease, likely due to 10-fold smaller cohort size compared to NSABP study (10). No racial disparity in DRFS was found in TN disease, consistent with published studies.

One of the key strengths of the study lies in the inclusion of patients who have access to similar clinical care and controlled treatment settings, which to ensure that our findings are generalizable and applicable to a broader population. Furthermore, the comparable distribution of key demographic and clinical factors, such as age, time to distant recurrence, stage, and lymph node status in Black and white patients is a significant strength, as it helps to minimize the potential for confounding factors that could skew our results.

To address potential confounding factors, we utilized a multivariate Cox regression model, which allowed us to more accurately assess the relationship between markers and the metastasis risk. This approach further enhances the robustness and reliability of our findings.

However, it is important to mention the limitations. The cohort size when separated by subtype is relatively small. Additionally, the absence of pre-chemotherapy samples and matched pre-post chemotherapy samples for individual patients precluded the study of baseline and fold changes of pro-metastatic tumor markers and TMEM doorways, which may have provided further insight into the mechanisms driving the observed associations. Despite these limitations, our study provides valuable insights into the potential use of TMEM doorways as a metastasis predictor, also a potential mechanism for racial disparity, highlighting the need for further research in this area to fully elucidate the underlying mechanisms involved.

Findings of this research provide an important perspective regarding difference in NAC response in patients with diverse geographic ancestry. Here, we have demonstrated that racial disparity in survival in residual ER+/HER2- subset (10) may be related to high-TMEM doorways in Black compared to white women. Furthermore, our study suggests that in addition to well established prognostic factors (tumor size, lymph node status), TMEM doorway score could be added as a marker of distant spread in patients with breast carcinoma. In clinical practice, we can use non-invasive imaging approaches to measure and follow up TMEM doorway score with TMEM-MRI activity (88) which can help clinicians to plan the systemic therapy and use therapeutic agents blocking TMEM doorway activity to slow down the metastatic

dissemination (87), which might ultimately guide us to overcome racial disparity in breast carcinoma.

6. CONCLUSION AND RECOMMENDATION

In primary breast cancer, TMEM doorways serve as portals of entry into the blood vasculature for systemic cancer cell dissemination. High-TMEM doorway density is associated with a higher distant recurrence risk in patients with primary breast cancers that have not previously been treated with chemotherapy.

NAC induces pro-metastatic changes in the tumor microenvironment that result in a greater TMEM doorway density. Here we demonstrate that TMEM doorway density score in residual primary breast cancer after NAC is a prognostic biomarker for distant recurrence, and that residual primary tumors after NAC in Black women have higher TMEM doorway density score than in white women, suggesting a previously unrecognized factor that might be contributing to racial disparities in breast carcinoma outcomes.

Furthermore, future studies could be designed to add inhibitors of TMEM doorway activity to standard-of-care adjuvant and neoadjuvant clinical trials with a proportionate number of underrepresented racial minority patients to determine if this approach would diminish racial disparity in outcome in breast cancer.

As a follow-up on this study, we will focus on one of the limitations of this thesis study: the absence of tissue samples from pre-NAC and matched tissue samples before and after NAC from same patient. We will focus on two new questions:

1. Do breast cancers from Black women have higher TMEM doorway score at baseline (i.e., before the start of chemotherapy) relative to white women?
2. Is there a racial disparity in the fold-change in TMEM doorway score before NAC versus after NAC?

These two questions are crucial to address limitations of this study to understand main mechanism: first, whether there are baseline differences in host biology between racial groups that might be contribute to observed disparities in pro-metastatic tumor markers second, whether there are differences in response to chemotherapy in Black patients. Answering these questions will provide important insights into the underlying factors that contribute to racial disparities in outcome.

Furthermore, future studies could be designed to add inhibitors of TMEM doorway activity to standard-of-care adjuvant and neoadjuvant clinical trials with a proportionate number of underrepresented racial minority patients to determine if this approach would diminish racial disparity in outcome in breast cancer.

7. REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin. 2022;72(1):7–33.

2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023;73(1):17–48.

3. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al. Breast cancer statistics, 2019. CA Cancer J Clin. 2019;69(6):438–51.

4. DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. CA Cancer J Clin. 2016 Feb;66(1):31–42.

5. Sparano JA, Wang M, Zhao F, Stearns V, Martino S, Ligibel JA, et al. Race and hormone receptor-positive breast cancer outcomes in a randomized chemotherapy trial. J Natl Cancer Inst. 2012 Mar 7;104(5):406–14.

6. Schneider BP, Li L, Radovich M, Shen F, Miller KD, Flockhart DA, et al. Genome-Wide Association Studies for Taxane-Induced Peripheral Neuropathy in ECOG-5103 and ECOG-1199. Clin Cancer Res Off J Am Assoc Cancer Res. 2015 Nov 15;21(22):5082–91.

7. Schneider BP, Shen F, Jiang G, O'Neill A, Radovich M, Li L, et al. Impact of Genetic Ancestry on Outcomes in ECOG-ACRIN-E5103. JCO Precis Oncol. 2017;2017.

8. Kabat GC, Ginsberg M, Sparano JA, Rohan TE. Risk of Recurrence and Mortality in a Multi-Ethnic Breast Cancer Population. J Racial Ethn Health Disparities. 2017 Dec;4(6):1181–8.

9. Albain KS, Gray RJ, Makower DF, Faghih A, Hayes DF, Geyer CE, et al. Race, Ethnicity, and Clinical Outcomes in Hormone Receptor-Positive, HER2-Negative, Node-Negative Breast Cancer in the Randomized TAILORx Trial. J Natl Cancer Inst. 2021 Apr 6;113(4):390–9.

10. Kim G, Pastoriza JM, Qin J, Lin J, Karagiannis GS, Condeelis JS, et al. Racial disparity in distant recurrence-free survival in patients with localized breast cancer: A pooled analysis of National Surgical Adjuvant Breast and Bowel Project trials. Cancer. 2022 Jul 15;128(14):2728–35.

11. Kim G, Pastoriza JM, Condeelis JS, Sparano JA, Filippou PS, Karagiannis GS, et al. The Contribution of Race to Breast Tumor Microenvironment Composition and Disease Progression. Front Oncol. 2020 Jun 30;10:1022.

12. Hughes R, Qian BZ, Rowan C, Muthana M, Keklikoglou I, Olson OC, et al. Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy. Cancer Res. 2015 Sep 1;75(17):3479–91.

13. Chen L, Li J, Wang F, Dai C, Wu F, Liu X, et al. Tie2 Expression on Macrophages Is Required for Blood Vessel Reconstruction and Tumor Relapse after Chemotherapy. Cancer Res. 2016 Dec 1;76(23):6828–38.

14. Chang YS, Jalgaonkar SP, Middleton JD, Hai T. Stress-inducible gene Atf3 in the noncancer host cells contributes to chemotherapy-exacerbated breast cancer metastasis. Proc Natl Acad Sci U S A. 2017 Aug 22;114(34):E7159–68.

15. Karagiannis GS, Pastoriza JM, Wang Y, Harney AS, Entenberg D, Pignatelli J, et al. Neoadjuvant chemotherapy induces breast cancer metastasis through a TMEM-mediated mechanism. Sci Transl Med. 2017 Jul 5;9(397):eaan0026.

16. Robinson BD, Sica GL, Liu YF, Rohan TE, Gertler FB, Condeelis JS, et al. Tumor Microenvironment of Metastasis in Human Breast Carcinoma: A Potential Prognostic Marker Linked to Hematogenous Dissemination. Clin Cancer Res Off J Am Assoc Cancer Res. 2009 Apr 1;15(7):2433–41.

17. Rohan TE, Xue X, Lin HM, D'Alfonso TM, Ginter PS, Oktay MH, et al. Tumor Microenvironment of Metastasis and Risk of Distant Metastasis of Breast Cancer. JNCI J Natl Cancer Inst. 2014 Jun 3;106(8):dju136.

18. Harney AS, Arwert EN, Entenberg D, Wang Y, Guo P, Qian BZ, et al. Real-Time Imaging Reveals Local, Transient Vascular Permeability, and Tumor Cell Intravasation Stimulated by TIE2hi Macrophage-Derived VEGFA. Cancer Discov. 2015 Sep;5(9):932–43.

19. Pignatelli J, Bravo-Cordero JJ, Roh-Johnson M, Gandhi SJ, Wang Y, Chen X, et al. Macrophage-dependent tumor cell transendothelial migration is mediated by Notch1/MenaINV-initiated invadopodium formation. Sci Rep. 2016 Dec;6(1):37874.

20. Arwert EN, Harney AS, Entenberg D, Wang Y, Sahai E, Pollard JW, et al. A Unidirectional Transition from Migratory to Perivascular Macrophage Is Required for Tumor Cell Intravasation. Cell Rep. 2018 May 2;23(5):1239–48.

21. Karagiannis GS, Pastoriza JM, Borriello L, Jafari R, Coste A, Condeelis JS, et al. Assessing Tumor Microenvironment of Metastasis Doorway-Mediated Vascular Permeability Associated with Cancer Cell Dissemination using Intravital Imaging and Fixed Tissue Analysis. J Vis Exp JoVE. 2019 Jun 26;(148).

22. Sparano JA, Gray R, Oktay MH, Entenberg D, Rohan T, Xue X, et al. A metastasis biomarker (MetaSite Breast™ Score) is associated with distant recurrence in hormone receptor-positive, HER2-negative early-stage breast cancer. NPJ Breast Cancer. 2017 Nov 8;3:42.

23. Sharma VP, Tang B, Wang Y, Duran CL, Karagiannis GS, Xue EA, et al. Live tumor imaging shows macrophage induction and TMEM-mediated enrichment of cancer stem cells during metastatic dissemination. Nat Commun. 2021 Dec 15;12(1):7300.

24. Nassar D, Blanpain C. Cancer Stem Cells: Basic Concepts and Therapeutic Implications. Annu Rev Pathol. 2016 May 23;11:47–76.

25. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209–49.

26. Caughran J, Braun TM, Breslin TM, Smith DR, Kreinbrink JL, Parish GK, et al. The Effect of the 2009 USPSTF breast cancer screening recommendations on breast cancer in Michigan: A longitudinal study. Breast J. 2018 Sep;24(5):730–7.

27. Waks AG, Winer EP. Breast Cancer Treatment: A Review. JAMA. 2019 Jan 22;321(3):288–300.

28. Plevritis SK, Munoz D, Kurian AW, Stout NK, Alagoz O, Near AM, et al. Association of Screening and Treatment With Breast Cancer Mortality by Molecular Subtype in US Women, 2000-2012. JAMA. 2018 Jan 9;319(2):154–64.

29. Costa RLB, Gradishar WJ. Triple-Negative Breast Cancer: Current Practice and Future Directions. J Oncol Pract. 2017 May;13(5):301–3.

30. Kennecke H, Yerushalmi R, Woods R, Cheang MCU, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol Off J Am Soc Clin Oncol. 2010 Jul 10;28(20):3271–7.

31. Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999-2004. Breast J. 2009;15(6):593–602.

32. van Maaren MC, de Munck L, de Bock GH, Jobsen JJ, van Dalen T, Linn SC, et al. 10 year survival after breast-conserving surgery plus radiotherapy compared with mastectomy in early breast cancer in the Netherlands: a population-based study. Lancet Oncol. 2016 Aug;17(8):1158–70.

33. Blichert-Toft M, Nielsen M, Düring M, Møller S, Rank F, Overgaard M, et al. Long-term results of breast conserving surgery vs. mastectomy for early stage invasive breast cancer: 20-year follow-up of the Danish randomized DBCG-82TM protocol. Acta Oncol Stockh Swed. 2008;47(4):672–81.

34. Brewster AM, Hortobagyi GN, Broglio KR, Kau SW, Santa-Maria CA, Arun B, et al. Residual risk of breast cancer recurrence 5 years after adjuvant therapy. J Natl Cancer Inst. 2008 Aug 20;100(16):1179–83.

35. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015 Feb 19;372(8):724–34.

36. Sawaki M, Shien T, Iwata H. TNM classification of malignant tumors (Breast Cancer Study Group). Jpn J Clin Oncol. 2019 Mar 1;49(3):228–31.

37. Giuliano AE, Connolly JL, Edge SB, Mittendorf EA, Rugo HS, Solin LJ, et al. Breast Cancer-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017 Jul 8;67(4):290–303.

38. Woeste MR, Bhutiani N, Donaldson M, McMasters KM, Ajkay N. Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery. J Surg Oncol. 2021 Feb;123(2):439–45.

39. Korde LA, Somerfield MR, Carey LA, Crews JR, Denduluri N, Hwang ES, et al. Neoadjuvant Chemotherapy, Endocrine Therapy, and Targeted Therapy for Breast Cancer: ASCO Guideline. J Clin Oncol Off J Am Soc Clin Oncol. 2021 May 1;39(13):1485–505.

40. Wolmark N, Wang J, Mamounas E, Bryant J, Fisher B. Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. J Natl Cancer Inst Monogr. 2001;(30):96–102.

41. Pagani O, Senkus E, Wood W, Colleoni M, Cufer T, Kyriakides S, et al. International guidelines for management of metastatic breast cancer: can metastatic breast cancer be cured? J Natl Cancer Inst. 2010 Apr 7;102(7):456–63.

42. Beslija S, Bonneterre J, Burstein HJ, Cocquyt V, Gnant M, Heinemann V, et al. Third consensus on medical treatment of metastatic breast cancer. Ann Oncol Off J Eur Soc Med Oncol. 2009 Nov;20(11):1771–85.

43. Chia SK, Speers CH, D'yachkova Y, Kang A, Malfair-Taylor S, Barnett J, et al. The impact of new chemotherapeutic and hormone agents on survival in a population-based cohort of women with metastatic breast cancer. Cancer. 2007 Sep 1;110(5):973–9.

44. Stockler M, Wilcken NR, Ghersi D, Simes RJ. Systematic reviews of chemotherapy and endocrine therapy in metastatic breast cancer. Cancer Treat Rev. 2000 Jun;26(3):151–68.

45. Wojcik BE, Spinks MK, Optenberg SA. Breast carcinoma survival analysis for African American and white women in an equal-access health care system. Cancer. 1998 Apr 1;82(7):1310–8.

46. Jatoi I, Becher H, Leake CR. Widening disparity in survival between white and African-American patients with breast carcinoma treated in the U. S. Department of Defense Healthcare system. Cancer. 2003 Sep 1;98(5):894–9.

47. Menashe I, Anderson WF, Jatoi I, Rosenberg PS. Underlying causes of the black-white racial disparity in breast cancer mortality: a population-based analysis. J Natl Cancer Inst. 2009 Jul 15;101(14):993–1000.

48. Joslyn SA, West MM. Racial differences in breast carcinoma survival. Cancer. 2000;88(1):114–23.

49. Partridge AH, Wang PS, Winer EP, Avorn J. Nonadherence to adjuvant tamoxifen therapy in women with primary breast cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2003 Feb 15;21(4):602–6.

50. Tammemagi CM, Nerenz D, Neslund-Dudas C, Feldkamp C, Nathanson D. Comorbidity and survival disparities among black and white patients with breast cancer. JAMA. 2005 Oct 12;294(14):1765–72.

51. Hershman D, McBride R, Jacobson JS, Lamerato L, Roberts K, Grann VR, et al. Racial disparities in treatment and survival among women with early-stage breast cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2005 Sep 20;23(27):6639–46.

52. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006 Jun 7;295(21):2492–502.

53. Scott LC, Mobley LR, Kuo TM, Il'yasova D. Update on triple-negative breast cancer disparities for the United States: A population-based study from the United States Cancer Statistics database, 2010 through 2014. Cancer. 2019 Oct 1;125(19):3412–7.

54. Mittal S, Brown NJ, Holen I. The breast tumor microenvironment: role in cancer development, progression and response to therapy. Expert Rev Mol Diagn. 2018 Mar;18(3):227–43.

55. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol. 2014;5:514.

56. Mills CD. Anatomy of a discovery: m1 and m2 macrophages. Front Immunol. 2015;6:212.

57. Koru-Sengul T, Santander AM, Miao F, Sanchez LG, Jorda M, Glück S, et al. Breast cancers from black women exhibit higher numbers of immunosuppressive macrophages with proliferative activity and of crown-like structures associated with lower survival compared to non-black Latinas and Caucasians. Breast Cancer Res Treat. 2016 Jul;158(1):113–26.

58. Martin DN, Boersma BJ, Yi M, Reimers M, Howe TM, Yfantis HG, et al. Differences in the tumor microenvironment between African-American and European-American breast cancer patients. PloS One. 2009;4(2):e4531.

59. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell. 2009 Sep 8;16(3):183–94.

60. Grann VR, Bowman N, Joseph C, Wei Y, Horwitz MS, Jacobson JS, et al. Neutropenia in 6 ethnic groups from the Caribbean and the U.S. Cancer. 2008 Aug 15;113(4):854–60.

61. O'Meara T, Safonov A, Casadevall D, Qing T, Silber A, Killelea B, et al. Immune microenvironment of triple-negative breast cancer in African-American and Caucasian women. Breast Cancer Res Treat. 2019 May;175(1):247–59.

62. Bergenfelz C, Leandersson K. The Generation and Identity of Human Myeloid-Derived Suppressor Cells. Front Oncol. 2020;10:109.

63. Wesolowski R, Duggan MC, Stiff A, Markowitz J, Trikha P, Levine KM, et al. Circulating myeloid-derived suppressor cells increase in patients undergoing neo-adjuvant chemotherapy for breast cancer. Cancer Immunol Immunother CII. 2017 Nov;66(11):1437–47.

64. Bohling SD, Allison KH. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. Mod Pathol Off J U S Can Acad Pathol Inc. 2008 Dec;21(12):1527–32.

65. Ohara M, Yamaguchi Y, Matsuura K, Murakami S, Arihiro K, Okada M. Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer. Cancer Immunol Immunother CII. 2009 Mar;58(3):441–7.

66. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol Off J Am Soc Clin Oncol. 2013 Mar 1;31(7):860–7.

67. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol Off J Eur Soc Med Oncol. 2014 Aug;25(8):1544–50.

68. Karagiannis GS, Goswami S, Jones JG, Oktay MH, Condeelis JS. Signatures of breast cancer metastasis at a glance. J Cell Sci. 2016 May 1;129(9):1751–8.

69. Brown M, Assen FP, Leithner A, Abe J, Schachner H, Asfour G, et al. Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice. Science. 2018 Mar 23;359(6382):1408–11.

70. Pereira ER, Kedrin D, Seano G, Gautier O, Meijer EFJ, Jones D, et al. Lymph node metastases can invade local blood vessels, exit the node and colonize distant organs in mice. Science. 2018 Mar 23;359(6382):1403–7.

71. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000 Sep 14;407(6801):249–57.

72. Kimijima I, Ohtake T, Sagara H, Watanabe T, Takenoshita S. Scattered fat invasion: an indicator for poor prognosis in premenopausal, and for positive estrogen receptor in postmenopausal breast cancer patients. Oncology. 2000;59 Suppl 1:25–30.

73. Yamaguchi J, Ohtani H, Nakamura K, Shimokawa I, Kanematsu T. Prognostic impact of marginal adipose tissue invasion in ductal carcinoma of the breast. Am J Clin Pathol. 2008 Sep;130(3):382–8.

74. Byrd AS, Toth AT, Stanford FC. Racial Disparities in Obesity Treatment. Curr Obes Rep. 2018 Jun;7(2):130–8.

75. Harvey AE, Lashinger LM, Hursting SD. The growing challenge of obesity and cancer: an inflammatory issue. Ann N Y Acad Sci. 2011 Jul;1229:45–52.

76. Mayr FB, Spiel AO, Leitner JM, Firbas C, Kliegel T, Jilma B. Ethnic differences in plasma levels of interleukin-8 (IL-8) and granulocyte colony stimulating factor (G-CSF). Transl Res J Lab Clin Med. 2007 Jan;149(1):10–4.

77. Van Dyke AL, Cote ML, Wenzlaff AS, Land S, Schwartz AG. Cytokine SNPs: Comparison of allele frequencies by race and implications for future studies. Cytokine. 2009 May;46(2):236–44.

78. Stewart PA, Luks J, Roycik MD, Sang QXA, Zhang J. Differentially expressed transcripts and dysregulated signaling pathways and networks in African American breast cancer. PloS One. 2013;8(12):e82460.

79. Wyckoff JB, Wang Y, Lin EY, Li J feng, Goswami S, Stanley ER, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res. 2007 Mar 15;67(6):2649–56.

80. Duran CL, Borriello L, Karagiannis GS, Entenberg D, Oktay MH, Condeelis JS. Targeting Tie2 in the Tumor Microenvironment: From Angiogenesis to Dissemination. Cancers. 2021 Nov 16;13(22):5730.

81. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res. 2005 Jun 15;65(12):5278–83.

82. Roussos ET, Goswami S, Balsamo M, Wang Y, Stobezki R, Adler E, et al. Mena invasive (MenaINV) and Mena11a isoforms play distinct roles in breast cancer cell cohesion and association with TMEM. Clin Exp Metastasis. 2011 Aug;28(6):515–27.

83. Gertler F, Condeelis J. Metastasis: tumor cells becoming MENAcing. Trends Cell Biol. 2011 Feb;21(2):81–90.

84. Leung E, Xue A, Wang Y, Rougerie P, Sharma VP, Eddy R, et al. Blood vessel endothelium-directed tumor cell streaming in breast tumors requires the HGF/C-Met signaling pathway. Oncogene. 2017 May 11;36(19):2680–92.

85. Eddy RJ, Weidmann MD, Sharma VP, Condeelis JS. Tumor Cell Invadopodia: Invasive Protrusions that Orchestrate Metastasis. Trends Cell Biol. 2017 Aug;27(8):595–607.

86. Roussos ET, Balsamo M, Alford SK, Wyckoff JB, Gligorijevic B, Wang Y, et al. Mena invasive (MenaINV) promotes multicellular streaming motility and transendothelial migration in a mouse model of breast cancer. J Cell Sci. 2011 Jul 1;124(Pt 13):2120–31.

87. Harney AS, Karagiannis GS, Pignatelli J, Smith BD, Kadioglu E, Wise SC, et al. The selective Tie2 inhibitor rebastinib blocks recruitment and function of Tie2Hi macrophages in breast cancer and pancreatic neuroendocrine tumors. Mol Cancer Ther. 2017 Nov;16(11):2486–501.

88. Karagiannis GS, Bianchi A, Sanchez LR, Ambadipudi K, Cui MH, Anampa JM, et al. Assessment of MRI to estimate metastatic dissemination risk and prometastatic effects of chemotherapy. NPJ Breast Cancer. 2022 Sep 2;8(1):101.

89. Zhao J. Cancer stem cells and chemoresistance: The smartest survives the raid. Pharmacol Ther. 2016 Apr;160:145–58.

90. Lee CH, Hsieh JCH, Wu TMH, Yeh TS, Wang HM, Lin YC, et al. Baseline circulating stem-like cells predict survival in patients with metastatic breast Cancer. BMC Cancer. 2019 Dec 2;19(1):1167.

91. Zhou L, Jiang Y, Yan T, Di G, Shen Z, Shao Z, et al. The prognostic role of cancer stem cells in breast cancer: a meta-analysis of published literatures. Breast Cancer Res Treat. 2010 Aug 1;122(3):795–801.

92. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. Cell Stem Cell. 2007 Nov 15;1(5):555–67.

93. Morimoto K, Kim SJ, Tanei T, Shimazu K, Tanji Y, Taguchi T, et al. Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression. Cancer Sci. 2009;100(6):1062–8.

94. Neumeister V, Agarwal S, Bordeaux J, Camp RL, Rimm DL. In Situ Identification of Putative Cancer Stem Cells by Multiplexing ALDH1, CD44, and Cytokeratin Identifies Breast Cancer Patients with Poor Prognosis. Am J Pathol. 2010 May 1;176(5):2131–8.

95. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin Cancer Res Off J Am Assoc Cancer Res. 2005 Feb 1;11(3):1154–9.

96. Ahmed MAH, Aleskandarany MA, Rakha EA, Moustafa RZA, Benhasouna A, Nolan C, et al. A CD44−/CD24+ phenotype is a poor prognostic marker in early invasive breast cancer. Breast Cancer Res Treat. 2012 Jun 1;133(3):979–95.

97. Bengochea A, de Souza MM, Lefrançois L, Le Roux E, Galy O, Chemin I, et al. Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. Br J Cancer. 2008 Jul 8;99(1):143–50.

98. Merle P, Kim M, Herrmann M, Gupte A, Lefrançois L, Califano S, et al. Oncogenic role of the frizzled-7/beta-catenin pathway in hepatocellular carcinoma. J Hepatol. 2005 Nov;43(5):854–62.

99. Lefebvre V, Dumitriu B, Penzo-Méndez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int J Biochem Cell Biol. 2007;39(12):2195–214.

100. Kadaja M, Keyes BE, Lin M, Pasolli HA, Genander M, Polak L, et al. SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. Genes Dev. 2014 Feb 15;28(4):328–41.

101. Christin JR, Wang C, Chung CY, Liu Y, Dravis C, Tang W, et al. Stem Cell Determinant SOX9 Promotes Lineage Plasticity and Progression in Basal-like Breast Cancer. Cell Rep. 2020 Jun 9;31(10):107742.

102. Guo W, Keckesova Z, Donaher JL, Shibue T, Tischler V, Reinhardt F, et al. Slug and Sox9 cooperatively determine the mammary stem cell state. Cell. 2012 Mar 2;148(5):1015–28.

103. Richtig G, Aigelsreiter A, Schwarzenbacher D, Ress AL, Adiprasito JB, Stiegelbauer V, et al. SOX9 is a proliferation and stem cell factor in hepatocellular carcinoma and possess widespread prognostic significance in different cancer types. PloS One. 2017;12(11):e0187814.

104. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005 Aug 17;97(16):1180–4.

105. McShane LM, Hayes DF. Publication of tumor marker research results: the necessity for complete and transparent reporting. J Clin Oncol Off J Am Soc Clin Oncol. 2012 Dec 1;30(34):4223–32.

106. Entenberg D, Oktay MH, D'Alfonso T, Ginter PS, Robinson BD, Xue X, et al. Validation of an Automated Quantitative Digital Pathology Approach for Scoring TMEM, a Prognostic Biomarker for Metastasis. Cancers. 2020 Mar 31;12(4):E846.

107. Ye X, Oktay MH, Xue X, Rohan TE, Ginter PS, D'Alfonso T, et al. Combining TMEM Doorway Score and MenaCalc Score Improves the Prediction of Distant Recurrence Risk in HR+/HER2− Breast Cancer Patients. Cancers. 2022 Jan;14(9):2168.

108. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jun 28;9(7):676–82.

109. Arganda-Carreras I, Kaynig V, Rueden C, Eliceiri KW, Schindelin J, Cardona A, et al. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. Bioinforma Oxf Engl. 2017 Aug 1;33(15):2424–6.

110. Pignatelli J, Goswami S, Jones JG, Rohan TE, Pieri E, Chen X, et al. Invasive breast carcinoma cells from patients exhibit MenaINV- and macrophage-dependent transendothelial migration. Sci Signal. 2014 Nov 25;7(353):ra112.

111. DeMichele A, Yee D, Esserman L. Mechanisms of Resistance to Neoadjuvant Chemotherapy in Breast Cancer. N Engl J Med. 2017 Dec 7;377(23):2287–9.

112. Wang C, Lin Y, Zhu H, Zhou Y, Mao F, Huang X, et al. The Prognostic and Clinical Value of Tumor-Associated Macrophages in Patients With Breast Cancer: A Systematic Review and Meta-Analysis. Front Oncol. 2022;12:905846.

113. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol. 2002 Mar;196(3):254–65.

114. Lee AH, Happerfield LC, Bobrow LG, Millis RR. Angiogenesis and inflammation in invasive carcinoma of the breast. J Clin Pathol. 1997 Aug;50(8):669–73.

115. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res. 1996 Oct 15;56(20):4625–9.

116. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med. 1991 Jan 3;324(1):1–8.

117. Albain KS, Unger JM, Crowley JJ, Coltman CA, Hershman DL. Racial disparities in cancer survival among randomized clinical trials patients of the Southwest Oncology Group. J Natl Cancer Inst. 2009 Jul 15;101(14):984–92.

8. SUPPLEMENTARY DOCUMENTS

SUPPLEMENT-1-2: Ethics Committee Approval for Thesis Study

SUPPLEMENT-3-4: Thesis Study Originality Report (Turnitin Report)

EINSTEIN Albert Einstein College of Medicine | Office of Human Research Affairs | Montefiore

Notification of Amendment Approval

Date: August 04, 2021

Principal Investigator: Maja H Oktay

Study Title:	Evaluation of prognostic and predictive bio-markers in formalin-fixed paraffin-embedded breast cancer specimens		
IRB #:	2017-8158	**Reference #:**	
Amendment Approval Date:	08/04/2021	**Study Expiration Date:**	06/08/2021

This amendment, , was reviewed and approved by expedited review under 45 CFR 46.110 and 21 CFR 56.110.

The following individual was removed as Key Personnel: Saeed Asiry

For a list of all currently approved documents, follow these steps: Go to Study Assistant – My Studies and open the study – Click on Informed Consent to obtain a list of approved consent documents and Other Study Documents for a list of other approved documents.

Reminders

All changes to a study must receive IRB approval before they are implemented. The only exception to the requirement for prior IRB review and approval is when the changes are necessary to eliminate apparent immediate hazards to the subject (45 CFR 46.103(b)(4), 21 CFR 56.108(a)). In such cases, report the actions taken as a reportable event.

Reportable Events must be reported to the IRB in compliance with the Einstein IRB policy.

Expiration Notice: IRB approval for this study is limited to the period specified above. In order to gain re-approval, you must submit a Progress Report prior to the expiration of the study. When the research is completed, submit a final Progress Report. The iRIS system will generate an email notification 60 days prior to the expiration of this study's approval. However, it is your responsibility to ensure that a Progress Report has been submitted by the required time.

SUPPLEMENT-2

Maja Oktay, MD, PhD
Professor of Pathology,
Montefiore Medical Center

October 10, 2022.

To whom it may concern,

I guarantee that the ethical committee approval is being obtained for Burcu Karadal, MD who is an M.D.-Ph.D. student enrolled in Tumor Biology and Immunology doctoral program of Hacettepe University Basic Oncology Institute under the supervision of Assoc. Prof. Gurcan Gunaydin for her thesis entitled “Racial Disparity in Distant Recurrence-Free Survival and Tumor Microenvironment of Residual Breast Cancer after Neoadjuvant Chemotherapy”. The document containing information regarding the ethical committee approval is provided with this document.

Sincerely,

Maja H. Oktay

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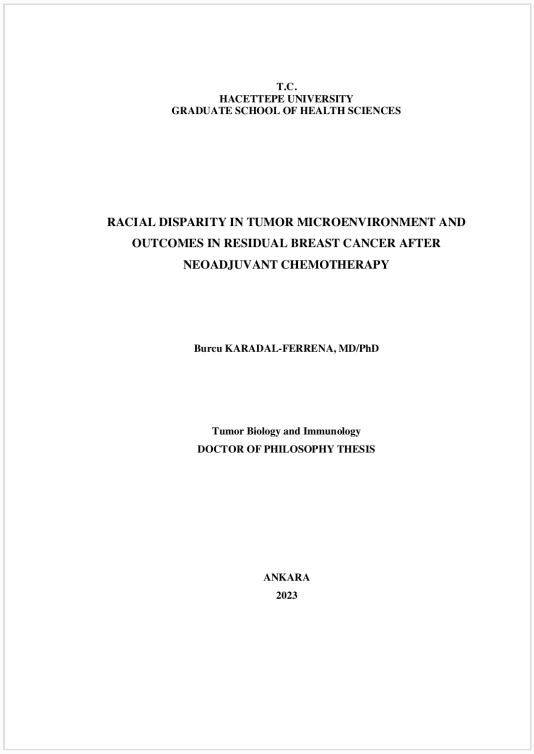

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GRADUATE SCHOOL OF HEALTH SCIENCES

RACIAL DISPARITY IN TUMOR MICROENVIRONMENT AND OUTCOMES IN RESIDUAL BREAST CANCER AFTER NEOADJUVANT CHEMOTHERAPY

Burcu KARADAL-FERRENA, MD/PhD

Tumor Biology and Immunology
DOCTOR OF PHILOSOPHY THESIS

ANKARA
2023

SUPPLEMENT-4

Racial Disparity in Tumor Microenvironment and Outcomes in Residual Breast Cancer after Neoadjuvant Chemotherapy

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1	Burcu Karadal, Gina Kim, Ved Sharma, Jessica Pastoriza et al. "Abstract GS1-02: Racial disparity in tumor microenvironment and outcomes in residual breast cancer treated with neoadjuvant chemotherapy", Cancer Research, 2023 Publication	1%
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