Actionable Biology and Prognostic Biomarkers in Breast Cancer Patients of African Descent

Nikita Wright

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ACTIONABLE BIOLOGY AND PROGNOSTIC BIOMARKERS IN BREAST CANCER PATIENTS OF AFRICAN DESCENT

by

NIKITA WRIGHT

Under the Direction of Ritu Aneja, PhD

ABSTRACT

Women of African descent are disproportionately affected by breast cancer relative to European women. Africans and African-Americans are more likely to acquire aggressive breast cancer phenotypes such as triple negative breast cancer (TNBC), which lacks expression of pharmacologically-targetable targets such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Given breast cancer patients of African descent are predominantly triple negative, they are often refractory to hormone and HER2-targeted systemic therapies in the clinic, underlying their more aggressive disease course and poorer prognosis relative to women of other ethnic backgrounds. My work seeks to identify actionable biomarkers in patients of African descent, who are predominantly triple-negative and thus lack targeted treatment options and robust risk-predictive biomarkers. Herein, I present a three-pronged approach to examine inherent differences in tumor biology among racially-distinct
breast tumors. I have examined surrogates of intratumor heterogeneity (mitotic propensity and centrosome amplification), the tumor immune microenvironment (tumor-infiltrating lymphocytes), and drivers of cell proliferation (human epidermal growth factor receptor family) among racially-distinct breast cancer patients and their potential as risk-prognostic biomarkers and alternative therapeutic targets. These facets of aggressive tumor biology have been linked to the acquisition of aggressive cellular phenotypes and drug resistance/relapse in breast cancer suggesting racial disparities in these biomarkers could perhaps be underlying the divergence in mortality rates. Furthermore, these key aspects of aggressive tumor biology (drivers of intratumor heterogeneity, tumor-infiltrating lymphocytes, and drivers of cell proliferation) can be evaluated in clinical samples using techniques such as immunohistochemistry and H&E staining as well as therapeutically targeted through rationally-designed agents such as putative centrosome declustering agents, immunotherapeutic intervention, monoclonal antibodies, and receptor agonists. Hence, my work suggests clinically-facile risk-prognostic and actionable biomarkers for patients of African descent, which may aid in reducing the global racial disparity in breast cancer. Furthermore, this work has broader clinical implications by extending prognostic biomarkers and actionable targets for aggressive breast cancer patients, irrespective of ethnicity, as well as paves the path for alternative avenues of addressing the global racially disparate burden in breast cancer such as metabolic, epigenetic, and proteomic approaches. Moreover, our work may further encourage the implementation of personalized treatment in the clinic based on each patient’s distinct tumor molecular profile.

INDEX WORDS: Triple negative breast cancer, African, Risk-prognostic, Biomarkers, Intratumor heterogeneity, Tumor immune microenvironment
ACTIONABLE BIOLOGY AND PROGNOSTIC BIOMARKERS IN BREAST CANCER
PATIENTS OF AFRICAN DESCENT

by

NIKITA WRIGHT

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ACTIONABLE BIOLOGY AND PROGNOSTIC BIOMARKERS IN BREAST CANCER
PATIENTS OF AFRICAN DESCENT

by

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May 2019
DEDICATION

This dissertation is dedicated to my heavenly father as well as my loving and supportive family who have imparted the strength, courage, confidence, and faith necessary to achieve my lifelong dream of completing my PhD and participating in cutting-edge cancer research. I would especially like to dedicate this dissertation to my late grandfather and grand uncles who passed from prostate cancer unexpectedly and inspired the basis for my thesis.
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1 INTRODUCTION

1.1 The racially disparate burden in breast cancer

At least 1 in 8 women in the United States (US) will acquire breast cancer (BC) in their lifetime(1). BC reigns as the number one form of invasive cancer diagnosed among women in the United States (US) and ranks second place, behind lung cancer, among the leading causes of death from cancer among women today(1). Women of African descent are disproportionately afflicted and affected by BC compared to women of European descent in the US. African-American (2) women exhibit significantly higher BC incidence rates than European-American (3) women before the age of 40(4). However, among all ages, AA women are more likely to die from BC than EA, Hispanic, and Native American women with death rates as much as 60% higher in some states such as Louisiana and Mississippi(4, 5). Furthermore, AA women tend to be diagnosed and die from BC at a much younger age compared to their EA counterparts(4). Moreover, BC patients of African ancestry experience a more aggressive clinical disease course as AAs exhibit lower proportions of localized BC and higher proportions of regional and distant-stage BC than EAs and other ethnic groups(4). Hence, AAs are more likely to be diagnosed with advanced staged BC than EAs among BC patients(6, 7). The prevalence of more aggressive BC subtypes is higher among AA compared to EA patients. Estrogen receptor-negative, progesterone receptor (PR)-negative, and triple negative BC (TNBC) have been reported to be more prevalent among AA compared to EA patients(8). TNBC, defined by a lack of ER, PR, and human epidermal growth factor receptor 2 (HER2), was recently reported to be twice as high among AA (24 per 100,000) compared to EA (12 per 100,000) women leaving AA patients with a lack of pharmacologically-targetable treatment options(4). Racial disparity in clinical outcomes persists within BC subtypes. Chen and colleagues observed that AA women exhibit a 40-70%
higher risk of developing stage IV disease than EA women across all BC subtypes(9). Among luminal-A like and luminal-B like BC patients, AAs were found to exhibit a 76% and 56% higher mortality rates than EAs, respectively(9). Black women were reported to exhibit significantly worse overall survival (OS) and progression-free survival (PFS) than white women among ER-positive BC patients(10). Although controversial, some studies have reported a more aggressive disease course and a poorer prognosis in AAs compared to EAs among TNBC patients(11-13).

Distinctions in inherent tumor biology have been suspected to underlie the racial disparate burden in BC. Thus, cancer health disparity research has largely focused on identifying pharmacologically-targetable biomarkers differentially-expressed among racially-distinct BC patients or strongly associated with African ancestry that can enhance risk-prognostication and reduce the disproportionately higher mortality rates observed among patients of African descent. However, many of the targeted therapeutics rationally designed for these biomarkers may perform well in preclinical studies but often perform dismally once they reach advanced clinical trials or elicit a significant response in only a small percentage of BC patients(14). One of the primary factors underlying this poor performance is the high interpatient and intratumoral heterogeneity rampant among the BC landscape, which interferes with the efficacy of these targeted agents(14-17). These novel agents are also often accompanied by toxic, harmful side effects that exacerbate the patient’s previously compromised health(18-20). Moreover, validating detection methods with high specificity and sensitivity, identifying appropriate cut-offs, lack of robust drug response biomarkers, limited understanding of drug mechanism of action, lack of pharmokinetic/pharmocodynamic studies, high costs of drug development, interobserver discordance, and variability in tissue fixation, sample procuring methods, antibodies,
immunohistochemical (IHC) staining methods, and resources all present significant challenges in successfully integrating targeted therapeutics into the clinic(14, 19-28). Furthermore, the time consuming, costly, and inefficient preclinical and clinical trial process presents further challenges in clinical implementation. Thus, AA BC patients are left with limited effective treatment options.

1.2 Triple negative breast cancer

The clinical management of TNBC continues to confound clinicians. Accounting for a small subset (10-20%) of all BC cases, TNBC patients experience notably lower survival rates than non-TNBC patients(11, 29, 30). The defining feature of this subtype is its lack of ER, PR, and HER2 overexpression(31); therefore, the only treatment options for TNBC are conventional chemotherapy, surgery and radiation therapy. TNBC is notorious for its extensive interpatient heterogeneity and intratumoral heterogeneity (32) that collude to produce poor clinical outcomes(33). In fact, TNBC is renowned for its aggressive clinical disease course and its higher prevalence among women of younger age and African descent(29, 34-36). Higher visceral and cerebral metastasis and local relapse rates typify their clinical course(37, 38). Furthermore, TNBC tumors display more unfavorable clinico-pathological features upon presentation such as larger tumor size, higher nuclear grade, higher stage, higher mitotic index, higher Ki67 proliferation index, and lymph node involvement compared to other breast cancer subtypes(34, 39, 40). These tumors also possess a higher likelihood of exhibiting distant recurrence within the first five years of diagnosis(34). These statistics have highlighted the need for finding new treatment modalities to improve TNBC outcomes. However, these efforts have been met with a stalemate in the clinic owing to the heterogeneous nature of the disease. Neoadjuvant chemotherapy is the first-line treatment choice for TNBCs due to their relatively higher
chemosensitivity and pathological complete response (pCR) rates compared to non-TNBC patients(35). Despite reportedly higher pCR rates, TNBC patients tend to experience significantly reduced progression-free survival (PFS) and lower overall survival (OS) within 3 years post-treatment compared to non-TNBC patients(41). This contradiction, often termed by clinicians and researchers as “the triple-negative paradox”, may in part be explained by the small percentage of TNBC patients that actually fall into the pCR group(34, 41). Only 30% of TNBC patients that undergo anthracycline and taxane-based cytotoxic chemotherapy prior to surgery achieve pCR and experience improved DFS rates(42). In addition, among patients with residual disease, TNBC patients experience higher relapse and death rates than non-TNBC patients within the first three years of follow-up(43-45). This outcome may partially be reflected by the high administration of adjuvant endocrine therapy for patients with luminal tumors. Thus, there is an urgent need for alternative targeted treatment options for this subset of BC patients. Despite a surge of preclinical and clinical studies investigating promising therapeutic targets for TNBC patients over the past decades, as of date, none have been approved.

1.2.1 Heterogeneous landscape of triple negative breast cancer

Perhaps the most groundbreaking attempt to subcategorize TNBCs according to common molecular features was undertaken by Lehmann and colleagues(46). Lehmann and his colleagues analyzed gene expression profiles of human TNBC tumor samples and conducted consensus clustering on the most differentially expressed genes to segment the subtype into seven unique clusters sharing common gene expression profiles. Six stable clusters and one unstable cluster was classified by Lehmann et. al as seven distinct TNBC subtypes characterized by shared gene ontologies and unique enriched canonical pathways. These seven molecular TNBC subtypes were labeled basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory,
mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), and unstable (UNS). The basal-like 1 (BL1) and basal-like 2 (BL2) subtypes stratify the intrinsic basal-like BC molecular subtype. These subgroups of basal-like BCs were found to be enriched in components of the cell cycle, DNA replication pathways, DNA damage response, and exhibit upregulation of genes associated with proliferation such as Ki67. The BL2 subtype is particularly enriched in growth factor receptor expression such as EGFR, Insulin-like growth factor (IGFR), and hepatocyte growth factor (MET). The IM subgroup is enriched for gene ontologies involved in fundamental signaling immune pathways such as T cell and B cell receptor signaling, natural killer cell pathway, and cytokine signaling. Additionally, this group has been observed to display increased antigen processing and presentation (46). The M and MSL subtypes are both enriched in genes that regulate cell motility, extracellular matrix receptor interaction, and cell differentiation including enhanced Wnt/β-catenin and TGF-β signaling. However, the MSL subtype differentiates itself from the M subtype with increased expression of genes in pathways that promote growth factor (i.e. EGFR, platelet-derived growth factor (PDGF)), calcium, and extracellular signal-regulated kinase signaling. In addition, MSL exhibits high activity of genes involved in angiogenesis and epithelial-mesenchymal transition. Perhaps the most distinguishing feature of the MSL subtype from the M subtype is a reduced expression levels of markers associated with cell proliferation along with elevated expression of stem cell and mesenchymal stem cell genes. Although ER negative, LAR subtype gene expression analysis revealed enhanced androgen and estrogen metabolic pathways. Particularly, increased expression of androgen receptor and its downstream targets was observed and further supported by enhanced nuclear AR immunohistochemical staining. Additionally, hierarchical clustering revealed that the LAR subtype exhibit a luminal-like gene signature making them resemble luminal ER positive
breast cancer. Lehmann and colleagues also uncovered subtype-specific sensitivities among the TNBCs to conventional therapeutic agents(46). They identified TNBC cell lines that share homogenous gene ontologies with each of the major TNBC subtypes through conducting gene expression profiling and clustering analysis. Their work yielded a comprehensive panel of TNBC cell lines representative of the six identified molecular TNBC subtypes. They utilized the panel to analyze differential drug response between the subtypes to traditional therapeutic agents administered in clinics. Cell viability assays revealed that the cell lines characterized as basal-like displayed significantly higher sensitivity to cisplatin compared to mesenchymal- and LAR-like lines likely due to their enrichment in DNA damage response markers. As one may suspect, the AR-dependent LAR-like cell lines displayed significantly higher sensitivity to the AR antagonist, bicalutamide, than the basal-like lines. These results suggest that the increased AR signaling present in LAR tumors permit this subgroup of TNBC patients to be selectively susceptible to anti-androgen targeted therapy. This clinically translatable information is valuable and warrants further investigation to discriminate TNBC subtypes according to their favorable therapeutic response to guide clinical decision-making.

Androgen receptor is expressed in approximately 10-43% of TNBCs depending on the cutoff of positivity used and thus, has emerged as a promising therapeutic target for TNBC patients(47). AR knockdown and inhibition with enzalutamide among AR-expressing TNBCs has resulted in reduced proliferation, migration, invasion and increased apoptosis in vitro and in vivo(48-50). Traina and colleagues also observed that enzalutamide elicited a clinical benefit rate (CBR) of 25% at 24 weeks and a median progression-free survival (PFS) of 14.7 weeks among advanced AR-positive TNBC patients in a nonrandomized phase II clinical trial as opposed to patients exhibiting less AR expression, who exhibited a CBR of only 20% at 24 weeks and a
median PFS of 12.6 weeks (unpublished data). Another phase II clinical trial using the AR antagonist, bicalutamide, elicited a CBR rate of 19% at 24 weeks and a median PFS of 12 weeks among AR-positive TNBC patients (51). However, the remaining roughly 67-90% of TNBCs lack AR expression deeming the disease a “quadruple threat” and is often referred to as quadruple negative breast cancer (QNBC). Thus, this subgroup of TNBCs are more immune to novel AR-targeted therapeutic agents compared to AR-expressing TNBCs and some studies have reported a worse prognosis among QNBCs compared to TNBCs (52-57). Hence, some QNBCs exhibit even more of a dire need for alternative therapeutic options and risk-predictive biomarkers than AR-positive TNBCs. TNBC has been a hot topic of cutting-edge research over the past decade in which a plethora of ongoing preclinical and clinical trials have yielded promising biomarkers and therapeutic targets. However, recent evidence suggests genetic differences between TNBC and QNBC tumors and although the prognostic role of AR in TNBC remains controversial, many studies have revealed that a lack of AR expression confers a more aggressive disease course. However, leading-edge research on alternative biomarkers and treatment strategies for QNBC patients remain scarce. Thus, there is an urgent need for increased preclinical and clinical QNBC research to uncover novel actionable molecular targets for this unique group of TNBC patients.

1.2.2 The racially disparate burden in triple negative breast cancer

Intriguingly, racial disparities exist within the inherently aggressive TNBC subtype. As previously mentioned, pre-menopausal African-American (2) women are overwhelmingly more afflicted with TNBC compared to women of other ethnicities, which is primarily underlying their substantially lower survival rates and poorer clinical outcomes (41, 58). Moreover, studies show higher incidence rates and earlier age of onset of TNBC in native African women compared to
AA women(12, 59). Moreover, accumulating evidence suggests that even among TNBCs, AAs experience poorer clinical outcomes such as lower OS and PFS than EAs owing to more unfavorable clinico-pathological features such as larger tumor size, higher proliferation, more extensive lymph node involvement, and presentation at a younger age(12, 13, 60). In addition, ITH was recently found to be greater in AA compared to European-American (3) TNBC tumors(61). Further evidence has uncovered that AA TNBCs harbor more aggressive TNBC subtypes such as BL1 and MSL while EA TNBCs harbor more favorable TNBC subtypes such as LAR(60, 61). Interestingly, Gasparini and colleagues observed that loss of AR was more prevalent among AA compared to EA TNBC patients as well as associated with more unfavorable clinico-pathological features and a poorer prognosis(62). Furthermore, Davis et al. discovered that AR loss was more associated with AA compared to EA TNBC tumors(63). These findings are consistent with reports of a greater prevalence of basal-like TNBC, which lacks AR expression and reduced sensitivity to AR antagonists, among AA compared to EA TNBCs (46). These unsettling statistics necessitate further investigation into the potential molecular drivers underpinning disparities in tumor biology between AA and EA TNBCs.

Several studies are addressing inherent biological differences between AA and EA TNBCs. A comparative analysis revealed higher Ki-67 and c-Kit expression and lower CK5, CK8, CK19, CD44 and AR expression in AA than EA TNBC patients(60). Linder et al., transcriationally profiled AA and EA TNBC samples and observed increased loss of BRCA1 expression and upregulation of IGFR and VEGF in AA compared to EA tumors(64). The group also found higher IGF-1 and VEGF activity and tumor vascularization in AAs compared to EAs among TNBC tumors. Gene-expression studies have uncovered considerably more upregulation of Wnt-β-catenin signaling in TNBC patients of African descent compared to TNBC patients of
European descent(12). Nalwoga et al., detected notably more enrichment of aldehyde dehydrogenase 1 (ALDH1), a cell surface marker associated with cancer stem cells, in TNBC tumors of African origin compared to TNBC tumors of non-African origin(65). Therapies targeting the previously mentioned inherent molecular differences distinguishing AA from EA TNBCs, such as PARP, IGFR, VEGF, and Wnt inhibitors, may potentially aid in attenuating the ethnic disparity in TNBC. However, these rationally-designed agents have yet been shown to perform robustly in TNBC patients or have been approved for targeted TNBC treatment. Thus, further investigation is warranted to uncover robust therapeutic targets and risk-prognostic and/or predictive biomarkers for AA TNBC patients.

1.3 Intratumoral heterogeneity

ITH has indisputably become one of the chief culprits underlying challenges in successfully treating and managing BC. Wang and colleagues claim that “no two cancer cells within the same cancer have the same genome”(66). Thus, BC cells are able to resist or become immune to conventional treatment strategies, such as chemotherapy, as well as rationally designed novel targeted therapies. Thus, intratumor diversity may underlie therapeutic resistance, metastasis, and relapse, which all characterize BC aggressiveness(67). Keenan and colleagues reported that AAs exhibit greater intratumor heterogeneity than EAs among both BC and TNBC tumors, which may be underlying the racial disparate burden in these diseases(61). Hence, it may be worthy to investigate disparities in drivers of ITH between AA and EA breast tumors or target the “root” of racial disparities in BC as a promising alternative strategy to alleviating the burden. Centrosome amplification (CA) and mitotic propensity (MP) have been considered to be two key drivers of ITH as they collectively foster chromosomal instability (CIN), which promotes ITH(67). Herein, my research proposes to investigate differences in these two drivers
of ITH between AA and EA BCs to rationalize the racial disparate burden in BC and uncover their potential as robust prognostic biomarkers and/or therapeutic targets in AA BC patients.

1.3.1 Centrosome amplification: Driver of intramoral heterogeneity

The centrosome is the microtubule-organizing center of the cell and plays a key role in cell shape, cell polarity, cell motility, cell adhesion, organelle transport, and assembly of the mitotic spindle to ensure normal bipolar cell or genome division (68, 69). Normal cells harbor 1-2 centrosomes depending on the phase of the cell cycle it is in. However, cancer cells typically exhibit CA or an increase in the number (numerical CA) or size (structural CA) of centrosomes which can sporadically occur as a result of dyregulation of the centrosome duplication pathway, de novo centrosome biogenesis, and/or cytokinesis failure (70). Thus, supernumerary centrosomes can compromise mitotic fidelity and interfere with proper segregation of chromosomes into daughter cells. As a result, aneuploidy or chromosomal instability arises which can pave the path for the generation of clonal diversity and aggressive karyotypes (71, 72). Centrosomal aberrations have been shown to positively correlate with ploidy and chromosomal instability (71, 72). Cells burdened with extra centrosomes often cluster their centrosomes at opposite poles of the cell to form a “pseudo biopolar spindle” in lieu of a lethal multi-polar spindle in order to ensure cell survival and allow genomically unstable cells to successfully replicate and thrive (73). Centrosome clustering has been significantly associated with worse OS and recurrence-free survival (RFS) (71). Excess centrosomes can also interfere with cell polarity and thus, enhance cell migration and progression (74, 75). Thus, CA has been linked to breast tumorigenesis and aggressiveness. Approximately, 80% of breast carcinomas have been reported to display CA (72). Centrosomal aberrations in breast tumors have been significantly associated with high grade and stage, lymph node metastasis, TNBC phenotype, and poor clinical outcomes.
such as overall survival (OS) and progression-free survival (PFS) (71, 72, 76, 77). As previously mentioned, these aggressive characteristics are associated with BC patients of African ancestry. Thus, it may be worthy to investigate differences in centrosomal profiles between AA and EA breast tumors as a suspect in the racial disparate burden in BC. Establishing differences in the incidence and severity of CA between AA and EA TNBC patients may provide compelling evidence for rationalizing differences in tumor progression and the divergence in clinical outcomes between the ethnic groups. Furthermore, uncovering this inherent tumor biological disparity between the racially-distinct patients may establish CA as a novel racial disparity biomarker in BC and a promising target for therapeutic intervention. CA is a cancer-cell specific trait that distinguishes them from normal, healthy cells. Thus, targeting CA offers a minimally cytotoxic therapeutic strategy that selectivity targets cancer cells burdened with severe centrosomal aberrations. Conveniently, there are already rationally designed drugs that selectively kill cancer cells harboring CA and are currently under clinical evaluation as promising anti-cancer therapeutics. These novel agents include putative centrosome declustering drugs such as griseofulvin and noscapine, commercially available HSET inhibitors such as CW069 and AZ82, and poly-ADP ribose polymerase inhibitors such as GF-15(2, 78-81). Furthermore, non-invasive methods that can detect centrosomal status (i.e. fine-needle aspirate cytology, immunohistochemistry) may demonstrate feasibility in the clinic for selecting patients that will exhibit susceptibility to these agents(77, 82). Validating associations between the severity level of CA with tumor growth, metastasis, and chemoresistance in preclinical and clinical BC models may illuminate upstream or downstream targets of CA that may be driving more aggressive BCs and variances in tumor progression between racially-distinct patients. Moreover, this elucidation may lay the groundwork for future studies to investigate differences
in molecular signaling pathways preceding the onset of CA or the mechanisms extra centrosomes exploit to drive more aggressive tumor behavior in AA compared to EA TNBC patients. Furthermore, CA exists in precancerous lesions and has been demonstrated to act as a precursor to breast tumorigenesis. Thus, evaluating centrosomal profiles among early-stage AA BC patients may serve as a risk-predictive biomarker for aggressive disease.

1.3.2 Mitotic propensity: Engine of intratumoral heterogeneity

MP, or turnover rate of proliferating cells in a tumor, is another important component fueling ITH. Tumors with a high propensity are more prone to erroneous mitoses and consequently chromosomal instability. As a result, increased clonal diversity and the generation of aggressive cellular phenotypes can arise. Indices routinely utilized in the clinic to measure tumor proliferation include mitotic index (MI) and Ki-67 proliferation index. MI and KI have proven to be useful as autonomous indices in the clinic as a high MI and KI among early stage tumors have been reported to be synonymous with a poor patient prognosis in BC. However, both indices are gleaned from separate fields and thus the precise propensity of a tumor cell to double among the proliferating cell population remains elusive. MI is deduced by computing the quantity of mitotic (M phase) cells per 10 high-powered microscopic fields whereas, KI is determined as the percentage of positive Ki-67 nuclear stained proliferating (G1, S, G2, and M phases) carcinoma cells in low-powered microscopic fields. A higher MI and KI have been reported among AA compared to EA breast tumors. Furthermore, extraneous factors often interfere with accurately quantitating mitotic figures in patient tumor samples and a lack of consensus on an appropriate cut-off and discrepancies in Ki-67 immunohistochemical (IHC) staining still persists. Thus, our group recently proposed a novel metric that rationalized the inconsistencies and limitations of MI and KI as autonomous prognosticators. The
metric cleverly integrates MI and KI onto the same measurement scale to ascertain the frequency of mitotic cells among Ki-67 positive cycling cells or mitotic propensity, termed mitosis: proliferation (M:P ratio). Hence, our metric provides an additional layer of risk-predictive information than MI and KI alone. My research proposes to investigate differences in M:P ratio or cycling kinetics between AA and EA breast tumors to (i) rationalize their differences in ITH and metastatic propensity and (ii) determine if M:P ratio can enhance risk-prognostication for AA BC patients at risk for faster tumor kinetic progression.

1.4 Tumor immune microenvironment

Accumulating evidence suggest that the interplay between the breast tumor and its microenvironment plays a critical role in tumor progression and therapeutic responses. The breast microenvironment provides a supportive niche for tumor growth and is composed of the extracellular matrix, stromal cells, fibroblasts, adipocytes, endothelial cells, and immune cells(90). Immune cells infiltrate the tumor upon neoplastic transformation to elicit an antitumoral or protumoral inflammatory response. Primarily lymphocytes, macrophages, mast cells, and neutrophils comprise the inflammatory defense army that infiltrate the incipient tumor to mount an attack or facilitate tumor progression(91). Thus, these inflammatory cells harbor dual roles in BC initiation and progression by secreting cytokines, growth factors, chemokines, oxygen intermediates to stimulate proliferation, apoptotic prevention or promotion factors, morphogenesis factors, and inducing mutagenic changes that promote DNA damage in tumor cells(91). Tumor associated macrophages (TAMs), neutrophils, T, and B lymphocytes harbor both pro-tumorigenic and tumoricidal properties however, natural killer (NK) lymphocytes are the only tumor infiltrating immune cells that harbor solely anti-tumorigenic properties. Tumor infiltrating lymphocytes (TILs) have been recognized as a bonified biomarker of the anti-tumoral
immune response, have been proven to harbor both prognostic and predictive value in BC and
tNBC, and have become one of the primary targets of cancer immunotherapy today. Thus, TILs
will be the primary focus of my dissertation.

1.4.1 **Tumor infiltrating lymphocytes: Critical mediators of the antitumoral immune
response**

TILs are white blood cells or T, B, and NK lymphocytes that migrate to the tumor stroma
or within the tumor upon neoplastic transformation to eradicate tumor cells and control tumor
progression. In BC, TILs primarily consists of T cells (~75%) and specifically CD8+ cytotoxic T
cells, CD4+ helper T cells, and CD4+ regulatory T cells (T regs)(92, 93). Cytotoxic T
lymphocytes kill tumor cells by inducing apoptosis. T cells express T cell receptors (TCRs) that
recognize neoantigens on cancer cells and induce programmed cell death by (i) releasing the
cytotoxin perforin to inject granzymes into the cytoplasm and trigger the caspase cascade
pathway through their serine protease function and by (ii) expressing their surface protein, FAS
ligand, which can bind to Fas on the cancer cell and recruit the death-induced signaling complex
and Fas-associated death domain to activate the caspase cascade reaction. TILs are associated
with more aggressive BC such as high grade, high stage, high Ki-67, lymph node metastasis, and
younger age at diagnosis(93). Furthermore, TIL infiltration is significantly more observed in
TNBC compared to non-TNBCs(92). Particularly, CD8+ T lymphocytes inversely correlates
with ER and PR expression and their density is significantly higher in TNBCs compared to non-
TNBCs(94, 95). However, a presence of TILs have been associated with a significantly better
prognosis in BC and TNBC. TILs have been shown to be an independent predictor of improved
pathological complete response (pCR) following neoadjuvant chemotherapy (NAC) in both BC
and TNBC as well as better OS and disease-free survival (DFS) among non-NAC treated TNBC
patients(96, 97). High TIL levels have also been significantly associated with improved overall survival (OS), increased metastasis-free survival (MFS), and reduced distance recurrence in TNBC(96). Hence, the presence of TILs plays a critical role in disease course and survival outcomes among BC and TNBC patients.

1.4.2 Racial disparities in the tumor microenvironment: Suspect in the racially disparate burden in BC

Emerging evidence suggest disparities in the breast tumor immune microenvironment exists between AA and EA breast tumors, which has been suspected to be contributing to the racial disparate burden in BC. Tumor stromal genes such as PSPHL, CXCL10, and CXCL11 have been reported to be significantly higher in AA compared to EA BC patients(98). Multiple groups have revealed significantly higher levels of pro-inflammatory cytokines such as resistin, leptin, interleukin 6 (IL-6) as well as the resistin receptor, CAP1, in AA compared to EA patients(99-103). Martin and colleagues also observed significantly higher expression levels of vascular endothelial growth factor (VEGF) and syndecan-1 (known inducers of angiogenesis) in AA compared to EA BC patients(98). In addition, Martin et. al observed greater microvessel density in AA compared to EA BC patients(98). TAMs have also been reported by several groups to be significantly higher in AA compared to EA BC patients(98, 104, 105). Tripathi and colleagues also discovered an enriched immune gene signature in the T and B cell response pathways among AA QNBC tumors suggesting increased anti-tumor immunity among AA QNBC patients (unpublished data). Hence, this evidence suggests disparities between AAs and EAs exists in the breast tumor microenvironment, which may be underlying the racial disparity in BC. However, the role of racial disparities in the tumor microenvironment in the racial disparate burden in BC, and especially in TNBC, remains poorly explored. Particularly, racial
disparities in TIL levels between AA and EA breast tumors remain elusive. In this dissertation, I investigate differences in TILs between AA and EA TNBC patients and their potential as robust risk-prognostic/predictive biomarker and therapeutic target for AA patients at risk for a poorer prognosis.

1.5 Tumor cell proliferation

Aggressive BC is often characterized by what it lacks such as the absence of the hormone and growth receptors, ER, PR, AR, and HER2 that promote BC proliferation and consequently a lack of risk-prognostic and targeted treatment options. Furthermore, as previously discussed, BC patients of African descent tend to lack expression of these tumor proliferation markers, exempting them from being eligible to receive conventional treatments targeting these biomarkers, which is a major underlying factor in their observed disparate clinical outcomes compared to patients of other ethnicities. Hence, it may be advantageous to investigate the role of other growth receptors in aggressive BC subtypes that lack expression of these traditional proliferation biomarkers such as TNBC and QNBC among AA BC patients as alternative risk-prognostic and/or targeted treatment options.

1.5.1 Human epidermal growth factor receptor family

The human epidermal growth factor receptor (EGFR) family consists of four structurally related tyrosine kinase receptors (EGFR/HER1, HER2, HER3, and HER4) that collectively stimulate growth factor signaling pathways that play critical roles in cell proliferation, growth, survival, and differentiation, such the PI3 kinase, Ras-Raf-MAPK, JNK, and PLCγ pathways(106-108). Aberrant expression of these receptors can also promote loss of cell adhesion and polarity and initiate invasion and angiogenesis and has been associated with human malignancies(109-111). Activation of these receptors and their signaling pathway via ligand
binding is mediated through hetero- or homo- dimerization of these receptors followed by subsequent phosphorylation of tyrosine residues on the intracellular tyrosine kinase domain. HER2 preferentially dimerizes with EGFR. HER2 is overexpressed in approximately 20-25% of all BCs and is a well-established predictive biomarker and therapeutic target in BC. However, owing to the lack of HER2 amplification in TNBCs and QNBCs it may be advantageous to investigate other members of the EGFR family as viable risk-predictive biomarkers or therapeutic targets for TNBC patients and patients of African ancestry. In this dissertation, I will investigate the role of the other three EGFR family members (EGFR, HER3, and HER4) in the global racial disparate burden and their potential as risk-prognostic/predictive biomarkers and/or therapeutic targets among ethnically-distinct TNBC patient populations.

1.5.1.1 EGFR

EGFR is overexpressed in at least 50% of TNBCs, which is notably higher compared to other BC subtypes. Ligands for EGFR include EGF, ampiregulin, transforming growth factor (TGF)-α, betacellulin, heparin-binding EGF, and epiregulin. EGFR expression is significantly more associated with young age, lower hormone receptor expression levels, high grade, high proliferation, genomic instability, and lymphocytic infiltration. EGFR has expression has also correlated with higher risk of relapse among adjuvant treated BC patients. Moreover, high EGFR copy number, immunoreactivity and expression have been shown to be independent prognostic indicators of poorer overall survival (OS) and disease-free survival (DFS) in TNBC, suggesting EGFR to be a potentially targetable and risk-prognostic biomarker in TNBC. Researchers at Caris Research Institute recently reported that EGFR IHC expression was higher among AR-negative compared to AR-positive TNBCs suggesting increased cellular growth and proliferation in QNBCs compared to TNBCs as well.
Anti-EGFR agents such as tyrosine kinase inhibitors (i.e. gefitinib and erlotinib) and anti-EGFR monoclonal antibodies (i.e. cetuximab) are currently being tested in TNBC patients in phase I/II clinical trials as monotherapy and in combination with chemotherapy and have elicited improved response in some patients(121). Thus, EGFR may serve as a promising risk-prognostic biomarker and therapeutic target for TNBC and QNBC patients as well as patients of African ancestry who exhibit a high prevalence of these diseases.

1.5.1.2 HER3

HER3 is unique in that it is the only receptor in the family that is catalytically inactive and requires dimerization with other members in order to be active(122). The receptor heterodimerizes with EGFR to stimulate the PI3K signaling pathway(122). Its overexpression has been reported in approximately 20-30% of invasive breast carcinomas(123). Yamaguchi and colleagues recently observed that HER3 signaling promotes upregulation or programmed cell death ligand 1 (PD-L1) which plays a critical role in suppression of anti-tumor immunity (unpublished data). HER3 has also been reported to limit patient sensitivity to EGFR-targeted agents such as cetuximab by increasing EGFR-HER3 heterodimerization and activating downstream signaling pathways in BC(124). Reports on the prognostic role of HER3 in BC remain conflicted however, it has been associated with poorer overall survival (OS) and DFS in TNBC according to one study (122, 125). Our group also recently reported that combined EGFR-HER3 score predicts worse BC-specific survival (BCSS) and increased distant metastasis after adjusting for age and stage among TNBC patients (unpublished data). A high EGFR-HER3 score among chemotherapy-treated patients in this cohort was associated with high IHC expression of luminal cytokeratins, DNA damage response proteins, and P-cadherin. Hence, it may be worthy to investigate the role of HER3 in the racial disparate burden in TNBC.
1.5.1.3 HER4

HER4 is detected in less than half of BCs and generally characterized by antiproliferative and pro-apoptotic activity(126). This EGFR family member has been reported to be downregulated in 18-75% of BCs and upregulated in 7-29% of cases(127). It is also unique in that it is the only member of the family that exist in four juxtamembrane (JM) and cytoplasmic (CYT) isoforms (JM-a/CYT1, JM-a/CYT2, JM-b/CYT1, and JM-b/CYT2) due to alternative mRNA splicing and one of these isoforms (JM-a) can be cleaved by tumor necrosis factor (TNF)-α converting enzyme (TACE) and γ-secretase and released into the cytoplasm as a soluble HER4 intracellular domain (4ICD) that can translocate to the nucleus(128-130). Ligands for HER4 include the neuregulins, epiregulin, betacellulin, and heparin-binding EGF(114). The receptor displays a diverse range of roles including modulating cell proliferation, cell cycle arrest, and pro-apoptotic pathways(131, 132). The 4ICD of HER4 promotes proliferation of ER-α positive BC cells and apoptosis of BC cells through the cell-killing BH3 domain(133). Thus, HER4 is often associated with a favorable prognosis in BC and specifically prolonged event-free survival and OS among TNBCs(122, 134, 135). However, Kim et. al recently reported that high HER4 levels are significantly associated with poor 5-year disease relapse free survival in TNBC(84). HER4 has been reported to positively correlate with ER-positive BC and be associated with less recurrence and mortality among ER-positive patients(131, 136).

Upregulation of HER4 gene expression has even been shown to predict response to neoadjuvant chemotherapy (NAC) in BC(137). In addition, HER4 has been associated with low proliferative indices, cell growth inhibition, and low histological grade as well as been observed to antagonize the influence of HER2 on clinical outcome(127). HER4 mRNA expression levels were found to be significantly lower among AR-negative compared to AR-positive TNBC cases(62). Hence,
lack of HER4 expression may be a marker of aggressive BC and serve as a novel prognostic biomarker this patient population.

1.5.1.4 *Retinoic acid receptor-α*

Retinoic acid (RA), a derivative of retinol of vitamin A, influence cell growth, differentiation, and death through binding to members of the retinoic acid receptor (124) subfamily comprised of RARα, RARβ, and RARγ, which belong to the nuclear receptor superfamily of transcription factors(138). RARs heterodimerize to members of the retinoic X receptor (RXR) subfamily to initiate RA-mediated signaling. RAR is activated by both all-trans and 9-cis RA, whereas RXR can only be activated by 9-cis RA(139). The RAR-RXR dimer translocates to the nucleus and regulates transcription of target genes by binding to specific RA response elements (RAREs) on the gene promoter. Unlike the EGFR family, RA signaling through RARs has been reported to elicit differentiation as well as antiproliferative, pro-apoptotic, and anti-oxidant activity(138). Thus, aberrant RA signaling has been linked to breast tumorigenesis and RA and its derivatives (retinoids) are often exploited as potential chemotherapeutic or chemopreventative agents. However, ER-negative BCs and TNBCs have been reported to express lower levels of RARα compared to non-TNBCs which may reflect their enhanced proliferative activity(140). The role of RARα in TNBC and particularly in the racially disparate burden remains elusive. Hence, my dissertation work explores the potential role of this biomarker as a driver of the racial disparity in TNBC incidence and mortality between patients of African and European descent.

1.6 References

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2 DISTINCTIONS IN BREAST TUMOR RECURRENCE PATTERNS POST-
THERAPY AMONG RACIALLY-DISTINCT POPULATIONS

2.1 Abstract

Clinical studies have revealed a higher risk of breast tumor recurrence in African-American (2) patients compared to European-American (EA) patients, contributing to the alarming inequality in clinical outcomes among the ethnic groups. However, distinctions in recurrence patterns upon receiving hormone, radiation, and/or chemotherapy between the races remain poorly characterized. We compared patterns and rates (per 1000 cancer patients per 1 year) of recurrence following each form of treatment between AA (n=1850) and EA breast cancer patients (n=7931) from a cohort of patients (n=10504) treated between 2005-2015 at Northside Hospital in Atlanta, GA. Among patients who received any combination of adjuvant therapy, AA displayed higher overall rates of recurrence than EA (p=0.015; HR: 1.699; CI: 1.108-2.606). Furthermore, recurrence rates were higher in AA than EA among stage I (p=0.031; HR: 1.736; CI: 1.052-2.864) and T1 classified patients (p=0.003; HR: 2.009; CI: 1.263-3.197). Interestingly, among patients who received neoadjuvant chemotherapy, AA displayed higher rates of local recurrence than EA (p=0.024; HR: 7.134; CI: 1.295-39.313). Our analysis revealed higher incidence rates of recurrence in AA compared to EA among patients that received any combination of adjuvant therapy. Moreover, our data demonstrates an increased risk of tumor recurrence in AA than EA among patients diagnosed with minimally invasive disease. This is the first clinical study to suggest that neoadjuvant chemotherapy improves breast cancer recurrence rates and patterns in AA.

2.2 Introduction

The significant divide in breast cancer mortality between African-American (2) and European-American (EA) patients remains a challenge for clinicians. Despite a similar number of
reported incidences of breast cancer among AA and EA women, AAs experience notably higher severity in clinical outcomes and exhibit a 40% higher death rate than EAs among premenopausal and menopausal breast cancer patients (1-3). Recurrent breast cancer has impeded successful management of the disease for decades and is one of the primary factors for this racial division in prognosis (4). Statistics demonstrate that approximately 40% of all breast cancer survivors will experience a recurrence episode during their lifetime, which has been suggested to play a principal role in breast cancer mortality (4, 5). Clinical studies have revealed a higher risk of recurrence in AA compared to EA, presumably contributing to the inequality in clinical outcomes among the ethnic groups (1). This statistic has provided an impetus for clinicians to devise and implement robust prognosticative measures to preclude recurrence in AA breast cancer patients. However, distinctions in recurrence rates and patterns following various forms of treatment between the races have not been thoroughly evaluated. This warrants more investigation to potentially attenuate the observed racial disparity in recurrence in the clinic. Hence, we conducted a large institutional study based in Atlanta, Georgia, in which we analyzed rates and patterns of tumor recurrence post hormone, radiation, and chemotherapy among AA and EA breast cancer patients. This retrospective clinical study uncovered previously unrecognized distinctions in recurrence patterns following each conventional form of treatment among racially distinct breast populations and may impart valuable clinical insight into preclusive measures for mitigating the ethnic disparity in breast tumor recurrence.

2.3 Results

2.3.1 Clinico-pathological characteristics of patients

The demographics, breast clinico-pathological characteristics, therapies administered and patterns of recurrence among the patients in the cohort are illustrated in Fig 1. From this cohort of
10,504 NH patients, 225 were recorded as having experienced a recurrence episode and 6,009 were determined as displaying no breast tumor recurrence. The remaining patients did not have recorded data indicating presence of recurrence or lack thereof. Among patients displaying recurrence, higher risk of recurrence was more prevalent among younger patients (p<0.0001) (Fig 1A). Among patients with no missing recurrence data, approximately 61% of patients who experienced recurrence were under the age of 48, compared to only 39% who did not experience any recurrence. Among breast clinico-pathological characteristics, recurrence was significantly more associated with higher nuclear grade, NGH grade, stage, as well as T, N, and M classifications (p<0.0001) (Fig 1B). Moreover, recurrence was weakly associated with lymph node metastasis with roughly 35% of patients with recurrence displaying a positive nodal status compared to only 15% of non-recurrence patients (p=0.121). Regarding treatment, there were statistical significant differences in the distribution of recurrence and non-recurrence patients who were administered neoadjuvant and adjuvant chemotherapy, hormone therapy, and a combination of adjuvant therapies (p<0.0001) (Fig 1C). There was a weak statistical significant difference between the proportion of recurrence and non-recurrence patients that received radiation therapy (p=0.065). Please visit Supplementary Table 1 for details.

2.3.2 Clinico-pathological characteristics among racially distinct patients

Among the NH patients exhibiting recurrence, the demographics, breast clinico-pathological features, and therapies administered were compared between AA (n=49) and EA (n=166) as shown in Fig 2. Regarding patient demographic characteristics, a test of hypothesis for population differences revealed a weak statistical significant difference in age at diagnosis between the races (p=0.145) (Fig 2A). Approximately 51% of AA were diagnosed under the age of 48, compared to only 35% of EA. No significant differences in either clinico-pathological
characteristics or treatments between the races were observed, likely owing to a significant reduction in patient numbers after stratification of recurrence patients by race (Fig 2B, C). Please visit Supplementary Table 2 for details.

2.3.3 Recurrence patterns among racially distinct patients

Recurrence rates and patterns, expressed in terms of incidence rates, were compared broadly between AA and EA patients (Table 1); the analysis indicated that AA exhibited higher overall tumor recurrence rates than EA (p=0.002; HR: 1.676; CI: 1.210-2.323). AA also displayed higher rates of distant recurrence than EA (p=0.023; HR: 1.699; CI: 1.075-2.684); however, these differences did not remain statistically significant after controlling for age, grade, and stage, likely owing to low patient numbers. Additionally, AA experienced higher rates of single tumor recurrence episodes than EA (p=0.003; HR: 1.758; CI: 1.208-2.557) and higher rates of distant recurrence to a single site than EA breast cancer patients (p=0.012; HR: 1.742; CI: 1.130-2.684), although statistical significance was not maintained after adjusting for age, grade, and stage.

2.3.4 Recurrence patterns among racially distinct patients following each form of treatment

Incidence rates and patterns of recurrence were compared between AA and EA after they received hormone, radiation, chemotherapy, and/or any combination of adjuvant therapy (Table 2). AA exhibited unadjusted higher rates of recurrence (p=0.041; HR: 1.612; CI: 1.021-2.545) and a trend towards higher incidence of distant tumor recurrence than EA post radiation therapy (p=0.065; HR: 1.732; CI: 0.967-3.100). However, statistical significance was lost after controlling for age, grade, and stage. The same trend of higher overall and distant recurrent was observed among recurrent patients who received hormone therapy and any combination of adjuvant therapies. Among patients who underwent hormone therapy, AA displayed stronger overall tendencies than EA to suffer from recurrence (p=0.112; HR: 1.541; CI: 0.906-2.623) and distant
recurrence (p=0.123; HR: 1.692; CI: 0.868-3.301). Following any combination of adjuvant therapy, AA displayed higher recurrence rates than EA after adjusting for age, grade, and stage (p=0.015; HR: 1.699; CI: 1.108-2.606). Moreover, AA displayed higher rates of distant recurrence than EA (p=0.003; HR: 2.164; 1.290-3.629) before adjusting for age, grade, and stage, as well as stronger tendencies toward regional recurrence (p=0.104; HR: 2.043; CI: 0.863-4.837) after receiving any combination of adjuvant therapy.

Quite interestingly however, among patients with recurrence that received neoadjuvant chemotherapy, this trend was reversed. AA displayed lower tendencies toward recurrence than EA patients; however, statistical significance was unable to be achieved owing to a low number of patients (n=40) recorded as receiving neoadjuvant chemotherapy and exhibiting recurrence. Furthermore, AA displayed lower tendencies toward regional and distant tumor recurrence (p=0.112; HR: 0.310; CI: 0.073-1.315) than EA patients. Moreover, AA displayed higher rates of local recurrence than EA after controlling for age, grade, and stage (p=0.024; HR: 7.134; CI: 1.295-39.313). These results suggest that aggressive recurrence rates and patterns may be attenuated in AA patients who received neoadjuvant chemotherapy. Additional studies with larger numbers of patients with recurrence that received neoadjuvant chemotherapy could further clarify the significance of this trend.

2.3.5 Recurrence rates among racially distinct breast cancer patients in different stages

Overall incidence rates of recurrence were compared between AA and EA in both early (I–II) and late stage (III-IV) breast cancer patients (Table 3). Our data revealed that AA displayed higher recurrence rates than EA among stage I patients (p=0.001; HR: 2.165; CI: 1.348-3.476), even after adjusting for age, grade, and stage (p=0.031; HR: 1.736; CI: 1.052-2.864). Among early stage (I-II) patients, AA also exhibited higher recurrence rates than EA (p=0.002; HR: 1.793; CI:
1.252-2.567), although statistical significance was only weakly maintained after controlling for age, grade, and stage (p=0.131; HR: 1.339; CI: 0.917-1.956). Furthermore, AA displayed higher recurrence rates than EA among T1 classified patients, irrespective of age, grade, and stage (p=0.003; HR: 2.009; CI: 1.263-3.197). Moreover, unadjusted models reveal that AA displayed higher rates of recurrence than EA among N0 (p=0.005; HR: 1.777; CI: 1.186-2.661) and M0 (p=0.002; HR: 1.682; CI: 1.210-2.338) classified patients. However, rates of recurrence were not significantly higher in AA as compared to EA among late stage patients. Thus, these results suggest that AA are at higher risk than EA for tumor recurrence among patients with non-invasive or minimally invasive breast cancer.

2.3.6 Survival outcomes among racially distinct patients displaying recurrence

Survival duration after initial recorded recurrence was compared between the racial groups (Fig 3). AA exhibited a non-statistically significant trend toward shorter survival time than EA after experiencing their first episode of recurrence (p=0.231) (Fig 3A). The average time until death was compared between EA and AA patients who experienced distant recurrences (Fig 3B). Interestingly, AA and EA patients exhibiting distant recurrence were comprised of similar percentages of alive patients, however AA (n=26) died considerably sooner than EA (n=80) (p=0.015). More precisely, AA patients who experienced distant recurrences died approximately one year earlier than EA distant recurrent patients. However patient numbers were too low to control for variables of age, grade, and stage.

2.4 Discussion

This clinical study is the first extensive investigation into the rates and patterns of tumor recurrence in breast cancer patients following conventional treatments among racially distinct populations. Our study has revealed notable distinctions in recurrence patterns among EA and AA
patients. First, AA displayed considerably higher rates of recurrence than EA (Table 1 and 2). Second, we observed higher severity in recurrence patterns displayed by AA for whom we discerned stronger trends in AA of tumor recurrence to regional and distant sites (Table 1 and 2). This trend was evident after patients received radiation, hormone, and any combination of adjuvant therapies. Overall, these observed trends were quite significant since local recurrence tends to elicit a more favorable clinical prognosis compared to distant recurrence, while the latter trends type precedes a poorer clinical prognosis. Triple negative breast cancer (TNBC) patients have been shown to display an increased risk for recurrence and particularly for recurrence to distant sites, while non-TNBC patients exhibit higher trends of recurrence to local sites (1,8). These findings parallel our observations of an increased risk of overall and especially distant recurrence in AA, as well as an increased risk of local recurrences in EA. This tendency reflects the well-reported higher incidence of TNBC phenotypes in AA patients and a higher prevalence of non-TNBC subtypes in EA patients (8,9). Furthermore, we observed a trend of a higher number of recurrence episodes in AA compared to EA. Additionally, we discerned stronger inclinations of distant recurrence to multiple organs in AA compared to EA. These observed aggressive recurrence patterns reveal that AA patients exhibit an increased prospect of a poor clinical prognosis, theoretically contributing to their higher mortality rates than EA patients. Recurrence rates were also found to be higher in AA than EA among early stage, minimally invasive breast cancer patients (Table 3). This data presents an intriguing paradox as advanced stage upon diagnosis is typically associated with increased risk for recurrence (10-13). Thus, these findings suggest that AA patients of all clinical stages should be closely evaluated for the prospect of tumor recurrence. Neoadjuvant chemotherapy seemed to reverse these observed recurrence trends (Table 2). Among patients who received neoadjuvant chemotherapy, AA displayed a lower rate of recurrence than
EA; however due to a low number of recorded patients that received neoadjuvant chemotherapy, statistical significance was diminished. In addition, higher incidences of aggressive recurrence patterns in AA were notably attenuated after these patients underwent neoadjuvant chemotherapy. This data suggest preoperative chemotherapy may reduce the severity of recurrence rates and patterns in AA patients. This study suggests that neoadjuvant chemotherapy should be recommended for AA patients who are at higher risk for developing recurrence. A recent clinical study reported that in fact, neoadjuvant chemotherapy is administered more frequently to AA than EA patients likely as a result of their higher prevalence of advanced stage, grade, and triple negative receptor status upon presentation (14). Owing to their robust prognostic value, the extent of lymph node involvement and tumor size warrant stringent evaluation upon diagnosis and serve as principal prognostic factors in assessing breast cancer recurrence proclivities in AA patients (1). Established supplementary prognostic factors often considered by clinicians, such as higher stage and grade upon initial diagnosis, lymphatic and vascular invasion, premenopausal status, and a TNBC phenotype, also merit thorough scrutiny for AA breast cancer patients (9,10-13,15-17). Hence, rigorous supervision of the prospect of recurrence is conceivably compulsory to palliate the elevated risk of recurrent breast cancer demonstrated by AA patients.

Although prior clinical studies have exposed disparities in recurrence risk among EA and AA, this study is one of the first to uncover distinctions in rates and patterns of tumor recurrences following conventional forms of breast cancer treatments among the racial groups, and thus highlights the need for further investigation and surveillance. Our comprehensive analysis has also illuminated previously unrecognized differences in the rates and patterns of recurrence post-chemotherapy among racially distinct populations by suggesting that AA respond better to neoadjuvant chemotherapy. Additionally, no study has yet elucidated the significantly higher risk
for recurrence among early stage AA patients. Overall, this study further advocates that race should be considered among the potential decisive risk factors in the clinic for recurrence. Awareness of the higher rate of recurrence in AA may compel clinicians to consider race as a critical factor in evaluating the prospect of the cancer returning after patients enter remission, and allow this factor to play a major role in treatment decisions. Hereinafter, enriched comprehensive screening programs and tailored treatment plans may be imperative to impede augmented risk of tumor recurrence and aggressive recurrence patterns in AA patients that may be reinforcing their poor clinical outcomes.

2.5 Methods

2.5.1 Study cohort

In this study, a large cohort of breast cancer patients treated at Northside Hospital (NH) in Atlanta, Georgia from 2005 to 2015, were examined. We received approval and permission by the institutional review board at Northside Hospital to access patient clinico-pathological information used in this study and have a written human subjects assurance on file. The demographics and clinico-pathological characteristics of each patient were recorded to generate a database of 10,504 patients. Patient demographic information recorded in the database included age at time of diagnosis and ethnicity. Age at diagnosis among patients was divided into three subgroups, comprised of patients below the age of 48 (premenopausal), over the age of 55 (postmenopausal), and in between (perimenopausal), to precisely describe menopausal status. The races of patients in the database were primarily comprised of African-Americans (AA) and European-Americans (EA). The “unknown/others” subcategory denote patients of all other ethnicities (excluding AA and EA) and patients lacking race information. Breast tumor characteristics that were recorded for each patient consisted of nuclear grade, Nottingham (NGH)
grade, stage, nodal status, T (primary tumor), N (lymph node metastasis) and M (distant metastasis) classifications. American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM Classification and Stage groupings for breast carcinoma will be used in this article. All patient treatments were recorded, including chemotherapy, hormone, and radiation therapy. Patients that underwent chemotherapy were subcategorized into neoadjuvant and adjuvant depending on the timing of treatment. Additionally, any combination of hormone, radiation, and chemotherapy that patients received was labeled as a combination of adjuvant therapies. Follow up data was collected to determine breast cancer recurrence episodes, as well as the site of recurrence, such as local, regional or distant sites. Local recurrences comprise recurrence of the tumor in the primary site. Regional recurrence encompasses recurrence of the breast cancer in adjacent lymph nodes. Distant recurrences involve metastatic breast cancer in remote organs such as distant lymph nodes, bone, liver or others.

2.5.2 Follow-up

Both follow-up of patients and initial diagnosis occurred between the years of 2005 and 2015. Initial diagnosis dates as well as treatment start and completion dates for any therapies were documented. Dates of last contact for all patients were recorded. Survival status (alive/dead) was reported for each patient along with survival time. Dates of first recurrence were noted. February 19, 2015 was the final follow-up for the last patient seen.

2.5.3 Statistical analysis

A significance level of 0.05 and 95% confidence intervals were selected for all analyses. Sample sizes were based on the available patients that comprised each category in the NH database and not power analysis. Chi-square tests were performed to examine significant differences in clinico-pathological characteristics, therapy administration, and recurrence characteristics.
between recurrence and non-recurrence patients as well as between AA and EA breast cancer patients. Recurrence rates were calculated as per 1000 person-years. Test statistics were computed using MATLAB (MATLAB and Statistics Toolbox Release 2015a, The MathWorks, Inc., Natick, Massachusetts, United States) program and 1-tailed univariate p-values were reported. One-tailed analysis was preferred over two-tailed for this particular study to adequately reflect the presumption that treatment is expected to improve patient outcome. Multivariate Cox proportional hazard models were computed to determine significant differences in recurrence rates and patterns between the racial groups (6, 7). These statistical models were additionally modified to control for variables of age, grade, and stage. The Kaplan Meier analysis was conducted in SAS 9.4 program to estimate survival function for AA and EA with recurrent disease over a 10-year period. A log-rank test was conducted to evaluate significance level for between-race differences in survival. Finally, a t-test was used to compare mean time from first recorded recurrence event until death among patients with distant recurrence.

2.6 Tables and Figures

Table 2.1 Broad-spectrum recurrence patterns among racially distinct populations.
<table>
<thead>
<tr>
<th></th>
<th>EA</th>
<th>AA</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>IR</td>
<td>n</td>
<td>IR</td>
</tr>
<tr>
<td>Overall</td>
<td>166</td>
<td>13.44</td>
<td>49</td>
<td>21.77</td>
</tr>
<tr>
<td>Recurrence site</td>
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<td></td>
<td></td>
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<tr>
<td>Local</td>
<td>48</td>
<td>3.89</td>
<td>12</td>
<td>5.33</td>
</tr>
<tr>
<td>Regional</td>
<td>27</td>
<td>2.19</td>
<td>10</td>
<td>4.44</td>
</tr>
<tr>
<td>Distant</td>
<td>84</td>
<td>6.8</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>Number of recurrences</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Single</td>
<td>131</td>
<td>10.6</td>
<td>41</td>
<td>18.21</td>
</tr>
<tr>
<td>Multiple</td>
<td>35</td>
<td>2.83</td>
<td>8</td>
<td>3.55</td>
</tr>
<tr>
<td>Distant recurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single site</td>
<td>73</td>
<td>5.91</td>
<td>24</td>
<td>10.66</td>
</tr>
<tr>
<td>Multiple sites</td>
<td>11</td>
<td>0.89</td>
<td>3</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Abbreviation: IR, incidence rate (1000 person-years).
Adjusted Cox hazard model variables: age at diagnosis, grade (1,2,3), and stage (I,II,III,IV).
*P values were calculated using the student t-test.
Table 2.2 Recurrence rates and patterns after receiving any form of treatment among racially distinct populations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EA</th>
<th>n</th>
<th>IR</th>
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<td></td>
<td></td>
<td>p value; HR (95% CI)</td>
<td>p value; HR (95% CI)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>85</td>
<td>22.73</td>
<td>23</td>
<td>26.66</td>
<td>0.466; 1.181 (0.755, 1.846)</td>
</tr>
<tr>
<td>Local</td>
<td>13</td>
<td>3.48</td>
<td>7</td>
<td>7.46</td>
<td>0.125; 2.053 (0.818, 5.151)</td>
</tr>
<tr>
<td>Regional</td>
<td>20</td>
<td>5.35</td>
<td>6</td>
<td>6.4</td>
<td>0.594; 1.284 (0.512, 3.219)</td>
</tr>
<tr>
<td>Distant</td>
<td>50</td>
<td>13.37</td>
<td>12</td>
<td>12.79</td>
<td>0.832; 0.934 (0.498, 1.751)</td>
</tr>
<tr>
<td>Neoadjuvant chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>32</td>
<td>28.95</td>
<td>6</td>
<td>19.77</td>
<td>0.373; 0.673 (0.281, 1.609)</td>
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<tr>
<td>Local</td>
<td>2</td>
<td>1.81</td>
<td>4</td>
<td>13.18</td>
<td>0.026; 6.857 (1.256, 37.447)</td>
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<tr>
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<td>7</td>
<td>6.33</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Distant</td>
<td>23</td>
<td>20.81</td>
<td>2</td>
<td>6.59</td>
<td>0.112; 0.310 (0.073, 1.315)</td>
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<td>Adjuvant chemotherapy</td>
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<td></td>
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</tr>
<tr>
<td>Overall</td>
<td>57</td>
<td>20.96</td>
<td>18</td>
<td>26.16</td>
<td>0.405; 1.253 (0.737, 2.130)</td>
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<tr>
<td>Local</td>
<td>12</td>
<td>4.41</td>
<td>3</td>
<td>4.36</td>
<td>0.865; 0.897 (0.255, 3.153)</td>
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<td>Regional</td>
<td>15</td>
<td>5.52</td>
<td>6</td>
<td>8.72</td>
<td>0.333; 1.598 (0.619, 4.125)</td>
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<tr>
<td>Distant</td>
<td>30</td>
<td>11.03</td>
<td>9</td>
<td>13.08</td>
<td>0.664; 1.179 (0.561, 2.480)</td>
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<td>Hormone therapy</td>
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<tr>
<td>Overall</td>
<td>69</td>
<td>10.45</td>
<td>17</td>
<td>15.94</td>
<td>0.112; 1.541 (0.906, 2.623)</td>
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<tr>
<td>Local</td>
<td>14</td>
<td>2.12</td>
<td>2</td>
<td>1.87</td>
<td>0.676; 0.731 (0.169, 3.172)</td>
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<tr>
<td>Regional</td>
<td>10</td>
<td>1.6</td>
<td>3</td>
<td>2.52</td>
<td>0.369; 1.854 (0.552, 4.959)</td>
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<td>Distant</td>
<td>40</td>
<td>6.06</td>
<td>11</td>
<td>10.31</td>
<td>0.123; 1.692 (0.868, 3.301)</td>
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<td>Radiation therapy</td>
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<tr>
<td>Overall</td>
<td>79</td>
<td>12.62</td>
<td>23</td>
<td>19.34</td>
<td>0.041; 1.612 (1.021, 2.545)</td>
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<tr>
<td>Local</td>
<td>22</td>
<td>3.52</td>
<td>6</td>
<td>5.04</td>
<td>0.450; 1.414 (0.575, 3.475)</td>
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<tr>
<td>Regional</td>
<td>10</td>
<td>1.6</td>
<td>3</td>
<td>2.52</td>
<td>0.532; 1.503 (0.419, 5.392)</td>
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<tr>
<td>Distant</td>
<td>47</td>
<td>7.51</td>
<td>15</td>
<td>12.61</td>
<td>0.065; 1.732 (0.967, 3.100)</td>
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<td>Adjuvant radiation, hormone, and chemotherapy</td>
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<tr>
<td>Overall</td>
<td>101</td>
<td>11.94</td>
<td>30</td>
<td>19.63</td>
<td>0.013; 1.678 (1.115, 2.524)</td>
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<tr>
<td>Local</td>
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<td>3.86</td>
<td>3</td>
<td>1.96</td>
<td>0.279; 0.520 (0.159, 1.698)</td>
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<td>7</td>
<td>4.58</td>
<td>0.104; 2.043 (0.863, 4.837)</td>
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<tr>
<td>Distant</td>
<td>51</td>
<td>6.03</td>
<td>20</td>
<td>13.09</td>
<td>0.003; 2.164 (1.290, 3.629)</td>
</tr>
</tbody>
</table>

Abbreviation: IR, incidence rate (1000 person-years). Adjusted Cox hazard model variables: age at diagnosis, grade (1,2,3), and stage (I,II,III,IV). *P values were calculated using the student t-test.
**Table 2.3 Overall recurrence rates among racially distinct staged breast cancer patients.**

<table>
<thead>
<tr>
<th></th>
<th>EA</th>
<th>AA</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>IR</td>
<td>Unadjusted Model</td>
<td>Adjusted Model</td>
</tr>
<tr>
<td><strong>Grouped stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (I-II)</td>
<td>130</td>
<td>11.14</td>
<td>0.002; 1.793 (1.252, 2.567)</td>
<td>0.131; 1.339 (0.917, 1.956)</td>
</tr>
<tr>
<td>Late (III-IV)</td>
<td>31</td>
<td>55.17</td>
<td>0.857; 0.934 (0.445, 1.962)</td>
<td>0.637; 0.823 (0.366, 1.850)</td>
</tr>
<tr>
<td><strong>Individual Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>70</td>
<td>7.76</td>
<td>0.001; 2.165 (1.348, 3.476)</td>
<td>0.031; 1.736 (1.052, 2.864)</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>22.67</td>
<td>0.447; 1.239 (0.713, 2.154)</td>
<td>0.823; 0.936 (0.523, 1.674)</td>
</tr>
<tr>
<td>III</td>
<td>25</td>
<td>48.01</td>
<td>0.902; 0.949 (0.410, 2.195)</td>
<td>0.590; 0.774 (0.306, 1.959)</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>145.8</td>
<td>0.822; 0.832 (0.167, 4.152)</td>
<td>0.967; 0.964 (0.168, 5.516)</td>
</tr>
<tr>
<td><strong>TNM Staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>2</td>
<td>130.83</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>T1</td>
<td>67</td>
<td>9.75</td>
<td>&lt;0.0001; 2.776 (1.781, 4.326)</td>
<td>0.003; 2.009 (1.263, 3.197)</td>
</tr>
<tr>
<td>T2</td>
<td>54</td>
<td>28.48</td>
<td>0.504; 0.801 (0.419, 1.534)</td>
<td>0.215; 0.647 (0.325, 1.287)</td>
</tr>
<tr>
<td>T3</td>
<td>12</td>
<td>49.67</td>
<td>0.215; 0.275 (0.035, 2.115)</td>
<td>0.161; 0.228 (0.029, 1.796)</td>
</tr>
<tr>
<td>T4</td>
<td>9</td>
<td>106.73</td>
<td>0.680; 1.282 (0.394, 4.173)</td>
<td>0.983; 1.015 (0.241, 4.270)</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>101</td>
<td>9.81</td>
<td>0.005; 1.777 (1.186, 2.661)</td>
<td>0.211; 1.319 (0.854, 2.037)</td>
</tr>
<tr>
<td>N1</td>
<td>44</td>
<td>27.05</td>
<td>0.201; 1.518 (0.801, 2.877)</td>
<td>0.828; 1.079 (0.545, 2.136)</td>
</tr>
<tr>
<td>N2</td>
<td>13</td>
<td>46.45</td>
<td>0.744; 1.207 (0.391, 3.719)</td>
<td>0.965; 0.970 (0.258, 3.646)</td>
</tr>
<tr>
<td>N3</td>
<td>5</td>
<td>55.06</td>
<td>0.742; 0.697 (0.081, 5.970)</td>
<td>0.974; 0.962 (0.095, 9.711)</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>157</td>
<td>12.82</td>
<td>0.002; 1.682 (1.210, 2.338)</td>
<td>0.288; 1.210 (0.851, 1.721)</td>
</tr>
<tr>
<td>M1</td>
<td>6</td>
<td>145.8</td>
<td>0.822; 0.832 (0.167, 4.152)</td>
<td>0.967; 0.964 (0.168, 5.518)</td>
</tr>
</tbody>
</table>

Abbreviation: IR, incidence rate (1000 person-years). Adjusted Cox hazard model variables: age at diagnosis, grade (1,2,3), and stage (I,II,III,IV). *P values were calculated using the student t-test.
Figure 2.1 NH demographics, breast clinico-pathological characteristics, and treatment compared between patients with or without tumor recurrence.

(A) The numbers and percentages of patients displaying demographic and (B) breast clinico-pathological characteristics were compared for patients with (n=225) and without recurrence (n=6009). (C) The numbers and percentages of recurrence for both recurrence and non-recurrence patients that underwent each form of conventional breast cancer treatment were also compared. Significant differences were observed between recurrence and non-recurrence patients displaying all clinico-pathological characteristics (p<0.0001), with the exception of nodal status (p=0.012). Significant differences were also observed between recurrence and non-recurrence patients receiving each form of treatment (p<0.0001), except for radiation therapy (p=0.065). A chi-square statistical analysis was used to generate p-values in order to determine significant differences in the proportion of patients exhibiting or not exhibiting recurrence in each category. For example, regarding grade, a p value of 0.02 represents a significant difference for the distribution of
recurrence and non-recurrence patients across all grades. Please refer to Supplementary Table 1 for details.

**(Figure 2.2)** **NH demographics, breast, clinico-pathological and treatment compared between AA and EA with tumor recurrence.**

(A) The demographic and (B) clinico-pathological characteristics of AA (n=49) and EA (n=166) patients with recurrence in the NH cohort were compared. (C) Treatment administration was also compared between AA and EA recurrent breast cancer patients. A chi-square statistical analysis was used to generate p-values in order to determine significant differences in the proportion of AA and EA patients exhibiting each characteristic and undergoing each form of treatment. No statistical significant differences were observed. Please refer to Supplementary Table 2 for details.
Figure 2.3 AA exhibit lower survival duration than EA among recurrent breast cancer patients.

(A) Survival time from first recurrence episode until death was compared between AA and EA breast cancer patients. Log-rank analysis was conducted to determine statistical differences between the racial groups. AA exhibited a non-significant lower survival time than EA (p=0.231). (B) The mean time (days) until death was compared between AA and EA breast cancer patients displaying distant recurrence. AA died notably sooner than EA patients (p=0.015). A t-test was performed to determine significant differences between the racial groups.

2.7 References


3 A NOVEL METRIC ILLUMINATES DISTINCTIONS IN CYCLING KINETICS AMONG RACIALLY-DISTINCT BREAST TUMORS

3.1 Abstract

Breast tumors in African-Americans (AAs) exhibit higher recurrence rates and faster kinetic progression to metastasis than those in European-Americans (EAs). This results in a stark ethnic disparity in breast cancer outcomes. Hence, enhancing understanding of cell cycle kinetics within breast tumors may illuminate motives underpinning racial differences in metastatic propensities. Current clinico-pathological prognostic markers that evaluate cell proliferation in breast carcinomas include mitotic index (MI) and Ki-67 index (KI). However, as autonomous prognosticators measured on distinct scales, MI and KI lack the ability to convey the proportion of mitotic cells among cycling cells, undermining their prognostic value. We performed a three-color immunofluorescence staining on paraffin-embedded AA (n=81) and EA (n=124) breast tumor tissue specimens from Northside Hospital to integrate mitotic cells and cycling cells into the same measurement scale. Phospho-histone H3 was used as a mitotic marker and Ki-67 as a cell proliferation marker. Stained samples were examined with confocal microscopy to determine the proportion of mitotic cells to Ki-67 positive cycling cells to yield a Mitosis: Proliferation (M:P) ratio. We observed higher M:P ratio in AA compared to EA breast tumor tissue specimens (p=0.002). We also observed higher M:P ratio in AA compared to grade-matched and stage-matched EA patients. AA displayed significantly higher M:P ratio than EA among early stage breast tumors (p=0.015). Furthermore, among the clinico-pathological parameters, age, race, grade, stage, and receptor status, multivariate analysis revealed that race was the only variable that exhibited a significant confounding influence on M:P ratio (p=0.042). Conclusion: A higher M:P Ratio likely reflects an increased risk of developing intratumoral heterogeneity and
producing aggressive clones; thus, a higher M:P ratio may provide an explanation for the observed greater metastatic propensity exhibited in AA compared to EA patients. Thus, our novel metric provides new insights into the KI-MI relationship in tumors, exposes previously unrecognized differences in cycling kinetics among early stage AA and EA breast tumors, and proffers additional metastatic risk predictive information currently unavailable in the clinic.

3.2 Introduction

Despite the vast array of cliniopathological prognostic markers available in clinics today, African Americans (AAs) exhibit a 39% higher mortality rate than EA breast cancer patients (4). This stark disparity is explicated by increased tumor metastasis and tumor infiltration among AA breast cancer patients (Yancy, et al., 2007). These circumstances underscore the urgent need for deeper insight into the biological properties of early stage and low-grade breast tumors. Faster tumor kinetic progression underlie the more aggressive behavior exhibited by AA compared to EA breast tumors (Yancy, et al., 2007). Thus, procurement of improved perceptions of cell cycle kinetics may be beneficial in discerning motives for enhanced metastatic risk in AA breast tumors. Previous studies have reported that early stage tumors demonstrate high mitotic turnover and this surge in mitotic events promotes an increase in erroneous cell divisions (Laroye and Minkin, 1991). Consequently, genomic integrity is compromised, fostering karyotypic diversity and spawning the emergence of aggressive clones harboring superior metastatic proficiency (Hanahan and Weinberg, et al., 2011). Thus, advanced insight into cell proliferation may be critical to disentangling the enigma of varying metastatic propensities among racially-distinct breast tumors. With a present lack of markers that can adequately assess risk for tumor metastasis, further examination of cell cycle mechanics may generate a promising new direction.
in enhancing efficacy of existing prognostic markers and illuminate differences among racially distinct breast tumors.

Existing cliniopathological prognosticators that elucidate cell cycle dynamics in breast carcinomas include mitotic index (MI) and Ki-67 proliferation index (KI). MI, a component of histological grade, signifies actively dividing cells or cells in M phase, thus capturing the frequency of mitotic occurrences within a tumor (Reyal, et al., 2012). KI, an independent prognostic marker, ascertains cells merely in the cell cycle (G1, S, G2, and M phases), thus capturing a holistic view of cell proliferation within lesions (Reyal, et al., 2012). Both indices proffer valuable information in stratifying patients into high-risk and low-risk groups for tumor metastasis and fatal outcomes. A high MI has been linked to an increased risk of death and recurrence irrespective of tumor size and lymph node metastasis and high Ki-67 has been associated with worse disease-free survival (DFS) and overall survival (OS) in breast cancer (85, 141-144). Illumination of mitotic frequency within early stage breast tumors may reflect the predisposition of incipient breast lesions acquiring aggressive traits, and thus offers vital metastatic risk-predictive information. Evaluation of cell proliferation within a tumor may expose inclinations of a tumor to spread and infiltrate distant sites within a patient. A high KI and MI among early stage breast tumors has been reported to be synonymous with a poor patient prognosis (Azambuja, et al., 2007, Laroye and Minkin, 1991, Medri, et al., 2003). Additionally, clinical studies have observed notably higher MI and Ki-67 expression in AA in comparison to EA primary breast tumors (Porter, et al., 2004). This knowledge may rationalize the considerably higher aggressive behavior exhibited by AA breast tumors than EA breast tumors, thus MI and KI serve as useful metastatic risk-predictive markers.
Despite the prognostic advantages of these independent parameters and the valuable role they play in metastatic risk assessment, MI and KI possess unsettling limitations that spark reservations in their ability to accurately assess metastatic dispositions of breast tumors. With both indices functioning autonomously, KI and MI portray an incomplete picture of the precise relationship of mitotic cells amongst proliferative cells by proffering information on distinct aspects of cell cycle progression within a tumor. KI is quantified as a percentage of nuclear Ki-67 stained invasive carcinoma cells observed in low-power microscopic fields (Reyal, et al., 2012). In contrast, MI is quantified as the number of mitotic cells per 10 high-power microscopic fields within the periphery of the tumor (Elston and Ellis, et al, 1991, Haapasalo, et al., 1989, Reyal, et al., 2012). The substantial variations in acquirement of these indices and the vastly different fields in which they are perceived deems it difficult to decipher the frequency of dividing cells among proliferating cells within breast tumors. This lack of depth poses a limited view of cell cycle dynamics within lesions and thus restricts the predictive power of these prognosticators for metastatic risk. Furthermore, discordancy between MI and KI scores has been observed in some studies. Fluctuations in cell cycle duration during M-phase can influence the number of mitoses visualized at different times within the same tumor. Hence, shorter cell cycle durations or a large fraction of growing cells may contribute to a faster tumor-doubling time thus, the correlation between the number of mitotic events and proliferation rate may be not be exactly linear(145). Lee and colleagues recently observed a poor correlation between MIB-1 (antibody against Ki-67 antigen) and mitotic and phosphorylated histone H3 (pHH3) scores among breast adenocarcinomas after adjusting for intratumoral heterogeneity(146). Factors such as differences in the proportion of time that a cell spends between the mitotic phase and interphase as well as variability in cell cycle time among different tissue and tumor types have
been suspected to underlie the discordance (146) Rossi and colleagues also observed a lack of correlation between MI and KI among breast cancer patients (147). In their study, patients with discrepancies between MI and KI exhibited a worse prognosis than patients with low MI and KI and a better prognosis than patients with high MI and KI. Thus, these findings stress the need for joint analyzation of these markers in order to improve risk-prognostication.

In addition to this narrow understanding of the MI and KI relationship in tumors, there exist many glitches in MI and KI as independent prognostic markers and illuminators of cell proliferation. The accuracy of quantifying mitotic figures within a patient tumor sample is consistently jeopardized by extraneous factors present within the tumor microenvironment such as, apoptotic bodies and piknotic nuclei, that create an impediment in distinguishing mitotic nuclei (Veras, et al., 2009). Delays in fixation of tumor tissue samples can attribute to a diminution in accuracy and reproducibility of mitotic counts (Cross, et al., 1990, Diest, et al., 2004). Furthermore, mitotic counts are often miscalculated owing to exclusion of prophase cells during acquirement of mitotic figures (Elston and Ellis, et al, 1991). Despite Ki-67 proliferation expression as an acceptable measure of breast cancer outcomes, uncertainties remain in its competence as an efficacious prognostic tool in the clinical arena. Ki-67 proliferation index lacks dependability with poorly defined and established cut-offs that can effectively stratify breast cancer patients into high and low risk groups for tumor proliferation, undermining its strength in clinical prognostication (Reyal, et al., 2012, Dowsett, et al., 2011). Moreover, there remains ample room for improvement in the acquisition of Ki-67 index owing to discrepancies in interpretation of Ki-67 immunohistochemical staining (Dowsett, et al., 2011). These shortcomings threaten the ability of the two indices to precisely assess cell cycle progression.
within breast tumors, thus impeding accurate evaluation of metastatic risk in breast cancer patients. Hence, this imprecision presents an obstacle in comprehending inconsistencies in metastatic rate among AA and EA breast tumors.

We have developed a novel metric that rationalizes the inconsistencies and limitations of MI and KI as autonomous prognosticators and the lack of depth these indices convey in elucidating cell cycle mechanics. The metric cleverly integrates MI and KI onto the same measurement scale to ascertain the frequency of mitotic cells among Ki-67 positive cycling cells, in which we have termed mitosis to proliferation (M:P) ratio. Hence, this novel metric allows clinicians to conveniently visualize and assess proliferation and mitotic counts in the same microscopic field simultaneously, increasing efficiency and rapidness of evaluating cell cycle kinetics among breast cancer patients. Thus, combining their measurement scales may significantly strengthen the correlation between MI and KI in the clinic. Furthermore, this improved index derives an opportunity to deduce the relationship between mitotic cells and proliferating cells within low grade breast tumors, providing an additional layer of predictive information that was previously unavailable. Thus, this clinically translatable prognostic tool may infer enhanced interpretations of cell cycle kinetics within early stage breast tumors and augment clinical inferences of metastatic predispositions of early stage breast lesions. This heightened comprehension of metastatic inclinations of incipient breast tumors may improve understanding of the perplexing dissimilar metastatic proclivities among AA and EA breast tumors. We analyzed differences in M:P ratio between AA and EA breast tumor tissue specimens from Northside Hospital (NH) in Atlanta, GA and patient-derived TNBC cell lines to evaluate the prognostic ability of M:P ratio among racially-distinct breast tumors. We postulate that M:P ratio may reveal notable variations in mitotic tendencies within AA and EA breast
tumors and consequently serve as a tool to enhance profiling of metastatic risk among racially distinct breast tumors. This enhanced scope of understanding of cell cycle kinetics within breast tumors, may aid in unraveling the mystery still puzzling pathologists today that of the substantially higher migratory potential harbored by AA breast tumors in comparison to EA breast tumors.

3.3 Results

3.3.1 Demographic and clinico-pathological variables among racially-distinct breast cancer patients

Descriptive characteristics of the NH patient cohort are provided in Table 1. We examined differences in clinico-pathological characteristics between AA (n=81) and EA (n=124) breast cancer patients observed at NH. We observed significant differences in nuclear grade between the races as 53.1% of AA and 37.1% of EA patients presented as grade 2 and 23.5% of AA and 42% of EA patients as grade 3 (p=0.02). We also observed significant differences in mitotic index in which 45.7% of AA and 31.45% of EA patients presented with a score of 2 and 8.6% of AA and 36.3% of EA patients with a score of 3 (p<0.0001). Furthermore, we observed weakly significant differences in stage in which 24.7% of AAs presented at late stage (III-IV) compared to 21% of EAs (p=0.09).

3.3.2 A novel metric may predict patient survival

We derived a novel metric that integrates MI and KI onto the same measurement scale to enhance our understanding of cycling kinetics and mitotic propensity among breast tumors. In order to combine the two indices into the same field, we performed a 3 color immunofluorescence staining on formalin-fixed paraffin-embedded (FFPE) AA and EA breast tumor tissue sections from Northside Hospital (NH) in Atlanta, GA as shown in Figure 1A.
Histone H3 is a nuclear core histone on DNA chromatin and its serine residues are phosphorylated during mitosis to facilitate chromosome condensation hence, metaphase chromosomes are heavily phosphorylated as opposed to interphase chromosomes (148, 149). Thus, the antibody against pHH3, has been show to strongly correlate with mitotic counts and mitotic activity index (MAI) (146, 150). Recently, pHH3 was shown to have significantly better reproducibility, predictability and better represent proliferation than Ki-67 and has better sensitivity for detecting mitosis than MAI among invasive breast carcinomas (151). Thus, pHH3 was used as a mitotic marker and Ki-67 as a proliferation marker. The nuclei were stained with propidium iodide. The stained samples were imaged using immunofluorescence confocal microscopy and approximately 10-12 fields were captured per specimen. The number of mitotic and proliferative cells were quantitated and integrated into our proposed mitosis: proliferation (M:P) ratio formula, which is the proportion of mitotic cells per field to the total number of Ki-67 positive cycling cells in the same field (Figure 1B).

3.3.3 **M:P ratio differentiates racially-distinct breast tumors**

M:P ratios were computed in AA (n=81) and EA (n=124) breast cancer patient specimens and compared between the racial groups. Interestingly, we observed that M:P ratio was significantly higher among AA compared to EA breast cancer patients (p=0.002) (Figure 2A). We also observed a trend of higher M:P ratio in AA compared to nuclear grade-matched EA breast cancer patients (Figure 2B). M:P ratio was weakly higher in AA compared to EA among grade 1 (p=0.1), grade 2 (p=0.08) and grade 3 (p=0.1) breast cancer patients. Furthermore, we observed significantly higher M:P ratio in AA compared to EA among early stage (I-IIa) breast cancer patients (p=0.015) but not among late stage (IIb-IV) patients (p=0.2) (Figure 2C). We performed a multivariate linear regression analysis to determine if demographic and clinic-
pathological variables such as age, race, nuclear grade, stage, and receptor status have a confounding influence on M:P ratio. Interestingly, we observed that race was the only covariate that exhibited a significant confounding influence on M:P ratio (p=0.042) (Table 2).

3.4 Discussion

The vast differences in tumor kinetic progression between breast cancer patients of African and European ancestry continue to challenge clinicians. Evaluating risk of metastatic progression among a heterogeneous breast cancer patient population remains imprecise with current clinico-pathological parameters measuring cell proliferation unable to provide an accurate assessment of the mitotic propensity of breast tumors. Our study proposes a novel metric termed M:P ratio that is able to glean an additional layer of risk-predictive information by capturing the frequency of actively dividing cells among the proliferative cell population, which has not yet been achieved in the clinic today. Our innovative metric combines the more widely accepted marker of mitosis, pHH3, and the most robust marker of proliferation today, Ki-67, onto the same uniform measurement scale to discern the cycling kinetics of breast tumors. To investigate the clinical utility of our novel metric among breast cancer patients of varying kinetic progression, we analyzed differences in M:P ratio between AA and EA breast cancer patients as they posit the ideal model system to assess cycling kinetics between aggressive and less aggressive breast tumors.

We observed that M:P ratio differentiates racially-distinct breast cancer patients and AA and EA TNBC patient-derived cell lines with a trend of higher M:P ratio among patients of African ancestry. M:P ratio was significantly higher in AA compared to EA among early-stage breast cancer patients suggesting that AAs may be exhibiting faster cycling kinetics during the early stages of the disease, which may be predisposing them to a poorer patient prognosis.
Interestingly, our multivariate regression analyses revealed that race was the only variable that exhibited a significant confounding influence on M:P ratio. Furthermore, M:P ratio may be able to predict a poorer patient prognosis among early-stage breast cancer patients as we found that M:P ratio negatively correlated with a poorer overall survival among low grade patients. Thus, M:P ratio may be able to serve as a risk-prognostic marker in the clinic and stratify early-stage patients into high- and low-risk groups to guide clinical-decision making and optimize treatment paths, especially for patients of African ancestry.

Hence, our study proposes a hitherto untapped parameter of cycling kinetics that may transform clinical oncology by refining measurement of cell proliferation in the clinic. MI and KI serve as conventional clinical measures of cell proliferation in the clinic today but present with many shortcomings prompting the urgent need for more robust clinically-applicable parameters that reconcile their weaknesses. M:P ratio serves as an innovative metric that successfully rationalizes the limitations of MI and KI as independent parameters and provides an additional layer of valuable risk-prognostic information currently unavailable in the clinic today. This novel metric is clinically-applicable as it can be measured through conventional immunohistochemical staining methods. Moreover, the unique index proffers clinicians a more efficient and less time consuming method to successfully assess cell proliferation among breast cancer patients by allowing visibility of mitotic and proliferating cell counts in one microscopic field. Hence, this novel methodology of employing one measurement scale may strengthen the correlation between mitotic and proliferating cell counts, reduce inter- and intra-observer variability and refine risk-prognostication. As mentioned earlier, recent studies have suggested that pHH3 marker is a more accurate assessment of mitosis in tumors compared to mitotic activity index and is more reproducible than Ki-67 alone thus, our novel index may glean a more
accurate assessment of the actively dividing cell population(151, 152). Furthermore, our study utilized the ideal model to investigate differences in cycling kinetics between aggressive and less aggressive breast tumors by examining disparities in M:P ratio between AA and EA breast cancer patients. Thus, our investigation further rationalizes the poorer clinical outcomes observed among AA patients and uncovers a novel risk-predictive metric that may enhance prognostication among patients of varying tumor kinetic progression. However, validation of the prognostic and predictive power of M:P ratio among additional cohorts will be necessary to implementing the metric into the clinic as standard routine assessment of cycling kinetics among breast cancer patients.

3.5 Methods

3.5.1 Breast tumor tissue specimens

Clinical data on breast cancer patients treated at Northside Hospital (NH) from 2005-2015 in Atlanta, Georgia was extensively mined from pathology reports. A plethora of breast clinico-pathological information and outcome data were extracted from pathology reports on 10,504 patients evaluated at NH. Demographic characteristics documented include race, gender, and age. Breast tumor clinico-pathological variables extracted include nuclear grade, mitotic score, stage, Nottingham grade, and Ki-67 percentage. The outcome data acquired include OS. Archival formalin-fixed, paraffin-embedded tissue specimens were provided by NH to accompany clinical patient data. Research Collaborative Agreements were instituted with NH who consented to supplementing our mined clinical patient data with tumor tissue specimens. Among these samples, 205 AA and EA breast cancer patient samples were isolated to conduct our study.
3.5.2 Immunofluorescence tissue staining and confocal microscopy

Formalin-fixed, paraffin-embedded tissue sections were incubated at 60°C-70°C for 2h, followed by 2 xylene washes (5 min each) and sequential ethanol washes (100%, 95%, 70% and 50%). Antigen retrieval was carried out in citric acid buffer (pH 6.0) at 98°C for 20 min. Slides were allowed to cool down and blocked in 5% BSA/PBS (30 min). Tissue sections were then incubated for 1h with a cocktail of rat anti-human pH3 antibody (1:500) (Abcam, Cambridge, UK) and mouse anti-human DM1A (α-tubulin, 1:1000) (Sigma-Aldrich, MO, USA) followed by donkey anti-rat Alexa 488 (1:2000) and goat anti-mouse Cy5 (1:2000) (Invitrogen, Grand Island, NY) secondary antibody incubation for 1h at room temperature. Slides were then washed 3 times in PBS followed by 1h incubation with rabbit anti-human Ki67 (1:1000) (Abcam, Cambridge, UK) and goat anti-rabbit Alexa 405 (Invitrogen, 1:2000) secondary antibody incubation for 1h at room temperature. Slides were again washed 3 times in PBS followed by incubation with Propidium Iodide (0.1ug/ml) for 15 min at room temperature and washing in PBS. Finally, coverslips were mounted on the slides using Prolong Antifade mounting medium (Invitrogen, Grand Island, NY). Zeiss LSM 700 confocal microscope was used to capture immunofluorescence images at 63X objective magnification. Approximately, 10-12 randomly selected fields were captured per sample.

3.5.3 Cell lines

All cell lines (MDA-MB-231, MDA-MB-468, MDA-MB-236, HCC1143, HCC1937, HCC1187, HCC38, HCC70, HCC1806, DU4475, MFM-223, BT-549, BT-20) were procured from American Type Cell Culture (ATCC) and cultured according to ATCC recommendations.

3.5.4 Cell lysates preparation and immunoblotting

Cell lysates were collected after cells reached ~80% confluence and lysed with 1x lysis buffer (Cell Signaling) as previously described(153). Protein concentration in cell lysates was
determined using Bradford Protein Assay (BioRad) and spectrometry. Cell lysates were resolved in 10% SDS-PAGE gels as previously described(154). SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) was used to visualize the bands. PARP, Hif-1α, and E-cadherin primary antibodies were obtained from BD Biosciences. Cyclin-E, Centrin-2, VEGF, Vimentin, uPAR, RAD51, MMP9, p53, and β-actin primary antibodies were obtained from Santa Cruz Biotech. γ-tubulin, PLK4, pericentrin, integrin-α2, MMP2, and ALDH1 primary antibodies were obtained from Abcam. γ-tubulin, PLK4, pericentrin, integrin-α2, MMP2, and ALDH1 primary antibodies were obtained from Abcam. Protein expression was semi-quantitated using densitometry with the ImageJ Software and normalized to β-actin.

3.5.5 Cell viability assay

All cell lines were treated with gefitinib (LC Laboratories) and carboplatin (Abcam). Sensitivities of cell lines to these drugs were assessed by performing MTT assay as previously described(155). IC50 values were computed using GraphPad Prism Software Inc.

3.5.6 Immunocytochemistry staining and FACS

AA and EA TNBC cell lines were seeded into triplicates and fixed in 70% ethanol once cells reached ~80% confluence. Cells were blocked with 2% bovine serum albumin (BSA) in 1x PBS and 0.05% Triton X-100 at room temperature for 1 h. Cells were then incubated in the antibodies anti-Ki-67 PE (BioLegend) and anti-pHH3 conjugated to AlexaFluor488 (Sigma) diluted in blocking buffer at 1:1000 for 45 min at 37°C. Antibodies were rinsed from cells with 1x PBS 5 times and then cells were incubated in 7-Aminoactinomycin D (7-AAD) (BioLegend) (0.25µg/ml) for 10 min at room temperature to stain DNA. The stained cells were subjected to FACS to isolate the populations of pH3 and Ki-67 positively stained cells for each cell line. The single cell population was first gated for the total number of 7-AAD positive cells. The
population of 7-AAD positive cells was then gated for the number of cells positive for Ki-67 and the percentage of cells positive for Ki-67 was determined. The cells positive for Ki-67 were then gated for cells positive for pHH3 and the percentage of cells positive for pHH3 was determined. FlowJo V10 software was used to analyze the data. Dot plots of side scatter area (SSC-A) were plotted.

3.5.7 Statistical Analyses

Chi-square tests were performed to analyze differences in breast clinico-pathological variables, biomarkers, and treatment information between AA and EA breast cancer patients. A one-tailed student t-test was performed to analyze significant differences in M:P ratios, protein expression, and drug sensitivities between 2 groups. A multivariate regression analysis was performed to evaluate potential confounding influences of demographic and clinico-pathological variables on M:P ratio. A linear regression plot was constructed to determine association of M:P ratio with overall survival (OS). SAS 9.4 program was used to perform hierarchical clustering to stratify TNBC cell lines into high and low M:P ratio subgroups by using Ward’s method to identify an ideal threshold value of 2.02. Results with p<0.05 were considered statistically significant.

3.6 Tables and Figures
Table 3.1 Clinico-pathological characteristics of AA and EA breast cancer patients
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<th>EA (n=124)</th>
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<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
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<tr>
<td>M:P ratio</td>
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Abbreviations: AA, African-American; EA, European-American; T, tumor size; N, lymph nodes metastasis; M, distant metastasis; *P* values were calculated using the Chi-square test.
Table 3.2 Multivariate regression analysis of demographic and clinico-pathological variables on M:P ratio

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<th>p-value</th>
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<td>0.688</td>
<td>0.505</td>
<td>-0.111</td>
<td>0.224</td>
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Abbreviations: CI, confidence interval; t Stat, test statistic

Figure 3.1 Quantitation of M:P ratio among breast tumors. Confocal micrographs of positive staining for pH3 (mitotic marker), Ki-67 (proliferation marker) and propodium iodide (PI) in breast tumor tissue specimens in same field from NH (A). M:P ratio equation integrating the number of mitotic and proliferating cells in same field to yield the proportion of mitotic cells among the proliferating cell population (B).
Figure 3.2 Comparison of M:P ratio among racially-distinct breast tumors. Comparison of M:P ratio among all (A), grade-matched (B) and stage-matched (C) AA and EA breast tumor tissue specimens. *p<0.05. **p<0.01.

3.7 References


4 CENTROSONE AMPLIFICATION IN BREAST CANCER: A BIOMARKER OF AGGRESSIVE DISEASE FEATURES AND RACIAL DISPARITIES

4.1 Abstract

Centrosome amplification (CA), a key property of malignant tumor cells, has emerged as a marker of breast cancer (BC) aggressiveness that is highly associated with triple-negative BC (TNBC). TNBC is a BC subtype that disproportionately afflicts African American (AA) women. However, the association of CA with TNBC molecular subtypes and self-reported race, the value of CA as an independent risk-predictive biomarker in early-stage TNBC, and the mechanisms by which CA may drive aggressive TNBC behavior remains under-studied. Using confocal microscopy, we quantitatively compared numerical CA profiles in a panel of race-annotated non-TNBC and TNBC cell lines of different subtypes based on AR expression and TNBC molecular subtypes. We then compared race-annotated patient non-TNBCs and TNBCs of different AR expression levels and molecular subtypes in terms of their CA20 - an established signature of 20 genes implicated in CA - and evaluated the prognostic value of CA20 in multivariate Cox models of disease-free survival (DFS) using public gene expression data. To discern mechanisms by which CA may fuel aggressive TNBC behavior, we induced CA in TNBC cell lines with aphidicolin and assessed expression of protein biomarkers of BC aggressiveness. Numerical CA was higher in TNBC than non-TNBC in vitro analyses and CA20 was higher among AA compared to EA BCs. Furthermore, high CA20 as a continuous variable predicted worse DFS in early-stage TNBC patients in adjusted analysis (p=0.03, hazard ratio=1.20). Induction of CA in TNBC cell lines upregulated expression of markers of angiogenesis and epithelial-mesenchymal transition (EMT) suggesting that CA may promote TNBC aggressiveness through these mechanisms. Collectively, these findings support an association between numerical CA and more aggressive TNBC features,
poorer DFS among early-stage BC patients and AA race. Our study also reveals novel mechanisms by which CA may promote aggressive TNBC behavior. Hence, our work suggests CA as a risk-predictive biomarker for TNBC patients that may also represent a viable therapeutic target.

4.2 Introduction

TNBC accounts for approximately 10-30% of invasive BC cases in the U.S. It is a unique BC subtype that lacks expression of the estrogen and progesterone receptors and amplification of the human epidermal growth factor receptor 2 (HER2/neu). The absence of these targets eliminates conventional endocrine therapy and HER2-targeted systemic treatments as therapeutic options and traditional chemotherapy is unable to eradicate most TNBCs. The disease is characterized by an aggressive clinical course, evidenced by its greater metastatic propensity, more unfavorable clinico-pathological characteristics upon presentation, and higher inter-patient and intratumor heterogeneity compared with non-TNBCs [1-3]. The disease is significantly more prevalent among premenopausal women and predominantly afflicts women of African ancestry, who experience poorer clinical outcomes than EA TNBCs. Currently, no reliable biomarkers or effective targeted treatments exist for TNBC patients; thus, AA TNBC patients often suffer abysmal outcomes. Novel biomarkers to risk-stratify TNBC patients, ideally ones that also represent possible therapeutic targets, are urgently needed to identify patients requiring more aggressive treatment regimens so that mortality rates can be reduced in these patients. CA is a hallmark of BC that causes ITH by fostering erroneous mitoses, which drive genetic instability and the generation of diverse karyotypes [4, 5]. It is well established that CA is associated with BC progression and imparts aggressive phenotypes, thus representing a possible anti-cancer target [6-11]. Furthermore, TNBCs harbor greater incidence and severity of CA than non-TNBCs [12, 13], although the potential prognostic and therapeutic value of CA in TNBC is unclear. The
goal of the present study was to shed light on the potential of CA as a biomarker of aggressive TNBC features and poor DFS, and mechanisms by which CA may promote TNBC progression, which could be exploited therapeutically.

4.3 Results

4.3.1 TNBC cell lines exhibit higher numerical CA than non-TNBC cell lines

Our group recently uncovered more extensive numerical CA and more severe structural centrosomal aberrations in several TNBC compared to non-TNBC cell lines [12]. In this study, we quantitated numerical centrosomal profiles in a more extensive panel of established cell lines derived from TNBCs (n=10) and non-TNBCs (n=2). We found a non-significant trend towards higher numerical CA in TNBC compared with non-TNBC cell lines (p=0.10) (Figures 1A and B). We also assessed the severity of numerical CA in each cell line (i.e., the percentage of cells with 3, 4, or 5+ centrosomes) and discovered that TNBC cell lines exhibit a non-significant trend towards more severe numerical CA, with a higher percentage of cells with 4 centrosomes (p=0.056) and ≥5 centrosomes (p=0.07) (Figure 1C). We immunoblotted TNBC and non-TNBC cell lines for expression of centrosomal proteins and CA markers. We observed significantly higher expression of γ-tubulin (p=0.049) and Cyclin-E (p=0.034) in TNBC compared to non-TNBC cell lines (Figure 1D). We also observed a trend of higher pericentrin expression in TNBC compared to non-TNBC cell lines (p=0.08). Furthermore, we assessed whether there were differences in expression of KIFC1, a minus-end directed microtubule binding protein that participates in centrosome clustering [23], between TNBC and non-TNBC cell lines. Interestingly, we observed a trend of higher KIFC1 expression in TNBC compared to non-TNBC cell lines (p=0.1).
4.3.2 Aggressive TNBC subtypes exhibit higher numerical CA than less aggressive TNBC subtypes

Accumulating evidence has exposed the vastly heterogeneous landscape of TNBC. Lehmann and colleagues uncovered six distinct intrinsic molecular subtypes in the disease based on shared gene ontologies (“TNBCtype”), including basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal stem cell-like (MSL), mesenchymal (M), immunomodulatory (IM), and luminal androgen receptor (LAR) subtypes [24]. The IM and MSL subtypes in patient TNBCs may reflect gene expression profiles of substantial infiltrating lymphocytes and stromal cells, respectively [19], which led to refinement of the classification scheme (“TNBCtype-4”) [19]. We compared centrosomal profiles between TNBC cell lines of different molecular subtypes (representative micrographs in Figure 2A). Interestingly, we observed that BL1 and MSL TNBC cell lines exhibited higher numerical CA than BL2, M, and LAR TNBC cell lines, with the exception of MDA-MB-438 cell line (p=0.016) (Figure 2B). BL1 and MSL TNBC cell lines also exhibited more severe numerical CA than BL2, MSL, M, and LAR TNBC cell lines, specifically, exhibiting a greater percentage of cells with ≥5 centrosomes (p=0.01) (Figure 2C). We also found that BL1 TNBC cell lines exhibited significantly higher numerical CA than BL2, MSL, M and LAR TNBC cell lines, with the exception of MDA-MB-468 cell line (p=0.036) (Figure 2D) and BL1 TNBC cell lines exhibited a trend towards a greater proportion of cells with 4 or ≥5 centrosomes (p=0.089 and 0.10, respectively) (Figure 2E). Furthermore, western blot analyses revealed a weakly significant trend of higher γ-tubulin expression in BL1 and MSL compared to BL1, MSL, M, LAR, and IM TNBC cell lines (p=0.059) (Figure 2F) and significantly higher γ-tubulin expression in BL1 compared to BL2, MSL, M, LAR, and IM TNBC cell lines (p=0.015) (Figure 2G). These results suggest that CA may be more extensive and severe in BL1 and/or MSL TNBCs.
4.3.3 Racial disparities in numerical CA exist between AA and EA TNBC cell lines

Women of African ancestry are 2-3 times more likely to develop TNBC than women of European ancestry [1]. Distinctions in inherent tumor biology between the races have been suggested to underlie this disparate burden; however, there is a limited understanding of the biological factors contributing to the racial disparity in TNBC. Thus, we were interested to see if disparities in centrosomal profiles exist between AA and EA TNBCs (representative micrographs in Figure 3A). Interestingly, we found that EA TNBC cell lines exhibited a greater percentage of cells with numerical CA than AA TNBC cell lines (p=0.01) (Figure 3B). Furthermore, EA TNBC cell lines exhibited more severe CA, with a greater percentage of cells with 4 (p=0.007) or ≥5 (p=0.029) centrosomes than AA TNBC cell lines (Figure 3C). However, immunoblotting for centrosome structural proteins and markers of CA revealed that AA TNBC cell lines exhibited higher expression of pericentrin (p=0.027) and Cyclin-E (p=0.024) (Figure 3D). Furthermore, we assessed whether there were differences in expression of KIFC1 between AA and EA TNBC cell lines. Interestingly, we found higher expression of this marker in AA than EA TNBC cell lines (p=0.05) (Figure 3D). This finding suggests that centrosome clustering may be more robust in AA than EA TNBC cell lines cells. Thus, we investigated the sensitivity of AA and EA TNBC cell lines to the centrosome declustering agent, griseofulvin (Figure 4). We observed that AA TNBC cell lines exhibited significantly lower IC50 values to griseofulvin than EA TNBC cell lines (79.5 vs. 105.7) (p=0.04), suggesting greater sensitivity to centrosome declustering agents among AA TNBCs and corroborating our findings of higher KIFC1 expression and increased centrosome clustering among AA compared to EA TNBC cell lines.
4.3.4 **CA20 is higher in more aggressive BC subtypes**

Previously, our group developed a transcriptional signature that may reflect CA and correlates with chromosomal instability and worse prognosis in multivariable models, referred to as the CA20 score, which is the sum of the normalized expression of 20 genes whose dysregulation causes CA [1]. To confirm our *in vitro* findings, we used publicly available microarray data to compute CA20 scores and determine differences between AA and EA BCs, TNBCs and non-TNBCs, and among TNBC subtypes. TCGA Breast dataset includes annotation about the variables of interest; thus, we queried it using Oncomine [2] to obtain normalized expression values for the CA20 genes, which were summed as previously described to derive CA20 scores [1]. CA20 was higher for AAs than EAs among all BC patients (*p*=0.008) (Figure 5A). However, AAs are predisposed to TNBC, which exhibits higher CA20 than non-TNBC [2]. CA20 is also associated with more aggressive disease features, such as higher tumor grade [18]. As a result, TNBC status and other aggressive disease features like TNBC status, higher tumor stage (for both TNBCs and non-TNBCs), HER2+ status (for non-TNBCs), low AR expression (for both TNBCs and non-TNBCs), and TNBC molecular subtype (for TNBCs) could confound analyses. Due to non-homogeneity of variances between BC subtypes, we only explored differences in CA20 within BC subtypes. We found a non-significant trend towards different mean CA20 by AJCC stage within non-TNBCs, which exhibited higher mean CA20 in higher-stage cases (*p*=0.095), and within TNBCs, which exhibited higher CA20 in lower-stage cases (*p*=0.063) (Figure 5Bi,ii). CA20 was also higher in HER2+ than HER2- non-TNBCs (*p*=0.004) (Figure 5C). Thus, amongst non-TNBCs, CA20 is higher in more aggressive subtypes.

We were interested to compare mean CA20 between racially distinct non-TNBCs and TNBCs after adjusting for potentially confounding factors. As CA20 was found to differ
significantly based on HER2 and AR statuses among non-TNBCs, and a non-significant trend was also noted for AJCC stage, we included these factors along with self-reported race in a generalized linear model to predict CA20. Tests of between-subjects effects revealed that AJCC stage was not a significant predictor \((p=0.37)\), whereas all other factors exhibited \(p<0.10\). Considering this finding and that CA20 did not significantly differ by stage in unadjusted models, were removed stage from the model and refit it. Among non-TNBCs, we found that CA20 was higher in AA than EA tumors \((p=0.054\), adjusted mean=26.41 vs. 22.65\) and HER2+ than HER2- tumors \((p=0.001\), adjusted mean=26.68 vs. 22.37\) \((\text{Table 1})\). Although the difference in the adjusted mean CA20 by race did not reach the threshold for significance, the observed power was low \((0.49)\), likely due to the small number of AA TNBCs \((n=11)\). By contrast, the observed powers for other factors and the corrected model were \(\geq 0.89\). In addition, full-factorial models did not reveal any interactions among factors \((p>0.20\) for all). Thus, among non-TNBCs, CA may be higher in breast tumors from AA patients and in the setting of HER2 positivity. When these factors are considered, CA20 does not differ by AJCC stage.

We also fit generalized linear models to predict CA20 among TNBCs adjusting for race and stage. Among TNBCs, AJCC stage did not significantly predict CA20 although a non-significant trend was noted \((p=0.075\), with the trend in the same direction as noted in unadjusted analyses). By contrast, CA20 did not differ by race according in the adjusted model \((p=0.92)\), but the analysis was severely underpowered to detect a racial difference \((\text{observed power}=0.051)\). Thus, we sought a different dataset to explore racial differences, the Yale TNBC dataset. The CA20 distribution was approximately normal for EA patients, whereas it was non-normal for AA patients \((p=0.013)\), exhibiting two distinct peaks, which we suspected existed due to some underlying grouping, such as lymph node status. Indeed, a trend towards different nodal status by
race was found (p=0.092), with AAs exhibiting a higher frequency of node-positive disease. In addition, CA20 was higher in node-positive than node-negative TNBCs from AA patients (p=0.016) (Figure 5D), suggesting it could be a confounding factor in analyses of racial differences. Because only 2 node-positive EA TNBCs were present in the dataset, we decided to focus exclusively on node-negative disease in exploring racial differences in CA20 using this dataset. Among node-negative TNBCs, we found that CA20 was higher in EA than AA cases (p=0.034, mean rank=22.00 vs. 14.63) (Figure 5E). Thus, in the case of node-negativity, EA TNBCs may exhibit higher CA20 than AA TNBCs, which supports our in vitro results of higher numerical CA in EA compared with AA among TNBC cell lines.

Unlike the TCGA data, the Yale data showed that mean CA20 may differ by TNBCType6 (p<10^-6). Specifically, the MSL and LAR subtypes exhibited lower CA20 than the other TNBC subtypes (p<0.05 for all per Tukey’s HSD post-hoc testing, Figure 5F; Table 2). These results support our in vitro finding of higher numerical CA among BL1 TNBCs. Lower CA20 among LAR TNBCs also corroborates our in vitro results of higher CA being associated with TNBCs lacking AR expression. We compared mean CA20 between node-negative AA and EA TNBCs after adjusting for TNBCType and did not detect a significant difference (p=0.21), although the mean was higher in EA than AA TNBCs; however, the analysis was underpowered to detect a difference (observed power=0.24).

4.3.5 High CA20 predicts poorer survival among early stage BC patients

Finally, we were interested to test the ability of CA20 to stratify early-stage BCs regardless of subtype in terms of DFS. In univariate Cox models, higher CA20 score (continuous and categorical) predicted significantly worse DFS (p=0.026, HR=1.047, and p=0.013, HR=2.85, respectively) (Table 3). In multivariate Cox models, only CA20 (continuous or dichotomized by
the mean) was a significant predictor, with all other covariates eliminated from the final model. This finding suggests that higher CA20, rather than the other covariates considered, uniquely confers poor DFS among early-stage BC patients.

4.3.6 *CA promotes aggressiveness in TNBC in vitro*

CA has long been associated with chromosomal instability, aneuploidy, and mitotic spindle abnormalities [31]. Greater centrosomal aberrations has recently been associated with enhanced cell migration in BC patient specimens as well as in cultured cell lines [32]. However, the precise role of CA in TNBC remains elusive. We induced CA in TNBC cell lines, MDA-MB-468 and MDA-MB-231, and immunoblotted cells for markers of angiogenesis and EMT to investigate the role of CA in TNBC (*Figure 6*). Upregulation of CA was confirmed through immunoblotting for centrosome structural proteins and CA markers for which we observed ≥4-fold increased expression. Interestingly, in the MDA-MB-468 cell line there was ~2.7-fold increase in expression of angiogenesis markers (*Fig 6A*) and ~2.1-fold increase in expression of EMT markers with the exception of E-cadherin, which exhibited an ~1.5 fold decrease in expression (*Fig 6B*). Our results also suggest more robust upregulation of angiogenesis and metastasis markers upon induction of CA in the AA TNBC cell line, MDA-MB-468, compared with the EA TNBC cell line, MDA-MB-231. These observations suggest that CA may be promoting disease progression in AA and EA TNBCs via different mechanisms.

4.4 **Discussion**

The limited availability of prognostic biomarkers and therapeutic strategies for TNBC contributes to the significant disparity in outcomes between TNBC and non-TNBC patients and underscores the need for (a) novel, alternative biomarkers that can predict the risk of a more aggressive disease course in patients, and (b) new and robust therapeutic targets to slow or prevent
disease progression in TNBC patients. Our study investigated the role of CA in TNBC to support the viability of CA as a potential risk-predictive biomarker in TNBC. A strength of our study is that a variety of TNBC cell lines of different molecular subtypes and derived from racially-distinct patients was used, although a limitation of any study of cell lines is generalizability to patient populations. Indeed, our group recently reported discordance in CA between patient tumors and patient-derived cancer cell lines [15]. Thus, to corroborate our in vitro work, we analyzed patient tumor data as well, another strength of our study, although it may have been underpowered to detect racial differences among TNBCs. At present, we are not aware of any publicly available microarray datasets with a large sample of race-annotated TNBCs, for which there is an urgent unmet need.

Our study is the first study to compare CA between molecular TNBC subtypes, uncovering more extensive and severe numerical CA among BL1 and MSL TNBC cell lines, which was corroborated by analysis of patient TNBCs. Our results are consistent with previous studies reporting the BL1 TNBC subtype to be associated with AA race [22, 33]. Keenan and colleagues found that BL1 and MSL tumors were more present among AA compared to EA TNBCs. Lindner’s group reported that transcriptional features of BL1 subtype correspond to AA TNBC tumors and transcriptional features of MSL and LAR correspond to EA TNBC tumors. AA TNBC cell lines also exhibited evidence of enhanced clustering, which may empower cancer cells with enhanced migratory ability and foster the acquisition of more aggressive cellular phenotypes [28, 34-36]. Given the literature detailing the role of CA and clustering in promoting aggressive behavior of TNBC cells and our finding that higher CA20 is an independent predictor of poor DFS in early-stage TNBC, targeting TNBCs with numerical CA and centrosome clustering may be a promising therapeutic strategy. Collectively, our findings suggest that AA TNBCs may be more susceptible
to centrosome declustering drugs than EA TNBCs, and it is imperative that preclinical models testing declustering drugs consider race.

The precise mechanisms in which CA may be promoting aggressive TNBC remain under investigation. To gain deeper insights into how CA may be contributing to rapid progression in TNBC, we induced CA in TNBC cell lines and evaluated the effect on various markers of BC aggressiveness. Interestingly, upon induction of CA we observed a significant upregulation of angiogenesis and EMT markers, suggesting a role of CA in upregulating these processes in TNBC. Our results corroborate our recent findings revealing that CA strongly correlates with expression of the metastasis marker, vimentin, in breast tumor tissue samples, as well as with enhanced migration and invasion in TNBC in vitro [30]. Our work is also the first to provide evidence of a direct association of CA and key angiogenesis molecules in TNBC cells suggesting novel mechanisms excessive centrosomes may be employing to promote tumor progression in TNBC. Interestingly, we also observed racial differences in these markers following induction of CA, with BC angiogenesis and EMT markers significantly more upregulated in the AA TNBC cell line. Our study lays the foundation for larger studies investigating whether mechanisms by which CA may promote TNBC progression differ based on race. Future studies evaluating the role of CA in TNBC patient specimens with accompanying patient clinico-pathological variables and clinical outcomes would be necessary to establish CA as a robust risk-predictive and prognostic biomarker for TNBC patients.

CA is a cancer-cell specific trait that distinguishes them from normal, healthy cells [46]. Thus, targeting CA offers a minimally cytotoxic therapeutic strategy that selectivity targets cancer cells burdened with severe centrosomal aberrations. Conveniently, there are already rationally designed drugs that selectively kill cancer cells harboring CA and are currently under clinical
evaluation as promising anti-cancer therapeutics. These novel agents include putative centrosome declustering drugs such as griseofulvin and noscapine, commercially available HSET inhibitors such as CW069 and AZ82, and poly-ADP ribose polymerase inhibitors such as PJ34 and GF-15 [28, 47-51]. Hence, these agents may aid in abrogating an aggressive disease course in TNBC patients, perhaps in particular AA TNBC patients. Furthermore, non-invasive methods that can detect centrosomal status (i.e. fine-needle aspirate cytology, immunohistochemistry) may demonstrate feasibility in the clinic for selecting TNBC patients that will exhibit susceptibility to these agents. Thus, assessing CA in the clinic may provide a novel strategy to stratify TNBC patients into high- and low-risk groups for an aggressive disease course and allow for optimization of treatment plans. According to our results, CA-targeting agents may be particularly promising therapeutic options for QNBC and BL1 TNBC patients. These agents may serve as a novel alternative therapeutic target for TNBC patients exempt from receiving AR-targeted therapy as well as potentially attenuate racial disparities in BC and TNBC.

4.5 Methods

4.5.1 BC cell lines

All cell lines, including TNBC cell lines (MDA-MB-231, MDA-MB-468, HCC1143, HCC1937, HCC38, HCC70, HCC1806, DU4475, MFM-223, BT-549, BT-20) and non-TNBC (MCF-7, T47D) cell lines, were procured from American Type Cell Culture (ATCC) and cultured according to ATCC recommendations.

4.5.2 Cell lysate preparation and immunoblotting

Cell lysates were collected after cells reached ~80% confluence and lysed with 1x lysis buffer (Cell Signaling) as previously described [14]. Protein concentration in cell lysates was determined using Bradford Protein Assay (BioRad) and spectrometry. Cell lysates were resolved
in 10% SDS-PAGE gels as previously described [15]. SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) was used to visualize the bands. PARP, Hif-1α, and E-cadherin primary antibodies were obtained from BD Biosciences. Cyclin-E, Centrin-2, VEGF, Vimentin, uPAR, RAD51, MMP9, p53, and β-actin primary antibodies were obtained from Santa Cruz Biotech. γ-tubulin, PLK4, pericentrin, integrin-α2, MMP2, and ALDHA1 primary antibodies were obtained from Abcam. p21 and CD44 antibodies were obtained from Cell Signaling. Secondary antibodies were obtained from Abcam. Protein expression was semi-quantitated using densitometry with the ImageJ Software and normalized to β-actin.

4.5.3 Immunofluorescence staining

Cells were seeded onto glass coverslips. After reaching ~80% confluence, cells were fixed in ice-cold methanol. Coverslips were blocked in 2% bovine serum albumin (BSA) in 1x PBS and 0.05% Triton X-100 at room temperature for 1 h. Coverslips were incubated in primary antibodies for γ-tubulin (Abcam) and α-tubulin (Sigma-Aldrich) diluted in blocking buffer at 1:1000 for 45 min in 37°C. Coverslips were incubated in Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary antibodies (Invitrogen) diluted in blocking buffer at 1:3000 for 40 min in 37°C. Primary and secondary antibodies were washed from cover slips with 1x PBS. Cover slips were incubated in Hoechst (Life Technologies) at room temperature for 10 min and mounted with Prolong Gold Antifade Reagent (Invitrogen). DU4475 is a suspension cell line and thus, was not assessed for numerical CA in this study.

4.5.4 Numerical CA Quantitation

Cells were imaged with the Zeiss LSC 700 confocal microscope using 63x objective (Oberkochen, Germany). All images were processed with Zen software (Oberkochen, Germany). Approximately 250-300 randomly selected cells were counted per cell line. The percentage of cells
exhibiting numerical CA (i.e., >2 centrosomes) and multipolar mitoses (i.e., mitotic spindles with >2 poles) were quantitated in each cell line. In addition, the severity of CA was assessed by quantitating the percentage of cells with 3, 4, or ≥5 centrosomes in each cell line.

4.5.5 Induction of CA

Each cell line was seeded in duplicate. After reaching ~70% confluence, cells were treated with 25 µM aphidicolin from *Nigrospora sphaerica* (Sigma-Aldrich) for 72 h to induce CA through S-phase arrest. Cells treated with solvent containing no drug were used as a negative control. Cell lysates were collected after 24-36 h. Induction of CA was confirmed through immunoblotting for centrosomal proteins (γ-tubulin, centrin-2, and pericentrin) and CA markers (Cyclin-E, PLK4).

4.5.6 Cell viability assay

AA (MDA-MB-468 and HCC1806) and EA (BT-20, HCC1143, and MDA-MB-231) TNBC cell lines were treated with griseofulvin (Sigma-Aldrich). Sensitivities of cell lines to griseofulvin were assessed through MTT assay as previously described[30]. IC50 values were computed using GraphPad Prism Software Inc. Griseofulvin concentrations versus percentage of cell survival was plotted for each cell line using GraphPad Prism Software, Inc.

4.5.7 Statistical analysis of in vitro data

A one-tailed student t-test or one-way ANOVA was performed for analyses comparing 2 or >2 groups, respectively. Results with *p*<0.05 were considered statistically significant.

4.5.7 CA20 analyses

Datasets: To determine whether CA20 differs between breast cancers from AAs and EAs, we queried the Cancer Genome Atlas (TCGA) breast dataset [16] using Oncomine [17] to obtain normalized expression values of CA20 genes, which were summed as previously described to
derive CA20 scores [18]. Metastases, normal breast samples, male breast cancers, and ductal carcinomas in situ were excluded, resulting in n=524 cases. Annotation about TNBC status, TNBC subtype, PAM50, and PAM50lite data were obtained from Lehmann et al. [19], except for one missing case (TCGA-E2-A14W), the annotation for which was obtained from cBioPortal [20, 21]. All other demographic and clinical annotation was obtained from cBioPortal. Publically available ER/PR/HER2 IHC data was used to determine TNBC status. HER2 status was determined by IHC results, but for cases with equivocal or indeterminate IHC values, FISH was used instead. The Yale TNBC dataset [22] was downloaded from GEO (accession GSE46581), and TNBCType6 values for this dataset were obtained using the TNBCtype tool [20].

**Statistical analysis:** Levene’s test was used to assess equality of variances between groups. The Shapiro-Wilk test was used to assess whether samples derived from normally distributed populations. Exploratory analysis of TCGA breast data revealed the CA20 distribution for EA TNBCs was non-normal, exhibiting negative skew and leptokurtosis; however, after trimming the bottom 5% of CA20 values (n=3 cases), the distribution was approximately normal, and so this 5% trimmed dataset was used for all subsequent analyses. Exploratory analysis of the Yale TNBC data revealed a single outlier in the BL1 TNBC subtype, so the outlier-trimmed dataset was used for all subsequent analyses. CA20 was compared between groups using parametric (independent-samples, 2-tailed t-test or one-way ANOVA) or non-parametric (Mann-Whitney or Kruskall-Wallis tests) depending on satisfaction of test assumptions. For significant ANOVA tests, Tukey’s HSD post-hoc testing was performed. The association between categorical variables (race, nodal status, and TNBC) was assessed using Fisher’s exact test due to the small sample size of TNBCs. The relationship between CA20 and AR expression was found to be non-monotonic based on examination of scatterplots; thus, AR groups were defined based on expression levels. To obtain
adjusted mean CA20 values for non-TNBCs, generalized linear models of main effects were fit after ensuring satisfaction of assumptions, with CA20 as the dependent variable and including potentially confounding factors (i.e., with p<0.10). DFS was computed as the time in months from initial treatment to the event (recurrence or progression) or last follow-up for censored cases. We tested the impact of high CA20 (continuous or categorized based on the mean) on DFS among early-stage (i.e., AJCC stage I/II) BC patients in univariate and multivariate Cox models adjusted for self-reported race and other potentially confounding factors, first ensuring non-violation of the proportional hazards assumption for all covariates by entering each one as a time-dependent covariate in univariate Cox models. Kaplan-Meier product-limit DFS estimates were plotted for categorical CA20 groups, and differences in DFS times were tested using the log-rank test. Univariate Cox models of DFS were fit including CA20 (continuous) or CA20 (mean as a cutpoint) for early-stage BC patients of all subtypes. In addition, multivariate Cox models were fit entering relevant clinicopathologic and demographic covariates, including ER, PR, and HER2 statuses; nodal status (+/-); tumor size (2 cm cutpoint); PAM50 type (Normal-Like, Luminal A, Luminal B, HER2-enriched, or Basal-Like); AR status (10th percentile cutpoint); race (AA or EA); and age at diagnosis (continuous), subject to backward stepwise elimination if p≥0.10. In addition, we tested the impact of CA20 score on DFS among early-stage TNBCs using Kaplan-Meier analysis with the log-rank test and univariate and Cox regression. Covariates with p<0.10 in univariate Cox models were entered into multivariate models. Observed power was computed using α=0.05. IBM SPSS v. 21 was used and results with p<0.05 were considered statistically significant.

4.6 Tables and Figures
Table 4.1 Adjusted mean difference in CA20 scores among non-TNBCs based on the race and HER2 and AR statuses

<table>
<thead>
<tr>
<th>Factor</th>
<th>Adjusted mean difference</th>
<th>Standard Error</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
<th>p-value</th>
<th>Observed Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>3.76</td>
<td>1.95</td>
<td>-0.07</td>
<td>7.59</td>
<td>0.054</td>
<td>0.49</td>
</tr>
<tr>
<td>HER2+</td>
<td>4.31</td>
<td>1.28</td>
<td>-6.82</td>
<td>-1.80</td>
<td>0.001</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Table 4.2 Turkey post-hoc testing of mean CA20 scores by TNBC subtypes.
CI=Confidence Interval; SE=Standard Error
### Table 4.3 Univariate and multivariate Cox models of disease-free survival based on CA20 score.

<table>
<thead>
<tr>
<th>TNBCType</th>
<th>Difference</th>
<th>SE</th>
<th>p</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL1 BL2</td>
<td>4.7499</td>
<td>3.4768</td>
<td>.747</td>
<td>-5.455</td>
<td>14.955</td>
</tr>
<tr>
<td>M</td>
<td>5.7671</td>
<td>2.8709</td>
<td>.349</td>
<td>-2.659</td>
<td>14.193</td>
</tr>
<tr>
<td>MSL</td>
<td>19.6127</td>
<td>3.1607</td>
<td>.000</td>
<td>10.336</td>
<td>28.890</td>
</tr>
<tr>
<td>IM</td>
<td>3.9741</td>
<td>2.7954</td>
<td>.714</td>
<td>-4.231</td>
<td>12.179</td>
</tr>
<tr>
<td>LAR</td>
<td>19.7339</td>
<td>4.0870</td>
<td>.000</td>
<td>7.738</td>
<td>31.730</td>
</tr>
<tr>
<td>BL2 BL1</td>
<td>-4.7499</td>
<td>3.4768</td>
<td>.747</td>
<td>-14.955</td>
<td>5.455</td>
</tr>
<tr>
<td>M</td>
<td>1.0172</td>
<td>3.3969</td>
<td>1.000</td>
<td>-8.953</td>
<td>10.987</td>
</tr>
<tr>
<td>IM</td>
<td>-.7758</td>
<td>3.3334</td>
<td>1.000</td>
<td>-10.560</td>
<td>9.008</td>
</tr>
<tr>
<td>LAR</td>
<td>14.9840</td>
<td>4.4722</td>
<td>.016</td>
<td>1.858</td>
<td>28.110</td>
</tr>
<tr>
<td>M</td>
<td>BL1</td>
<td>-5.7671</td>
<td>2.8709</td>
<td>.349</td>
<td>-14.193</td>
</tr>
<tr>
<td>M</td>
<td>BL2</td>
<td>-1.0172</td>
<td>3.3969</td>
<td>1.000</td>
<td>-10.987</td>
</tr>
<tr>
<td>MSL</td>
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<td>3.0726</td>
<td>.000</td>
<td>4.827</td>
<td>22.864</td>
</tr>
<tr>
<td>IM</td>
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<td>2.6954</td>
<td>.985</td>
<td>-9.704</td>
<td>6.118</td>
</tr>
<tr>
<td>LAR</td>
<td>13.9667</td>
<td>4.0192</td>
<td>.011</td>
<td>2.170</td>
<td>25.764</td>
</tr>
<tr>
<td>MSL</td>
<td>BL1</td>
<td>-19.6127</td>
<td>3.1607</td>
<td>.000</td>
<td>-28.890</td>
</tr>
<tr>
<td>MSL</td>
<td>BL2</td>
<td>-14.8628</td>
<td>3.6451</td>
<td>.002</td>
<td>-25.562</td>
</tr>
</tbody>
</table>
Only final model covariates are given. BC=Breast Cancer; CI=Confidence Interval; HR=Hazard Ratio; MV=Multivariate; UV=Univariate

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Model</th>
<th>Covariate</th>
<th>p</th>
<th>HR</th>
<th>95% CI for HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage BC</td>
<td>UV</td>
<td>CA20 (continuous)</td>
<td>0.026</td>
<td>1.05</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>MV</td>
<td>CA20 (categorical)</td>
<td>0.013</td>
<td>2.85</td>
<td>1.28</td>
</tr>
</tbody>
</table>
Figure 4.1 Quantitation of numerical CA in TNBC and non-TNBC cell lines.

A. Representative immunofluorescent confocal micrographs of TNBC and non-TNBC cell lines stained for centrosomes (γ-tubulin, green), microtubules (α-tubulin, red), and nuclei (using Hoechst, blue).

B. Comparison of the prevalence of numerical CA in TNBC and non-TNBC cell lines.

C. Comparison of the severity of numerical CA between TNBC and non-TNBC cell lines. 250-300 cells were counted per cell line.

D. Western blots comparing centrosomal protein and CA marker expression between TNBC and non-TNBC cell lines. Scale bar represents 5 µM.
Figure 4.2 Quantitation of numerical CA in TNBC cell lines of different molecular subtypes.

A. Representative immunofluorescent confocal micrographs of TNBC cell lines stained for centrosomes (γ-tubulin, green), microtubules (α-tubulin, red), and DNA (Hoechst, blue). B. 

...
Quantitation of the extent of numerical CA in BL1 and MSL cell lines compared to that in BL2, M, and LAR TNBC cell lines. C. Quantitation of the severity of CA (i.e., the percentage of cells with 3, 4, or 5+ centrosomes) in BL1 and MSL cell lines compared to that in BL2, M, and LAR TNBC cell lines. D. Quantitation of the prevalence of numerical CA in BL1 cell lines compared to its prevalence in cell lines from all other TNBC molecular subtypes. E. Quantitation of the severity of CA (i.e., the percentage of cells with 3, 4, or 5+ centrosomes) in BL1 cell lines and its comparison with that in all other TNBC subtypes. F. Western blots comparing γ-tubulin expression between BL1 and MSL and BL2, M, LAR, and IM TNBC cell lines. G. Western blots comparing γ-tubulin expression between BL1 and MSL, BL2, M, LAR, and IM TNBC cell lines. *p<0.05, **p≤0.01. Scale bar represents 5 µM.

Figure 4.3 Quantitation of numerical CA in AA and EA TNBC cell lines.
A. Representative immunofluorescent confocal micrographs of EA and AA TNBC cell lines stained for centrosomes (γ-tubulin, green), microtubules (α-tubulin, red), and DNA (Hoechst, blue). B. Quantitative comparison of the percentage of cells with numerical CA between EA and AA TNBC cell lines. C. Quantitative comparison of the severity of CA (i.e., the percentage of cells with 3, 4, or 5+ centrosomes) between EA and AA TNBC cell lines. D. Western blots in EA and AA TNBC cell lines of i. pericentrin, KIFC1, and Cyclin-E. *p<0.05, **p≤0.01.
Figure 4.4 Sensitivity of AA and EA TNBC cell lines to griseofulvin.

MTT assays were performed on AA and EA TNBC cell lines and IC50 values were compared between the racially-distinct cell lines. i. Comparison of IC50 values from griseofulvin treatment between AA and EA TNBC cell lines. ii. Plot of percent cell survival vs concentration (μM) in AA and EA TNBC cell lines. *p<0.05.
Figure 4.5 Differences in CA20 scores between groups of BC patients.  
A-C: TCGA data, D-F: Yale data. A. Boxplots of CA20 by race among all breast cancer subtypes (Mann-Whitney test p=0.008, mean rank=244.55 vs. 193.90; median=27.22 vs. 23.36; in AAs and EAs, respectively); B. Bar graphs of mean CA20 by AJCC stage in (i) non-TNBCs (ANOVA p=0.095, means=19.01, 21.60, 22.37, and 23.39, respectively, for stages I-IV, respectively) and (ii) TNBCs (ANOVA p=0.063, means=31.59, 32.63, 28.84, and 26.11, for stages I-IV, respectively). C. Boxplots of CA20 by HER2 status in non-TNBCs (Mann-Whitney test p=0.004, mean rank=160.1 vs 126.7; median =20.64 vs. 24.40, in HER- vs. HER2+ cases). D. Boxplots of CA20 by lymph node (LN) status in AA TNBCs (Mann-Whitney test p=0.016, mean rank=20.55 vs. 12.58; median=192.45 vs. 209.81; for LN- and LN+ cases, respectively). E. Boxplots of CA20
by race among LN- TNBCs (Man-Whitney test p=0.034, mean rank=14.63 vs. 22.00; median=192.45 vs. 203.10, for AAs and EAs, respectively). F. Means plot from ANOVA of CA20 by TNBCType. For all bar graphs, error bars reflect +/- 2X standard error. Open circles in boxplots represent outliers with >1.5X of the interquartile range.

Figure 4.6 Induction of CA in TNBC in vitro and expression of proteins involved in disease progression.

Western blots using MDA-MB-468 and MDA-MB-231 TNBC cell lines of proteins associated with A. angiogenesis and B. EMT. “C” denotes untreated cells (controls) and “T” denotes cells treated with aphidocolin for 72 h at 25 µM.
4.7 References


5 CENTROSOLE AMPLIFICATION AS A NOVEL BIOMARKER OF QUADRUPLE NEGATIVE BREAST CANCER DISEASE

5.1 Abstract

Centrosome amplification (CA), a key property of malignant tumor cells, has emerged as a marker of breast cancer (BC) aggressiveness that is highly associated with triple-negative BC (TNBC). Although controversial, the absence of androgen receptor (AR) expression in TNBC, or quadruple negative breast cancer (QNBC), has been reported to confer a more aggressive disease course. However, the role of CA in QNBC and the mechanisms by which CA may be driving aggressive QNBC behavior remains under-studied. Using confocal microscopy, we quantitatively compared numerical CA profiles in a panel of AR-positive and AR-negative TNBC cell lines. We then compared CA20 - an established signature of 20 genes implicated in CA – between QNBC and TNBC patients and evaluated the prognostic value of CA20 in multivariate Cox models of disease-free survival (DFS) using public gene expression data. To discern mechanisms by which CA may fuel aggressive QNBC behavior, we induced CA in a QNBC and TNBC cell line with aphidicolin and assessed aggressive BC biomarkers. Numerical CA was higher in AR-negative than AR-positive TNBC in in vitro analyses, findings that were substantiated by gene expression studies of CA20 in patient BCs. Furthermore, high CA20 as a continuous variable predicted worse DFS in early-stage TNBC patients in adjusted analysis (p=0.03, hazard ratio=1.20). Induction of CA in TNBC cell lines upregulated expression of markers of BC stem cells and the DNA damage response, suggesting that CA may promote TNBC aggressiveness through these mechanisms. Collectively, these findings support an association between numerical CA and QNBC disease and poorer DFS among early-stage TNBC patients. Our study also reveals novel mechanisms by which
CA may promote aggressive QNBC behavior. Hence, our work suggests CA as a potential risk-predictive biomarker for QNBC patients that may also represent a viable therapeutic target.

5.2 Introduction

TNBC accounts for approximately 10-30% of invasive BC cases in the U.S. It is a unique BC subtype that lacks expression of the estrogen and progesterone receptors and amplification of the human epidermal growth factor receptor 2 (HER2/neu). The absence of these targets eliminates conventional endocrine therapy and HER2-targeted systemic treatments as therapeutic options and traditional chemotherapy is unable to eradicate most TNBCs. The disease is characterized by an aggressive clinical course, evidenced by its greater metastatic propensity, more unfavorable clinico-pathological characteristics upon presentation, and higher inter-patient and intratumor heterogeneity compared with non-TNBCs [1-3]. The disease is significantly more prevalent among premenopausal women and predominantly afflicts women of African ancestry who, according to some studies, experience a more aggressive disease course and poorer clinical outcomes than EA TNBCs [1, 4, 5]. Currently, no reliable biomarkers or effective targeted treatments exist for TNBC thus, patients often suffer abysmal outcomes. Thus, alternative risk-prognostic and therapeutic targets are urgently needed to successfully manage the disease.

Androgen receptor (AR) has recently emerged as a promising alternative therapeutic target for TNBC patients. AR is expressed in approximately 10-43% of TNBCs depending on the threshold of positivity used[6]. Knockdown and inhibition of AR with enzalutamide among AR-expressing TNBCs resulted in a reduction in proliferation, migration, invasion and an increase in apoptosis[7-9]. Also, Traina and colleagues observed that enzaluatmide elicited a clinical benefit rate (CBR) of 25% at 24 weeks and a median progression-free survival (PFS) of 14.7 weeks among AR-positive TNBC patients but a CBR of only 20% at 24 weeks and a median PFS of 12.6 weeks
among patients with less AR expression in a nonrandomized phase II clinical trial (unpublished data). The AR antagonist, bicalutamide, also performed well in a phase II clinical trial as the drug elicited a CBR of 19% at 24 weeks and a median PFS of 12 weeks among AR-positive TNBC patients[10]. However, the remaining 76-90% of TNBCs that lack expression of AR, often referred to as quadruple negative breast cancer (QNBC), may not be as sensitive to these novel AR-targeted agents. Furthermore, some studies have reported that a lack of AR expression in TNBC confers a more aggressive disease course[11-16]. Moreover, a lack of AR expression has been observed to be more prevalent among TNBC patients of African compared to European ancestry[17]. Thus, this subgroup of TNBC patients are even more at a disadvantage than AR-positive TNBC patients and are in urgent need for alternative biomarkers and treatment options.

CA is a hallmark of BC that causes ITH by fostering erroneous mitoses, which drive genetic instability and the generation of diverse karyotypes [18, 19]. It is well established that CA is associated with BC progression and imparts aggressive phenotypes, thus representing a possible anti-cancer target [20-25]. Emerging evidence suggest that TNBCs harbor greater incidence and severity of CA than non-TNBCs and that CA underlies an aggressive disease course among TNBCs [26, 27]. However, the role of CA in QNBC and it’s a potential as an alternative biomarker and therapeutic target in the disease remains elusive. In this study, we investigate the potential prognostic and therapeutic value of CA in QNBC and mechanisms by which CA may promote QNBC progression, which could be exploited therapeutically.

5.3 Results

5.3.1 Numerical CA is higher in AR-negative compared to AR-positive TNBC cell lines

As previously mentioned, a subset of TNBCs lack expression of AR and are classified as AR-negative or QNBCs, which exhibit a more basal-like molecular subtype, are more common
among individuals of African ancestry, are not susceptible to AR-targeted therapy, and are characterized by poor DFS [41-43]. Hence, we were interested to determine if QNBCs exhibit more numerical CA than TNBCs (representative micrographs in Figure 1A). We compared numerical CA between QNBC and TNBC cell lines and found that numerical CA was higher in QNBC cell lines (p=0.050) (Figure 1B). We also found that QNBC cell lines exhibited a greater proportion of cells with ≥5 centrosomes than TNBC cell lines (p=0.037) (Figure 1C). We also tested whether numerical CA differs between QNBCs, TNBCs, and non-TNBCs and uncovered a non-significant trend towards increasing numerical CA with decreasing hormone receptor expression (p=0.10) (Figure 1D). We found that the percentage of cells with or ≥5 centrosomes increased with decreasing hormone receptor expression as well (p=0.039) (Figure 1E). The effects of AR signaling on centrosome homeostasis in BC are unknown, but our study suggests AR loss is associated with CA in TNBC.

5.3.2 CA20 is higher in AR-low compared to AR-high expressing TNBCs

Previously, our group developed a transcriptional signature that may reflect CA and correlates with chromosomal instability and worse prognosis in multivariable models, referred to as the CA20 score, which is the sum of the normalized expression of 20 genes whose dysregulation causes CA [1]. To confirm our in vitro findings, we used publicly available microarray data to compute CA20 scores. TCGA breast dataset includes annotation about the variables of interest; thus, we queried it using Oncomine [2] to obtain normalized expression values for the CA20 genes, which were summed as previously described to derive CA20 scores [1].

CA20 was higher in AR-low than AR-high non-TNBC cases (p=0.002) (Figure 3A; Table 1). Thus, amongst non-TNBCs, CA20 is higher in more aggressive subtypes. We also discovered that CA20 was higher in the AR-low group among TNBCs when the 10th percentile was used
(p=0.013, mean rank=56.88 vs. 36.32) (Figure 3B). This finding supports our in vitro results of higher CA being associated with QNBC.

5.3.3 High CA20 predicts poorer survival among early stage TNBC patients

Finally, we were interested in testing the ability of CA20 to stratify early-stage TNBCs regardless of subtype in terms of DFS. Among early-stage TNBCs, higher CA20 score (continuous) was associated with worse DFS in univariate analysis (p=0.040, HR=1.16) and multivariate analysis (p=0.013, HR=1.23) (Table 2) with the exception of age at diagnosis (p=0.11). The QNBC patient sample size was too small to analyze for survival. Thus, higher CA20 score (continuous) may offer reliable prognostic information among early-stage TNBC patients regardless of commonly considered clinicopathologic and demographic information. We suspected categorizing based on mean CA20 score among early-stage TNBCs was not ideal because TNBCs have very high CA20 scores (relative to non-TNBCs), and the threshold at which these high levels confer poor prognosis may be lower than the mean value among TNBCs.

5.3.4 CA promotes aggressiveness in QNBC in vitro

CA has long been associated with chromosomal instability, aneuploidy, and mitotic spindle abnormalities [45]. Greater centrosomal aberrations have recently been associated with enhanced cell migration in BC patient specimens as well as in cultured cell lines [46]. However, the precise role of CA in TNBC and QNBC remains elusive. We induced CA in the TNBC cell line, MDA-MB-468, and the QNBC cell line, MDA-MB-231, by arresting the cells in S-phase with aphidocolin treatment and immunoblotted each cell line for markers of stem cells (i.e. CD44, ALDHA1, Integrin-α) and DNA damage (PARP, p53, RAD51, p21) to investigate the role of CA in AR-low and AR-high expressing TNBC cell lines (Figure 4). Upregulation of CA was confirmed through immunoblotting for centrosome structural proteins and CA markers for which
we observed ≥4-fold increased expression. Interestingly, in the MDA-MB-468 cell line there was 
~2-fold increase in expression of stem cell markers (Fig 4A) and ~2.3 fold increase in expression 
of DNA damage markers (Fig 4B). In MDA-MD-231 cells there was a ~4-fold increase in DNA 
damage markers. The results suggest a potential role of CA in TNBC aggressiveness via this 
mechanism. Our results also suggest more robust upregulation of stem cell, angiogenesis, and 
metastasis markers upon induction of CA in the TNBC cell line, MDA-MB-468, compared with 
the QNBC cell line, MDA-MB-231. Conversely, there was notably more upregulation of DNA 
damage markers observed in the QNBC compared to the TNBC cell line.

5.4 Discussion

The recent emergence of QNBC disease has left this novel subgroup of TNBCs with an 
even more limited availability of prognostic biomarkers and therapeutic strategies than AR-
positive TNBC. Although, controversial, some studies suggest that lack of AR expression confers 
a more aggressive disease course which underscores the need for (a) novel, alternative biomarkers 
that can predict the risk of a more aggressive disease course and (b) new and robust therapeutic 
targets to slow or prevent disease progression in QNBC patients. Our study investigated the role 
of CA in QNBC to support the viability of CA as a potential risk-predictive biomarker and 
therapeutic target in QNBC. Indeed, our group recently reported discordance in CA between 
patient tumors and patient-derived cancer cell lines [29]. Thus, to corroborate our in vitro work, 
we analyzed patient tumor data as well.

Our study is the first to compare centrosome profiles in TNBCs based on AR status, 
uncovering more extensive and severe CA in the AR-negative subgroup.

The precise mechanisms in which CA may be promoting aggressiveness in QNBC remain 
elusive. To gain deeper insights into how CA may be contributing aggressive disease in QNBC,
we induced CA in MDA-MB-231 and MDA-MB-468 QNBC and TNBC cell lines, respectively and evaluated the effect on various markers of BC aggressiveness. Interestingly, upon induction of CA we observed a significant upregulation of stem cell, angiogenesis, DNA damage, and EMT markers in both cell lines, suggesting a role of CA in upregulating these processes in TNBC. Our work is consistent with previous literature reporting a link between CA and DNA damage in BC.

As previously mentioned, several studies have suggested that CA drives chromosomal instability to foster tumor progression [45, 48, 49]. Aberrant function of tumor suppressor genes involved in the DNA repair pathway, such as p53 and BRCA1/2, have been associated with amplified centrosomes and subsequent erroneous cell division in BC cell lines [18, 19, 45]. Our results also corroborate our recent findings revealing that CA strongly correlates with expression of the metastasis marker, vimentin, in breast tumor tissue samples, as well as with enhanced migration and invasion in TNBC in vitro [30]. Denu et. al recently observed a decrease in CD24 expression and increase in CD44 expression upon induction of CA in normal breast epithelial cells by PLK4 overexpression[27]. However, our work is also the first to provide evidence of a direct association of CA and key stem cell and angiogenesis molecules in TNBC cells suggesting novel mechanisms excessive centrosomes may be employing to promote tumor progression in TNBC. Approximately 30% of TNBCs are chemosensitive and achieve a complete pathological response to neoadjuvant chemotherapy, however the remaining 70% of TNBC patients often succumb to residual disease and relapse, which underlies their poorer clinical outcomes compared with other subtypes of BC patients [50, 51]. Cancer stem cells have been implicated in drug resistance and relapse from chemotherapy in BC [52-56]; thus, upregulation of stem cell markers upon induction of CA in TNBC cell lines may reveal a potential role of CA in chemoresistance in TNBC. Further validation of a link between CA and stem cell generation may support screening of early-stage TNBC patients
for centrosomal status to allow for early risk-prediction of patient response to chemotherapy. An early detection of an unfavorable response to chemotherapy may prompt clinicians to recommend the addition of CA-targeting agents to chemotherapy regimens to elicit a more robust response and prevent TNBC patients from experiencing relapse. However, DNA damage markers were notably more regulated in the QNBC compared to the TNBC cell line suggesting the DNA damage response pathway may be particularly more active in QNBC tumors.

CA is a cancer-cell specific trait that distinguishes them from normal, healthy cells [57]. Thus, targeting CA offers a minimally cytotoxic therapeutic strategy that selectivity targets cancer cells burdened with severe centrosomal aberrations. Conveniently, there are already rationally designed drugs that selectively kill cancer cells harboring CA and are currently under clinical evaluation as promising anti-cancer therapeutics. These novel agents include putative centrosome declustering drugs such as griseofulvin and noscapine, commercially available HSET inhibitors such as CW069 and AZ82, and poly-ADP ribose polymerase inhibitors such as PJ34 and GF-15 [58-63]. Hence, these agents may aid in abrogating an aggressive disease course in QNBC and BL1 TNBC patients in addition to platinum-based drugs. Furthermore, non-invasive methods that can detect centrosomal status (i.e. fine-needle aspirate cytology, immunohistochemistry) may demonstrate feasibility in the clinic for selecting QNBC patients that will exhibit susceptibility to these agents. Thus, assessing CA in the clinic may provide a novel strategy to stratify QNBC patients into high- and low-risk groups for an aggressive disease course and allow for optimization of treatment plans. Thus, these agents may serve as a novel alternative therapeutic target for TNBC patients exempt from receiving AR-targeted therapy. However, future studies evaluating the role of CA in QNBC patient specimens with accompanying patient clinico-pathological variables and clinical outcomes would be critical to establishing CA as a robust risk-predictive and prognostic
biomarker for QNBC patients.

5.5 Methods

5.5.1 BC cell lines

All cell lines, including TNBC cell lines (MDA-MB-231, MDA-MB-468, HCC1143, HCC1937, HCC38, HCC70, HCC1806, DU4475, MFM-223, BT-549, BT-20) and non-TNBC (MCF-7, T47D) cell lines, were procured from American Type Cell Culture (ATCC) and cultured according to ATCC recommendations.

5.5.2 Cell lysate preparation and immunoblotting

Cell lysates were collected after cells reached ~80% confluence and lysed with 1x lysis buffer (Cell Signaling) as previously described [28]. Protein concentration in cell lysates was determined using Bradford Protein Assay (BioRad) and spectrometry. Cell lysates were resolved in 10% SDS-PAGE gels as previously described [29]. SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) was used to visualize the bands. PARP, Hif-1α, and E-cadherin primary antibodies were obtained from BD Biosciences. Cyclin-E, Centrin-2, VEGF, Vimentin, uPAR, RAD51, MMP9, p53, and β-actin primary antibodies were obtained from Santa Cruz Biotech. γ-tubulin, PLK4, pericentrin, integrin-α2, MMP2, and ALDHA1 primary antibodies were obtained from Abcam. p21 and CD44 antibodies were obtained from Cell Signaling. Secondary antibodies were obtained from Abcam. Protein expression was semi-quantitated using densitometry with the ImageJ Software and normalized to β-actin.

5.5.3 Immunofluorescence staining

Cells were seeded onto glass coverslips. After reaching ~80% confluence, cells were fixed in ice-cold methanol. Coverslips were blocked in 2% bovine serum albumin (BSA) in 1x PBS and 0.05% Triton X-100 at room temperature for 1 h. Coverslips were incubated in primary antibodies
for γ-tubulin (Abcam) and α-tubulin (Sigma-Aldrich) diluted in blocking buffer at 1:1000 for 45 min in 37°C. Coverslips were incubated in Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary antibodies (Invitrogen) diluted in blocking buffer at 1:3000 for 40 min in 37°C. Primary and secondary antibodies were washed from cover slips with 1x PBS. Cover slips were incubated in Hoechst (Life Technologies) at room temperature for 10 min and mounted with Prolong Gold Antifade Reagent (Invitrogen). DU4475 is a suspension cell line and thus, was not assessed for numerical CA in this study.

5.5.4 Numerical CA Quantitation

Cells were imaged with the Zeiss LSC 700 confocal microscope using 63x objective (Oberkochen, Germany). All images were processed with Zen software (Oberkochen, Germany). Approximately 250-300 randomly selected cells were counted per cell line. The percentage of cells exhibiting numerical CA (i.e., >2 centrosomes) and multipolar mitoses (i.e., mitotic spindles with >2 poles) were quantitated in each cell line. In addition, the severity of CA was assessed by quantitating the percentage of cells with 3, 4, or ≥5 centrosomes in each cell line.

5.5.5 Cell viability assay

QNBC and TNBC cell lines were treated with griseofulvin (Sigma-Aldrich). Sensitivities of cell lines to griseofulvin were assessed through MTT assay as previously described[30]. IC50 values were computed using GraphPad Prism Software Inc. Griseofulvin concentrations versus percentage of cell survival was plotted for each cell line using GraphPad Prism Software, Inc.

5.5.6 Induction of CA

Each cell line was seeded in duplicate. After reaching ~70% confluence, cells were treated with 25 µM aphidicolin from Nigrospora sphaerica (Sigma-Aldrich) for 72 h to induce CA through S-phase arrest. Cells treated with solvent containing no drug were used as a negative
Cell lysates were collected after 24-36 h. Induction of CA was confirmed through immunoblotting for centrosomal proteins (γ-tubulin, centrin-2, and pericentrin) and CA markers (Cyclin-E, PLK4).

5.5.7 Statistical analysis of in vitro data

A one-tailed student t-test or one-way ANOVA was performed for analyses comparing 2 or >2 groups, respectively. Results with p<0.05 were considered statistically significant.

5.5.8 CA20 analyses

Datasets: To determine whether CA20 differs between QNBC and TNBC patients, we queried the Cancer Genome Atlas (TCGA) breast dataset [31] using Oncomine [32] to obtain normalized expression values of CA20 genes, which were summed as previously described to derive CA20 scores [33]. Metastases, normal breast samples, male breast cancers, and ductal carcinomas in situ were excluded, resulting in n=524 cases. Publically available ER/PR/HER2/AR IHC data was used to determine TNBC status. The Yale TNBC dataset [37] was downloaded from GEO (accession GSE46581) [35].

Statistical analysis: Levene’s test was used to assess equality of variances between groups. The Shapiro-Wilk test was used to assess whether samples derived from normally distributed populations. CA20 was compared between groups using parametric (independent-samples, 2-tailed t-test or one-way ANOVA) or non-parametric (Mann-Whitney or Kruskall-Wallis tests) depending on satisfaction of test assumptions. For significant ANOVA tests, Tukey’s HSD post-hoc testing was performed. The association between categorical variables (race, nodal status, and TNBC) was assessed using Fisher’s exact test due to the small sample size of TNBCs. The relationship between CA20 and AR expression was found to be non-monotonic based on examination of scatterplots; thus, AR groups were defined based on expression levels. DFS was
computed as the time in months from initial treatment to the event (recurrence or progression) or last follow-up for censored cases. We tested the impact of high CA20 (continuous or categorized based on the mean) on DFS among early-stage (i.e., AJCC stage I/II) TNBC patients in univariate and multivariate Cox models adjusted for self-reported race and other potentially confounding factors, first ensuring non-violation of the proportional hazards assumption for all covariates by entering each one as a time-dependent covariate in univariate Cox models. Univariate Cox models of DFS were fit including CA20 (continuous) or CA20 (mean as a cutpoint) for early-stage BC patients of all subtypes. In addition, multivariate Cox models were fit entering relevant clinicopathologic and demographic covariates, including ER, PR, and HER2 statuses; nodal status (+/-); tumor size (2 cm cutpoint); PAM50 type (Normal-Like, Luminal A, Luminal B, HER2-enriched, or Basal-Like); AR status (10th percentile cutpoint); and age at diagnosis (continuous), subject to backward stepwise elimination if p≥0.10. Covariates with p<0.10 in univariate Cox models were entered into multivariate models. Observed power was computed using α=0.05. IBM SPSS v. 21 was used and results with p<0.05 were considered statistically significant.

5.6 References
6 TUMOR-INfiltrating lymphocytes as risk-prognostic biomarker among early-stage african-american triple negative breast cancer patients

6.1 Abstract

Triple negative breast cancer (TNBC) is defined by a lack of pharmacologically-targetable breast cancer biomarkers and disproportionately afflicts and affects African-American (AA) women. Tumor-infiltrating lymphocytes (TILs) have been associated with better clinical outcomes, response to neoadjuvant chemotherapy (NAC) but more aggressive breast tumor features. Among formalin-fixed, paraffin-embedded resection samples, we compared stromal and peripheral TILs between early-stage AA and EA TNBC patients at Emory University Hospital in Atlanta, GA. We also compared proportions of infiltrating immune subsets and performed differential gene expression analyses between AA and EA TNBC patients in a publically available gene expression dataset. Among early-stage (I-II) patients (N=103), we observed more stromal TILs among AAs (N=71) compared to EAs (N=32) (p=0.02). Among early-stage AA patients, stromal TILs correlated negatively with age at diagnosis (ρ=-0.25; p=0.03) and androgen receptor expression (ρ=-0.26; p=0.04) and positively with BRCA1-associated protein (ρ=0.30; p=0.02) and programmed cell death protein 1 (ρ=0.56; p<0.0001). High peripheral TIL count predicted better 10-year overall survival (p=0.045; hazard ratio (HR): 0.27; 95% CI:0.08-0.97) and 10-year disease-free survival (p=0.027; HR:0.30; 95% CI:0.10-0.87) among early-stage AA patients in multivariate analyses. In silico analyses revealed greater proportions of T regulatory cells (p=0.046) among AA compared to EA TNBC samples as well as more upregulation of proinflammatory genes, gene ontologies, and biological pathways reflecting cytokine activity, lymphocyte differentiation, and immune effector processes among AA patients. Our findings
uncover previously unrecognized racial disparities in the tumor immune microenvironment among triple negative breast tumors and suggests that TILs may serve as a promising risk-prognostic biomarker for early-stage AA TNBC patients.

6.2 Introduction

Triple negative breast cancer (TNBC) is a highly aggressive breast cancer (BC) subtype that lacks expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) (1). Therefore, TNBC patients do not benefit from conventional endocrine and HER2-targeted systemic treatments leaving chemotherapy and radiation as the mainstay of treatments (2, 3). African-American (AA) women are 2-3 times more prone to developing TNBC and experiencing onset of the disease at a younger age compared to European-American (EA) women (4, 5). TNBC patients of African descent also exhibit more unfavorable clinico-pathological tumor characteristics, acquire more aggressive TNBC subtypes, and display poorer clinical outcomes compared to TNBC patients of European descent (2, 5-11). Novel, alternative biomarkers are urgently needed to manage an aggressive disease course and reduce mortality rates among this patient population.

Neoplastic transformation induces tumor-infiltrating lymphocytes (TILs) to migrate to the tumor site and control progression (12, 13). TILs that migrate to the tumor stroma are considered stromal TILs and TILs that migrate to the stroma adjacent to the invasive tumor front are considered to be peripheral TILs (14, 15). Stromal TILs have been associated with improved overall survival (OS), increased metastasis-free survival, reduced risk of relapse and reduced distance recurrence in TNBC (16). Furthermore, a high level of stromal TILs has been associated with improved pathological complete response (pCR) following neoadjuvant chemotherapy (NAC), validating TILs as a predictive biomarker in TNBC (15-17). In a non-NAC setting,
peripheral TILs have been associated with longer OS and disease-free survival (DFS) among TNBC patients in multivariate analyses (14). Paradoxically, the presence of TILs has been associated with more aggressive clinico-pathological tumor characteristics in BC such as high grade, high stage, high Ki-67, lymph node metastasis, a triple negative breast tumor phenotype, and young age at diagnosis (17-23). Thus, we were interested in evaluating if there was a disparity in the presence of TILs between AAs and EAs and if TILs can function as a risk-prognostic biomarker among TNBC patients of African ancestry. We compared the presence of stromal and peripheral TILs between AA and EA TNBC patients, in a non-NAC setting, observed at Emory University Hospital (EUH) in Atlanta, GA and assessed the predictive and prognostic value of TILs among these patient populations.

6.3 Results

6.3.1 Clinico-pathological characteristics among racially-distinct TNBC patients

We compared clinico-pathological characteristics between AA (N=87) and EA (N=34) TNBC patients observed at EUH (Table 1). We observed a significant difference in nuclear grade (p=0.04) between AA and EA TNBC patients, with 94.3% of AA and 82.4% of EA patients classified as grade 3. A significant difference was observed in mitotic index (p=0.0001) between the races wherein which 78.2% of AAs exhibited a score of 3 compared to 38.2% of EAs. We also observed significantly higher Nottingham grade in AA compared to EA TNBC patients (p=0.008), in which 85.1% of AAs were diagnosed at grade 3 compared to 58.8% of EAs. These findings corroborate previous studies reporting higher grade and mitotic index among AA compared to EA TNBCs. We also compared BC biomarker expression between AA and EA TNBC patients (Table 2). We observed significant differences in retinoic acid receptor-α (RARα) (p=0.02) and β-catenin (p=0.01) between the racial groups in which 50.6% of AAs and 67.7% of EAs were positive for
RARα expression and 80.5% of AAs and 50% of EAs were positive for β-catenin expression. Elevated levels of RARα have been associated with more ER-positive compared to ER-negative BCs (32). β-catenin has been shown to be required for tumorigenesis of TNBC cells through promoting migration, stemness, anchorage-independent growth, and chemosensitivity (33). Like TILs, β-catenin has been shown to be more upregulated in TNBCs compared to non-TNBCs and both nuclear and cytoplasmic β-catenin expression levels were recently found to positively correlate with stromal lymphocytic infiltration in BC, suggesting that Wnt/β-catenin signaling may be playing a critical role in BC anti-tumoral immunity (23). Thus, differential expression of these markers observed between AA and EA TNBCs may be playing a role in the disparity in clinical outcomes between the racial groups.

6.3.2 AAs harbor more TILs than EAs among early-stage TNBC patients

We analyzed differences in the presence of stromal and peripheral TILs between AA (N=87) and EA (N=34) TNBC patients (Figure 1). We discovered a trend of higher levels of stromal TILs in AAs compared to EAs among all TNBC patients (p=0.06) and significantly higher among non-adjuvant chemotherapy treated AA compared to EA patients (p=0.01) (Fig 1A). Interestingly, among early-stage (I-II) (N=103) TNBC patients, AAs (N=71) harbored significantly more stromal TILs than EAs (N=32) (p=0.02) however, not among late-stage (III-IIIC) (N=18) TNBC patients (p=0.62) (Fig 1B,C). Among non-adjuvant chemotherapy treated early-stage TNBC patients, stromal TILs were also higher in AAs compared to EAs (p=0.01) (Fig 1B). Among radiation and non-radiation treated early-stage TNBC patients, we observed a trend of high levels of stromal TILs in AAs than EAs (p=0.07) (Fig 1B). We did not observe significant differences in peripheral TILs between the races (data not shown).
6.3.3 *TILs are associated with more aggressive disease features among early-stage AA TNBC patients*

We examined associations between stromal TILs (Table 3) and peripheral TILs (Table 4) with demographic variables and clinico-pathological parameters among racially-distinct early-stage TNBC patients to elucidate the prognostic role of TILs among these patient populations. Among treated and non-treated early-stage AA and EA patients, stromal and peripheral positively correlated with aggressive clinico-pathological features such as higher tubule formation, nuclear grade, mitotic index, Ki-67 and Nottingham grade (p<0.05). However, among early-stage AA patients, stromal and peripheral TILs negatively correlated with age at diagnosis (ρ=-0.25; p=0.03 and ρ=-0.30; p=0.01, respectively) and among early-stage EA patients, high levels of stromal TILs were associated with increased lymph node positivity (ρ=0.43; p=0.02) and greater total lymph node involvement (ρ=0.45; p=0.01).

To glean additional insight into the role of TILs in TNBC, we examined if TILs are associated with biomarkers routinely assessed among BC patients in the clinic. Correlations of stromal and peripheral TILs with breast clinico-pathological biomarker expression among racially-distinct TNBC patients can be found in Tables 5 and 6, respectively. Among early-stage AA patients, stromal TILs negatively correlated with AR expression (ρ=-0.25; p=0.04) and positively correlated with PD-1 (ρ=0.56; p<0.0001) and BAP1 (ρ=0.3; p=0.02). However, among early-stage EA TNBC patients, stromal TILs positively correlated with BAP1 (ρ=0.44; p=0.03), PD-1 (ρ=0.43; p=0.02), nuclear HSET (ρ=0.50; p=0.02), and EGFR (ρ=0.58; p=0.004) expression. Similar associations were also observed with peripheral TILs except among adjuvant chemotherapy treated early-stage AA patients, peripheral TILs negatively correlated with protein regulator of cytokinesis 1 (PRC1) expression (ρ=-0.28; p=0.05) and among non-radiation treated
early-stage AA patients, peripheral TILs positively correlated with human epidermal growth factor receptor 4 (HER4) expression ($\rho=0.43; p=0.02$).

6.3.4 **High TIL levels predict better survival among early-stage AA TNBC patients**

We next investigated if the presence of TILs is associated with better or worse survival among all and racially-distinct TNBC patients. TILs were stratified into high and low subgroups and Kaplan-Meier analyses were performed to determine the impact of TILs on 10-year OS (Figure 2). We also assessed unadjusted and adjusted associations of peripheral TILs (Table 7) with 10-year OS using Cox proportional hazard regression models. Stromal TILs were unable to significantly predict OS (data not shown). However, we discovered that high peripheral TIL levels predicted better 10-year OS among all patients as well as among radiation treated patients after adjusting for age, Nottingham grade, and stage ($p=0.019$; hazard ratio (HR): 0.344; 95% CI:0.141-0.838 and $p=0.043$; HR:0.284; 95% CI:0.084-0.963, respectively). Among all AA patients, high peripheral TIL levels also predicted significantly longer 10-year OS in Kaplan-Meier analysis ($p=0.007$) (Fig 2A) and after controlling for age, Nottingham grade, and stage ($p=0.003$; HR:0.229; 95% CI:0.084-0.622). High peripheral TIL levels also predicted longer 10-year OS among adjuvant chemotherapy ($p=0.015$; HR:0.230; 95% CI:0.070-0.755) and radiation ($p=0.014$; HR:0.167; 95% CI:0.040-0.697) treated AA patients irrespective of age, Nottingham grade, and stage and in Kaplan-Meier analyses ($p<0.05$) (Fig 2B,C). Among early-stage AA patients, high peripheral TIL levels predicted better 10-year OS after adjusting for age, Nottingham, and stage ($p=0.045$; HR:0.273; 95% CI:0.077-0.969) and in Kaplan-Meier analyses ($p=0.05$) (Fig 2D). Furthermore, high peripheral TIL levels predicted better 10-year OS among non-adjuvant chemotherapy treated early-stage AA patients in multivariate ($p=0.041$; HR:0.031; 95% CI:0.001-0.865) and Kaplan-Meier analyses ($p=0.006$) (Fig 2E). High peripheral TIL levels also predicted
better 10-year OS among non-radiation treated early-stage AA patients in Kaplan-Meier analyses (p=0.04) (Fig 2F). Peripheral TILs were unable to significantly predict OS among EA TNBC patients (data not shown).

We also performed Kaplan-Meier analyses and computed Cox proportional hazard regression models to determine the impact of stromal and peripheral TIL levels on DFS over a 5- and 10-year period among TNBC patients. High stromal (Table 8) and peripheral (Table 9) TIL levels were able to predict significantly better 10-year DFS among radiated-treated AA patients in multivariate analyses (p=0.015; HR:0.230; 95% CI:0.070-0.755 and p=0.029; HR:0.31; 95% CI:0.11-0.89, respectively). High peripheral TIL levels were able to predict better 10-year DFS among all AA patients in multivariate analyses (p=0.035; HR:0.36; 95% CI:0.14-0.93) (Table 13). Among early-stage AA patients, high peripheral TIL levels were able to predict better 10-year DFS (p=0.027; HR:0.30; 95% CI:0.10-0.87) as well as among radiation-treated early-stage AA patients (p=0.022; HR:0.259; 95% CI:0.08-0.823) (Table 13). Our Kaplan-Meier analyses reveal that high peripheral TIL levels were able to predict significantly better 5-year DFS among radiation-treated early-stage AA patients (p=0.047) (Figure 3). Peripheral TILs were unable to significantly predict DFS among EA TNBC patients (data not shown).

6.3.5 Distinctions in TIL subsets between AA and EA TNBC patients

TILs represent a heterogeneous population of immune cells harboring pro-tumorigenic and anti-tumorigenic properties. To glean deeper insight into distinctions in the types of infiltrating immune cells present in the tumor microenvironment among racially-distinct triple negative breast tumors, we utilized the CYBERSORT tool and LM22 to estimate the relative proportions of 22 immune cell phenotypes among gene expression profiles from AA (N=41) and EA (N=87) TNBC patient tissue specimens in TCGA dataset and analyzed for significant differences between the
races (Figure 4). Interestingly, we observed AAs harbored a significantly higher proportion of T regulatory cells (Tregs) ($p=0.046$) and lower proportion of M2 macrophages ($p=0.04$) than EAs among TNBCs (Fig 4A). We also observed that AAs harbored a weakly significantly lower proportion of naïve B cells ($p=0.08$) and weakly significantly higher proportion of memory B cells ($p=0.09$) than EAs among TNBC samples (Fig 4B).

### 6.3.6 Differentially expressed immune-related genes, pathways and gene ontologies between AA and EA TNBC patients

We also investigated differential expression of immune-related genes, pathways, and gene ontologies between AA compared to EA TNBC patients isolated from TCGA breast dataset. Utilizing the DESeq2 software tool, we analyzed differences in expression of 15,942 genes between AA ($N=41$) and EA ($N=87$) TNBC patients in TCGA breast dataset. We observed that resistin (RETN), triggering receptor expressed on myeloid cells like 4 (TREML4), C-C motif chemokine ligand 3 like 1 (CCL3L1), and leukocyte associated immunoglobulin like receptor 2 (LAIR2) immune-related genes were significantly more upregulated among AA compared to EA TNBC patients ($p<0.00001$) (Figure 5). Log$_2$ fold changes can be found in Table 10. RETN and TREM-4 genes were expressed with a log$_2$ fold change of almost 2 times higher among AA compared to EA TNBCs. We also utilized the GAGE and Pathview packages to analyze differences in biological pathways or experimentally-derived differential expression sets and gene ontologies, respectively between AA and EA TNBCs in TCGA breast dataset. We observed significantly more upregulation of the proinflammatory expression sets, intestinal immune network for IgA production ($p=0.006$) and hematopoietic cell lineage ($p=0.028$), in AA compared to EA TNBC patients (Table 11). We also discovered significantly more upregulation of the proinflammatory gene ontologies hematopoietic or lymphoid organ development, hemopoiesis,
leukocyte differentiation, immune effector process, lymphocyte differentiation, and immune system development among AA compared to EA TNBC patients \( (p<0.001) \) (Table 12). Significant downregulation of immune-related genes and processes were not observed among AA compared to EA TNBCs (data not shown).

### 6.4 Discussion

TNBC remains a formidable challenge for clinicians worldwide, underscoring the urgent need for more promising pharmacologically-targetable and/or risk-predictive biomarkers. AA women are even more at a disadvantage as they exhibit notably higher TNBC incidence and mortality rates than women of other ethnicities. The advent of cancer immunology and studies investigating the role of the tumor microenvironment in tumor progression has birthed an array of alternative and promising immunological targets that may enhance clinical risk-prognostication and serve as viable therapeutic options for TNBC patients.

Our group discovered that stromal TILs were significantly higher in AAs compared to EAs among early-stage but not among late-stage TNBC patients suggesting racial disparities in TILs exist in the early-stages of the disease and a greater presence of TILs may be associated with African ancestry. This finding is consistent with previous studies reporting that TILs are associated with more aggressive breast tumor phenotypes such as TNBC status, advanced grade and stage, lymph node metastasis, and younger age at diagnosis, which are features typically associated with BC patients of African descent. Among early-stage AA TNBC patients, stromal and peripheral TILs were associated with younger age at diagnosis and aggressive tumor characteristics such as high nuclear grade, mitotic index, tubule formation, and Nottingham grade, which may rationalize younger age at diagnosis and a more aggressive disease course among AA compared to EA TNBC patients. Ma et. al reported that CD8+ T cells strongly correlated with stromal TIL in BC
suggested that CD8+ T cells, in particular, may be more prevalent among AA compared to EA TNBCs, although further investigation is needed (23).

Interestingly, we discovered that stromal and peripheral TILs were associated with lack of AR expression among early-stage AA TNBC patients suggesting that the increased presence of TILs may be associated with their higher prevalence of quadruple negative breast cancer (QNBC) among AA compared to EA TNBC patients (11). Stromal and peripheral TILs also positively correlated with PD-1 among early-stage AA TNBC patients. A high TIL count has been predictive of a positive response to anti-PD-1 or anti-PD-L1 antibodies (16). Hence, anti-PD-1/PD-L1 therapy may be beneficial for AA TNBC patients with high TIL counts and TILs may serve as a strong predictive biomarker for immune checkpoint therapy response for AA patients. Stromal and peripheral TILs also positively correlated with BAP1 among early-stage AA and EA TNBC patients suggesting a link between an anti-tumor inflammatory/immune response and DNA damage. Thus, TILs may also serve as a predictive biomarker for response to platinum-based agents such as cisplatin and carboplatin. Among early-stage AA TNBC patients, high peripheral TIL levels predicted longer 10-year OS in Kaplan-Meier analyses and after controlling for age, Nottingham grade, and stage suggesting that a high peripheral TIL count may be predictive of a better prognosis. We also observed that high peripheral TIL levels predicted longer 10-year OS among adjuvant chemotherapy- and radiation- treated AA TNBC patients and longer 5-year DFS among radiation-treated early-stage AA patients, suggesting peripheral TILs may be able to predict response to treatment in AA patients. Thus, the administration of adoptive cell therapy using TILs to TNBC patients of African ancestry harboring low levels of lymphocytic infiltration may be beneficial in improving their survival rates. A combination of TIL immunotherapy and PD-1/PD-L1 checkpoint blockade therapy may elicit a more robust response.
Our *in silico* analyses revealed a trend of lower proportions of naïve B cells and higher proportions of memory B cells in AA compared to EA TNBCs corroborating our *in vivo* findings of increased lymphocytic infiltration among AA TNBC patients. Furthermore, we observed significantly lower proportions of M2 macrophages in AA compared to EA TNBCs, although previous studies have reported higher counts of tumor-associated macrophages of a M2 phenotype in AA compared to EA breast tumors (34-36). M2 macrophages are immunosuppressive and implicated in interfering with the anti-tumoral immune response thus, our finding further suggest increased anti-tumor immune activity in AA compared to EA TNBCs (37). However, interestingly we observed a significantly greater proportion of Tregs among AA compared to EA TNBCs suggesting increased immunosuppressive activity among TNBC patients of African ancestry. Ma and colleagues recently showed that FOXP3+ Tregs strongly correlated with stromal TILs, and particularly CD8+ T cells, in BC suggesting that our observation of increased lymphocytic infiltration among AA compared to EA triple negative breast tumors may be associated with their higher levels of Tregs observed in our study (23). Perhaps a greater presence of suppressive T cells could be counteracting a greater presence of antitumoral TILs, still subjecting AAs to a worse clinical prognosis. Further investigation is warranted to determine how differences in the composition of the tumor immune microenvironments among racially-distinct TNBCs could be underlying the disparate clinical outcomes observed among AA patients.

Further *in silico* analyses by our group revealed that the proinflammatory genes, RETN, TREM-4, CCL3L1, and LAIR2, were significantly more upregulated in AA compared to EA TNBCs. RETN and TREM-4 genes were expressed almost 2 folds higher among AA compared to EA TNBCs. Stewart and colleagues also reported that RETN was expressed 2.25 folds higher in AA compared to EA BC patients in TCGA dataset (38). Martin *et. al* observed C-X-C motif
chemokine 10 and 11 being expressed 5.96 and 1.96 folds higher, respectively in AA compared to EA BC patients (34). We also observed more upregulation of proinflammatory KEGG pathways among AAs compared to EAs such as the intestinal immune network for IgA production and hematopoietic cell lineage as well as key proinflammatory gene ontologies such as lymphoid organ development, leukocyte and lymphocyte differentiation, hemopoiesis, and immune effector process.

Our study is the first to uncover a previously unrecognized disparity in an important member of the tumor immune microenvironment, TILs, between racially distinct TNBC patients and encourages further investigation of racial disparities in TILs among these patient populations. Our work provides valuable risk-prognostic information of TILs to better guide clinicians in the optimization of treatment paths for early-stage AA TNBC patients. These findings support the need for increased evaluation of TILs in the clinic for early-stage AA TNBC patients and suggest immunotherapy could be exploited as an effective therapeutic strategy for TNBC patients of African ancestry with low lymphocytic infiltration. However, robust validation of our findings in additional non-NAC and NAC treated patient cohorts as well as more characterization of immune cell subsets among racially-distinct triple negative breast tumors will be critical to achieving these aims.

6.5 Methods

6.5.1 Study Cohort

We analyzed a cohort of 121 TNBC patients treated at EUH in Atlanta, GA from 2002 (initial day of diagnosis) to 2016 (last day of contact). The percentage of stromal and peripheral TILs was assessed in hematoxylin- and eosin- stained patient specimens as described by Krishnamurti and colleagues (14). Stromal TILs were evaluated according to the International
TILs working group 2014 in which the percentage of stromal tissues occupied by infiltrating lymphocytes and plasma cells were estimated (24). Intratumoral lymphocytes, or lymphocytes in direct contact with tumor cells, were excluded from the evaluation as no patients in this study underwent NAC. Peripheral TILs were also evaluated as the percentage of stromal lymphocytes occupying the entire circumference of the invasive tumor front with a width of roughly one dozen tumor cells (14). The percentage of stromal and peripheral TILs were estimated in intervals including <5%, 5-10%, 11-50% and >50%. Patient demographic characteristics, clinicopathological variables, and BC biomarker status were recorded for each patient. Demographic characteristics include self-reported race and age at the time of diagnosis. This article referred to the 7th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM Classification and Stage groupings for BC (25). Expression of all BC biomarkers investigated in this study was determined through immunohistochemical (IHC) staining and scored as Hscore (percentage x intensity), Nper (nuclear percentage) and Nhscore (nuclear percentage x intensity). Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Information on patient treatment was recorded including adjuvant chemotherapy and radiation therapy.

6.5.2 Follow Up

Initial diagnosis and follow-up of patients occurred between 2002 and 2016. Initial dates of diagnoses, treatment start and completion dates, and last dates of contact were recorded for each patient. Survival status (alive/dead) was also recorded for each patient in addition to survival time. The date of last follow-up for the last patient seen is March 3, 2016.
6.5.3 Infiltrating Immune Cell Composition and Differential Gene Expression Determination

We queried The Cancer Genome Atlas (TCGA) breast dataset from the TCGA portal in Oncomine for all TNBC patients (26, 27). Publicly-available ER/PR/HER2 IHC data was used to determine TNBC status. We utilized the Cell type Identification By Estimating Relative Subsets of known RNA Transcripts (CIBERSORT) tool, which is a deconvolution algorithm that uses a set of reference gene expression values, to resolve intratumoral immune cell composition in gene expression data from tumor samples of mixed cell types (28). The Leukocyte gene signature matrix (LM22), which is comprised of 547 genes that resolves 22 human hematopoietic cell phenotypes [seven T cell types, naïve and memory B cells, plasma cells, natural killer (NK) cells, and myeloid subsets] in tumor samples, was used to identify proportions of each immune cell type in TNBC patients based on their gene expression profiles in the TCGA dataset (28). To avoid false positives, gene expression data was processed using 1000 permutations. As the input data was normalized, the quantile normalization option was disabled. According to the available race data, we selected 128 AA and EA samples out of 145 TNBC samples. We performed differential gene expression analysis between the ethnic groups using the DESeq2 software tool (29). Differentially expressed genes with a \( p \) value of \(<0.05\) and log2fold change of above +1 and below -1 were selected in this study. Differential expression analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontologies between the ethnic groups were predicted by GAGE and Pathview packages, respectively (30, 31). Heat maps were generated to depict the relative proportion of select immune cells and differentially expressed genes among each patient group of interest.

6.5.4 Statistical Analysis

The significance level for all analyses was \( p<0.05 \) with 95% confidence intervals (CIs).
Chi-square tests were performed to analyze differences in demographic characteristics, breast clinico-pathological variables and biomarker expression, as well as treatment information between AA and EA TNBC patients. SAS 9.4 program was used to generate test statistics and 2-tailed univariate p-values were reported. Wilcoxon rank-sum tests were performed to determine differences in the means of stromal and peripheral TILs between AA and EA patient populations. Pearson correlation coefficients ($\rho$) were computed to determine associations between stromal and peripheral TILs with demographic and breast clinico-pathological variables and biomarker expression among AA and EA TNBC patients. Spearman’s rank correlation coefficients ($\rho$) were computed for categorical covariates. Unadjusted and adjusted multivariate Cox proportional hazard regression models were computed to assess the impact of TILs on 10-year OS and DFS before and after controlling for age, Nottingham grade, and stage. The Kaplan-Meier analysis was conducted using SAS 9.4 program to estimate survival function for AA and EA TNBC patients over a 5- or 10- year period based on high and low TIL levels. A log-rank test was used to stratify stromal and peripheral TILs into high ($\geq 10$) and low ($<10$) groups to evaluate associations of a high and low presence of TILs with better or worse survival among racially-distinct TNBC patients. Wilcoxon rank-sum tests were performed to determine significant differences in the proportions of immune cell fractions between AA and EA TNBCs after generating CYBERSORT outputs. $P$-value cut-offs were not used. The Wald test was used to test for significance among differentially expressed genes between the racial groups using the DESeq2 software tool.

6.6 Tables and Figures
Table 6.1 Clinico-pathological characteristics of AA and EA TNBC patients observed at EUH
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*Abbreviations: AA, African American; EA, European American; T, tumor size; N, lymph node metastasis; M, distant metastasis.
*p values were calculated using the chi-square test.
Significant p values in table are in bold font.
Table 6.2 Breast cancer biomarker expression among AA and EA TNBC patients observed at EUH

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<td>12</td>
</tr>
<tr>
<td>Positive</td>
<td>39</td>
<td>44.83</td>
<td>15</td>
</tr>
<tr>
<td>Missing/Unknown</td>
<td>11</td>
<td>12.64</td>
<td>7</td>
</tr>
</tbody>
</table>

Abbreviations: AA, African-American; EA, European-American; AR, Androgen Receptor; FOXM1, Forkhead box 1 protein; PRC1, protein regulator of cytokinesis 1; BAK1, Retinoic acid receptor α; BAP1, BRCA1 associated protein 1; PD1, Programmed cell death protein 1; EGFR, epidermal growth factor receptor; HER3, human epidermal growth factor receptor 3; HER4, human epidermal growth factor receptor 4; Hscore (percentage x intensity); Nper (nuclear percentage), and Nhscore (nuclear percentage x intensity); N/A, non-applicable. *P values were calculated using the chi-square test. Significant p values in table are in bold font.
Table 6.3 Correlation of stromal TILs with demographic and clinico-pathological variables among early-stage racially-distinct TNBC patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Clinico-pathological variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age*</td>
</tr>
<tr>
<td>Early-stage AA</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage AA Chemo</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage AA Non-Chemo</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage AA Rad</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage AA Non-Rad</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage EA</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage EA Chemo</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage EA Non-Chemo</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage EA Rad</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Early-stage EA Non-Rad</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>p value</td>
</tr>
</tbody>
</table>

*Pearson correlation test (p)

*Spearman’s rank correlation test (p)

Abbreviations: Chemo, adjuvant chemotherapy; Rad, radiation; LN, lymph node; Pos, positive; Tot, total; Nott, Nottingham. Missing values are attributed to an insufficient number of patients to analyze.

Significant p values in table are in bold font.
Table 6.4 Correlation of peripheral TILs with demographic and clinico-pathological variables among early-stage racially-distinct TNBC patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age(^a)</th>
<th>Nuclear grade(^b)</th>
<th>Mitotic index(^b)</th>
<th>Nott. grade(^b)</th>
<th>Ki67%(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-stage AA</td>
<td>p</td>
<td>-0.3</td>
<td>0.12</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.01</td>
<td>0.31</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Early-stage AA Chemo</td>
<td>p</td>
<td>-0.28</td>
<td>0.13</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.03</td>
<td>0.3</td>
<td>0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>Early-stage AA Non-Chemo</td>
<td>p</td>
<td>-0.51</td>
<td>0.34</td>
<td>0.48</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.02</td>
<td>0.13</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Early-stage AA Radiation</td>
<td>p</td>
<td>-0.25</td>
<td>0.04</td>
<td>0.16</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.09</td>
<td>0.78</td>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>Early-stage AA Non-Rad</td>
<td>p</td>
<td>-0.44</td>
<td>0.37</td>
<td>0.36</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Early-stage EA</td>
<td>p</td>
<td>-0.32</td>
<td>0.4</td>
<td>0.48</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.07</td>
<td>0.02</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Early-stage EA Chemo</td>
<td>p</td>
<td>-0.34</td>
<td>0.5</td>
<td>0.42</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.07</td>
<td>0.01</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Early-stage EA Non-Chemo</td>
<td>p</td>
<td>-0.49</td>
<td>0.63</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>Early-stage EA Rad</td>
<td>p</td>
<td>-0.44</td>
<td>0.24</td>
<td>0.41</td>
<td>0.47</td>
</tr>
<tr>
<td>Early-stage EA Non-Rad</td>
<td>p</td>
<td>-0.13</td>
<td>0.74</td>
<td>0.8</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.73</td>
<td>0.01</td>
<td>0.07</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(^a\)Pearson correlation test (p)  
\(^b\)Spearman’s rank correlation test (p)  
Abbreviations: Chemo, adjuvant chemotherapy; Rad, radiation; Nott, Nottingham.  
Significant p values in table are in bold font.


Table 6.5 Correlations of stromal TILs with clinico-pathological biomarkers among early-stage racially-distinct TNBC patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>AR</th>
<th>HSET</th>
<th>EGFR</th>
<th>BAP1</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-stage AA</td>
<td>-0.255</td>
<td>-0.023</td>
<td>0.049</td>
<td>0.296</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.044</strong></td>
<td><strong>0.87</strong></td>
<td><strong>0.715</strong></td>
<td><strong>0.022</strong></td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>Early-stage AA Chemo</td>
<td>-0.258</td>
<td>-0.018</td>
<td>0.055</td>
<td>0.284</td>
<td>0.589</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.053</strong></td>
<td><strong>0.902</strong></td>
<td><strong>0.704</strong></td>
<td><strong>0.038</strong></td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>Early-stage AA Non-Chemo</td>
<td>-0.35</td>
<td>0.023</td>
<td>0.023</td>
<td>0.376</td>
<td>0.445</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.131</strong></td>
<td><strong>0.931</strong></td>
<td><strong>0.929</strong></td>
<td><strong>0.113</strong></td>
<td><strong>0.043</strong></td>
</tr>
<tr>
<td>Early-stage AA Rad</td>
<td>-0.191</td>
<td>0.036</td>
<td>-0.007</td>
<td>0.35</td>
<td>0.705</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.32</strong></td>
<td><strong>0.865</strong></td>
<td><strong>0.973</strong></td>
<td><strong>0.068</strong></td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>Early-stage AA Non-Rad</td>
<td>-0.235</td>
<td>0.084</td>
<td>0.018</td>
<td>0.314</td>
<td>0.672</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.134</strong></td>
<td><strong>0.623</strong></td>
<td><strong>0.917</strong></td>
<td><strong>0.048</strong></td>
<td><strong>&lt;.0001</strong></td>
</tr>
<tr>
<td>Early-stage EA</td>
<td>-0.223</td>
<td>0.495</td>
<td>0.576</td>
<td>0.445</td>
<td>0.427</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.284</strong></td>
<td><strong>0.019</strong></td>
<td><strong>0.004</strong></td>
<td><strong>0.03</strong></td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td>Early-stage EA Chemo</td>
<td>-0.164</td>
<td>0.473</td>
<td>0.575</td>
<td>0.394</td>
<td>0.412</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.454</strong></td>
<td><strong>0.03</strong></td>
<td><strong>0.005</strong></td>
<td><strong>0.07</strong></td>
<td><strong>0.026</strong></td>
</tr>
<tr>
<td>Early-stage EA Non-Chemo</td>
<td>-0.818</td>
<td>0.749</td>
<td>-0.669</td>
<td>0.34</td>
<td>0.305</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.09</strong></td>
<td><strong>0.145</strong></td>
<td><strong>0.217</strong></td>
<td><strong>0.455</strong></td>
<td><strong>0.425</strong></td>
</tr>
<tr>
<td>Early-stage EA Rad</td>
<td>-0.219</td>
<td>0.634</td>
<td>0.695</td>
<td>0.551</td>
<td>0.512</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.399</strong></td>
<td><strong>0.006</strong></td>
<td><strong>0.002</strong></td>
<td><strong>0.018</strong></td>
<td><strong>0.013</strong></td>
</tr>
<tr>
<td>Early-stage EA Non-Rad</td>
<td>-0.106</td>
<td>-0.318</td>
<td>0.123</td>
<td>0.479</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td><strong>0.802</strong></td>
<td><strong>0.602</strong></td>
<td><strong>0.817</strong></td>
<td><strong>0.337</strong></td>
<td><strong>0.048</strong></td>
</tr>
</tbody>
</table>

Abbreviations: ρ, pearson correlation coefficient; Chemo, adjuvant chemotherapy; Rad, radiation.

Significant ρ values in table are in bold font.
Table 6.6 Correlation of peripheral TILs with clinico-pathological biomarkers among racially-distinct TNBC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>AR</th>
<th>HSET</th>
<th>PRC1</th>
<th>BAP1</th>
<th>PD-1</th>
<th>HER4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-stage AA</td>
<td>-0.241</td>
<td>-0.063</td>
<td>-0.268</td>
<td>0.262</td>
<td>0.323</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>0.057</td>
<td>0.666</td>
<td>0.05</td>
<td>0.044</td>
<td>0.006</td>
<td>0.817</td>
</tr>
<tr>
<td>Early-stage AA Chemo</td>
<td>-0.263</td>
<td>-0.051</td>
<td>-0.284</td>
<td>0.271</td>
<td>0.35</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>0.048</td>
<td>0.732</td>
<td>0.051</td>
<td>0.047</td>
<td>0.004</td>
<td>0.673</td>
</tr>
<tr>
<td>Early-stage AA Non-Chemo</td>
<td>-0.364</td>
<td>0.068</td>
<td>-0.016</td>
<td>0.368</td>
<td>0.212</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>0.115</td>
<td>0.825</td>
<td>0.947</td>
<td>0.121</td>
<td>0.355</td>
<td>0.8</td>
</tr>
<tr>
<td>Early-stage AA Rad</td>
<td>-0.273</td>
<td>0.104</td>
<td>-0.26</td>
<td>0.264</td>
<td>0.432</td>
<td>-0.067</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.54</td>
<td>0.12</td>
<td>0.1</td>
<td>0.002</td>
<td>0.671</td>
</tr>
<tr>
<td>Early-stage AA Non-Rad</td>
<td>-0.263</td>
<td>-0.215</td>
<td>-0.286</td>
<td>0.324</td>
<td>0.125</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td>0.133</td>
<td>0.271</td>
<td>0.133</td>
<td>0.071</td>
<td>0.454</td>
<td>0.015</td>
</tr>
<tr>
<td>Early-stage EA</td>
<td>-0.295</td>
<td>0.572</td>
<td>-0.005</td>
<td>0.431</td>
<td>0.317</td>
<td>-0.133</td>
</tr>
<tr>
<td></td>
<td>0.153</td>
<td>0.005</td>
<td>0.983</td>
<td>0.036</td>
<td>0.077</td>
<td>0.517</td>
</tr>
<tr>
<td>Early-stage EA Chemo</td>
<td>-0.216</td>
<td>0.549</td>
<td>0.003</td>
<td>0.361</td>
<td>0.307</td>
<td>-0.179</td>
</tr>
<tr>
<td></td>
<td>0.323</td>
<td>0.01</td>
<td>0.989</td>
<td>0.099</td>
<td>0.105</td>
<td>0.403</td>
</tr>
<tr>
<td>Early-stage EA Non-Chemo</td>
<td>-0.645</td>
<td>0.64</td>
<td>-0.368</td>
<td>0.548</td>
<td>0.668</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>0.245</td>
<td>0.542</td>
<td>0.203</td>
<td>0.049</td>
<td>0.97</td>
</tr>
<tr>
<td>Early-stage EA Rad</td>
<td>-0.287</td>
<td>0.646</td>
<td>0.009</td>
<td>0.463</td>
<td>0.293</td>
<td>-0.063</td>
</tr>
<tr>
<td></td>
<td>0.264</td>
<td>0.005</td>
<td>0.971</td>
<td>0.053</td>
<td>0.174</td>
<td>0.805</td>
</tr>
<tr>
<td>Early-stage EA Non-Rad</td>
<td>-0.326</td>
<td>0.444</td>
<td>-</td>
<td>0.373</td>
<td>0.339</td>
<td>-0.292</td>
</tr>
<tr>
<td></td>
<td>0.431</td>
<td>0.454</td>
<td>-</td>
<td>0.41</td>
<td>0.337</td>
<td>0.482</td>
</tr>
</tbody>
</table>

Abbreviations: ρ, pearson correlation coefficient; Chemo, adjuvant chemotherapy; Rad, radiation. Missing values are attributed to an insufficient number of patients to analyze. Significant p values in table are in bold font.
Table 6.7 Prediction of peripheral TILs on 10-year OS among racially-distinct TNBC patients.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>p-value; HR (95% CI)</th>
<th>p-value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.1159; 0.518 (0.228, 1.176)</td>
<td>0.0189; 0.344 (0.141, 0.838)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.2790; 0.598 (0.236, 1.517)</td>
<td>0.0879; 0.403 (0.142, 1.144)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.2097; 0.422 (0.110, 1.624)</td>
<td>0.2426; 0.368 (0.069, 1.968)</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.1277; 0.428 (0.144, 1.275)</td>
<td>0.0434; 0.284 (0.084, 0.963)</td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.1761; 0.463 (0.152, 1.412)</td>
<td>0.1682; 0.380 (0.096, 1.505)</td>
<td></td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.3981; 0.633 (0.219, 1.828)</td>
<td>0.2861; 0.545 (0.179, 1.662)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.8389; 0.880 (0.258, 3.009)</td>
<td>0.9041; 0.923 (0.249, 3.415)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.1023; 0.234 (0.041, 1.336)</td>
<td>0.0887; 0.200 (0.031, 1.276)</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.4018; 0.504 (0.102, 2.499)</td>
<td>0.3716; 0.464 (0.086, 2.498)</td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.1227; 0.367 (0.103, 1.310)</td>
<td>0.1574; 0.371 (0.094, 1.467)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value; HR (95% CI)</td>
<td>p-value; HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.0106; 0.303 (0.121, 0.757)</td>
<td>0.0026; 0.229 (0.084, 0.622)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.0387; 0.326 (0.113, 0.994)</td>
<td>0.0154; 0.230 (0.070, 0.755)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.0449; 0.218 (0.049, 0.966)</td>
<td>0.0818; 0.147 (0.017, 1.274)</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.0228; 0.239 (0.070, 0.819)</td>
<td>0.0141; 0.167 (0.040, 0.697)</td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.0965; 0.358 (0.107, 1.202)</td>
<td>0.1399; 0.301 (0.061, 1.483)</td>
<td></td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.0653; 0.326 (0.099, 1.074)</td>
<td>0.0445; 0.273 (0.077, 0.969)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.2313; 0.428 (0.107, 1.716)</td>
<td>0.265; 0.429 (0.097, 1.900)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.0249; 0.075 (0.008, 0.721)</td>
<td>0.0408; 0.031 (0.001, 0.865)</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.1344; 0.255 (0.042, 1.527)</td>
<td>0.1159; 0.208 (0.029, 1.473)</td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.0524; 0.235 (0.054, 1.015)</td>
<td>0.1199; 0.283 (0.058, 1.389)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage.
Peripheral TILs were treated as a categorical variable.

Significant p values in table are in bold font.
Table 6.8 Prediction of stromal TILs on 10-year DFS among racially-distinct TNBC patients.
<table>
<thead>
<tr>
<th>All patients</th>
<th>p-value; HR (95% CI)</th>
<th>p-value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Unadjusted model</strong></td>
<td><strong>Adjusted model</strong></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.399; 0.58 (0.17, 2.02)</td>
<td>0.079; 0.31 (0.08, 1.14)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.253; 0.42 (0.09, 1.86)</td>
<td>0.093; 0.27 (0.05, 1.24)</td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.637; 1.78 (0.16, 19.6)</td>
<td>0.575; 0.40 (0.01, 9.62)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.189; 0.36 (0.08, 1.63)</td>
<td>0.047; 0.21 (0.04, 0.98)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.566; 2.01 (0.18, 22.2)</td>
<td>0.748; 0.59 (0.02, 13.9)</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.155; 0.22 (0.03, 1.75)</td>
<td>0.0607; 0.139 (0.018, 1.093)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.175; 0.24 (0.03, 1.88)</td>
<td>0.098; 0.17 (0.02, 1.38)</td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.997; 0.00 (-)</td>
<td>0.998; 0.00 (-)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.126; 0.20 (0.02, 1.56)</td>
<td>0.0546; 0.131 (0.016, 1.041)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.997; 0.00 (-)</td>
<td>0.997; 0.00 (-)</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value; HR (95% CI)</td>
<td>p-value; HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Unadjusted model</td>
<td>Adjusted model</td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.522; 0.65 (0.18, 2.36)</td>
<td>0.154; 0.38 (0.10, 1.42)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.3610; 0.493 (0.108, 2.250)</td>
<td>0.1968; 0.363 (0.078, 1.692)</td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.5600; 2.281 (0.142, 36.51)</td>
<td>1.000; 0.02 (-)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.298; 0.44 (0.09, 2.05)</td>
<td>0.123; 0.29 (0.06, 1.39)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.733; 1.51 (0.13, 16.7)</td>
<td>0.669; 0.51 (0.02, 11.0)</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.1779; 0.243 (0.031, 1.902)</td>
<td>0.097; 0.17 (0.02, 1.37)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.215; 0.27 (0.03, 2.14)</td>
<td>0.175; 0.23 (0.02, 1.90)</td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.997; 0.00 (-)</td>
<td>1.000; 0.11 (-)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.152; 0.21 (0.02, 1.75)</td>
<td>0.113; 0.18 (0.02, 1.49)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.997; 0.00 (-)</td>
<td>0.997; 0.00 (-)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. Overall TILs were treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.
Table 6.9 Prediction of peripheral TILs on 10-year DFS among racially-distinct TNBC patients.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$p$-value; HR (95% CI)</td>
<td>$p$-value; HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
<td></td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.330; 0.63 (0.24, 1.59)</td>
<td></td>
<td><strong>0.035; 0.36 (0.14, 0.93)</strong></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.484; 0.69 (0.24, 1.94)</td>
<td></td>
<td>0.164; 0.47 (0.17, 1.35)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.342; 0.31 (0.02, 3.45)</td>
<td></td>
<td>0.997; 0.00 ( - )</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.341; 0.61 (0.22, 1.68)</td>
<td></td>
<td><strong>0.029; 0.31 (0.11, 0.89)</strong></td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.949; 0.92 (0.08, 10.1)</td>
<td></td>
<td>0.334; 0.25 (0.01, 4.17)</td>
<td></td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.184; 0.49 (0.17, 1.40)</td>
<td></td>
<td><strong>0.027; 0.30 (0.10, 0.87)</strong></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.393; 0.60 (0.19, 1.91)</td>
<td></td>
<td>0.195; 0.46 (0.14, 1.48)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.997; 0.00 ( - )</td>
<td></td>
<td>0.998; 0.00 ( - )</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.2176; 0.490 (0.158, 1.523)</td>
<td></td>
<td><strong>0.0219; 0.259 (0.082, 0.823)</strong></td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.615; 0.49 (0.03, 7.85)</td>
<td></td>
<td>0.3801; 0.281 (0.016, 4.793)</td>
<td></td>
</tr>
<tr>
<td><strong>AA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$-value; HR (95% CI)</td>
<td>$p$-value; HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
<td></td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.308; 0.57 (0.20, 1.66)</td>
<td></td>
<td>0.111; 0.42 (0.14, 1.22)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.343; 0.57 (0.18, 1.80)</td>
<td></td>
<td>0.302; 0.54 (0.17, 1.72)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.607; 0.48 (0.03, 7.72)</td>
<td></td>
<td>1.000; 0.00 ( - )</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.245; 0.49 (0.15, 1.62)</td>
<td></td>
<td>0.135; 0.39 (0.11, 1.34)</td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.936; 0.90 (0.08, 9.99)</td>
<td></td>
<td>0.386; 0.29 (0.01, 4.74)</td>
<td></td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.195; 0.45 (0.13, 1.49)</td>
<td></td>
<td>0.120; 0.38 (0.11, 1.28)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.2856; 0.501 (0.141, 1.781)</td>
<td></td>
<td>0.4096; 0.584 (0.162, 2.099)</td>
<td></td>
</tr>
<tr>
<td>Non-Chemotherapy</td>
<td>0.998; 0.00 ( - )</td>
<td></td>
<td>1.000; 0.00 ( - )</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.102; 0.33 (0.08, 1.24)</td>
<td></td>
<td><strong>0.1144; 0.292 (0.063, 1.346)</strong></td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.613; 0.48 (0.03, 7.82)</td>
<td></td>
<td>0.427; 0.32 (0.01, 5.30)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. Overall TILs were treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.

Significant $p$ values and corresponding HR and confidence intervals in table are in bold font.
Table 6.10 Immune-related genes upregulated in AA compared to EA TNBC patients in TCGA dataset.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>log2FoldChange</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETN</td>
<td>resistin</td>
<td>1.890840792</td>
<td>4.97E-10</td>
</tr>
<tr>
<td>TREML4</td>
<td>triggering receptor expressed on myeloid cells like 4</td>
<td>1.677117556</td>
<td>2.34E-09</td>
</tr>
<tr>
<td>CCL3L1</td>
<td>C-C motif chemokine ligand 3 like 1</td>
<td>1.356316644</td>
<td>1.50E-06</td>
</tr>
<tr>
<td>LAIR2</td>
<td>leukocyte associated immunoglobulin like receptor 2</td>
<td>1.238761571</td>
<td>4.96E-06</td>
</tr>
</tbody>
</table>

Table 6.11 Immune-related pathways upregulated in AA compared to EA TNBC patients in TCGA dataset.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04672 Intestinal immune network for IgA production</td>
<td>0.006</td>
</tr>
<tr>
<td>hsa04640 Hematopoietic cell lineage</td>
<td>0.028</td>
</tr>
</tbody>
</table>
Figure 6.1 Comparison of stromal TILs among racially-distinct TNBC patients.
Comparison of stromal TILs among AA and EA TNBC patients (39), among early-stage AA and EA TNBC patients, (39) and late-stage AA and EA TNBC patients overall and with or without adjuvant chemotherapy (chemo) or radiation (rad) treatment. No record of not having received treatment for late-stage patients. *p<0.05.
Figure 6.2 Peripheral TILs predict OS among early-stage AA TNBC patients.
Peripheral TILs were stratified into high (≥10) and low (<10) subgroups using a log-rank test. Kaplan-Meier curves were generated to estimate impact of high and low peripheral TIL levels on survival over a 10-year period among AA TNBC patients. Prediction of high and low peripherals TILs on 10-year OS among (39) all AA patients, (39) adjuvant chemotherapy-treated AA patients, (39) radiation-treated AA patients, (39) early-stage AA patients, (39) non-adjuvant chemotherapy treated early-stage AA patients and (F) non-radiation treated early-stage AA patients.
Figure 6.3 Peripheral TILs predict DFS among early-stage AA TNBC patients.
Peripheral TILs were stratified into high (≥10) and low (<10) subgroups using a log-rank test. Kaplan-Meier curve was generated to estimate impact of high and low peripheral TIL levels on DFS over a 5-year period among early-stage AA TNBC patients treated with radiation.
Figure 6.4 Comparison of intratumoral immune cell fractions between AA and EA TNBC samples.

CIBERSORT tool was used to determine the proportions of distinct infiltrating immune cells among AA and EA TNBCs isolated from the TCGA breast dataset. No cut-offs for p-values were used. The color gradient from green to red represents the increasing fractions of intratumoral immune cells. (39) Immune cell fractions that showed statistically significant differences between the races with a greater proportion of Tregs and smaller proportion of M2 macrophages among AAs compared to EAs. (39) Immune cell fractions that showed weakly statistically significant differences (0.05>p>0.1) between the races with a smaller proportion of naïve B cells and greater proportion of memory B cells among AAs compared to EAs.
Figure 6.5 Differentially expressed immune-related genes among AA and EA TNBC samples. The DESeq2 tool was used to determine differences in the expression of immune-related genes between AA and EA TNBC patients isolated from the TCGA breast dataset. Genes that were significantly differentially expressed between the racially-distinct TNBC patients (p<0.05) and a log2-fold change of above +1 were included in the heat map to depict the relative expression levels of each gene. The color gradient from red to green represents increasing expression of each gene. RETN (resistin), LAIR2 (leukocyte associated immunoglobulin like receptor 2), TREML4 (triggering receptor expressed on myeloid cells like 4), and CCL3L1 (C-C motif chemokine ligand 3 like 1) genes were found to be significantly more upregulated in AA compared to EA samples (p<0.00001).

6.7 References


7 TUMOR-INFILTRATING LYMPHOCYTES AS RISK-PROGNOSTIC BIOMARKER AMONG EARLY-STAGE QUADRUPLE NEGATIVE BREAST CANCER PATIENTS

7.1 Abstract

Quadruple negative breast cancer (QNBC) is defined by a lack of estrogen and progesterone receptors, human epidermal growth factor receptor 2, and androgen receptor (AR). Some studies suggest the absence of AR expression in triple negative breast cancer (TNBC) confers a more aggressive disease course. We compared stromal and peripheral TILs between early-stage AR-negative and AR-positive TNBC patients at Emory Hospital in Atlanta, GA and analyzed a publically-available gene expression dataset for distinctions in infiltrating immune cell phenotypes between AR- low and high expressing TNBC patents. Among early-stage TNBC patients (N=94), we observed more stromal (p=0.002) and peripheral (p=0.018) TILs in AR-negatives (N=76) compared to AR-positives (N=18). Our in silico results suggest increased antitumoral immune activity among AR-low compared to AR-high expressing TNBCs. Among early-stage AR-negative patients, stromal and peripheral TILs correlated negatively with younger age at diagnosis (p<0.01). High peripheral TILs count was associated with longer 10-year disease-free survival among AR-negative patients in multivariate analyses (p=0.026; HR: 0.062). Our findings uncover previously unrecognized distinctions in the tumor immune microenvironment between AR-negative and AR-positive triple negative breast tumors and suggest that TILs may be a promising, alternative risk-prognostic biomarker that could be exploited therapeutically for early-stage QNBC patients.
7.2 Introduction

Triple negative breast cancer (TNBC) is an aggressive form of breast cancer defined by the absence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2/neu) expression. Thus, TNBC patients do not benefit from conventional endocrine therapy and HER2-targeted systemic treatments as therapeutic options \(^1,2\). Furthermore, anthracycline-taxane based chemotherapy is unable to completely eliminate most triple negative breast tumors. Quadruple negative breast cancers (QNBCs) represent a subgroup of TNBCs that lack androgen receptor expression. Approximately 45-88% of TNBCs lack nuclear AR expression \(^3,4\). Thus, unlike AR-positive TNBCs, QNBC patients are excluded from receiving AR-targeted therapy as an alternative treatment option. Although the prognostic role of AR in TNBC remains controversial, a lack of AR expression has been associated with poor differentiation, high clinical stage, high Ki-67, high mitotic index, increased lymphovascular invasion, increased risk of recurrence and distant metastasis, and worse 5-year disease-free survival (DFS) and overall survival \(^5-9\). Thus, novel, alternative risk-prognostic biomarkers and therapeutic targets are urgently needed to abrogate an aggressive disease course and avert a poor prognosis among this patient population.

Upon neoplastic transformation, tumor-infiltrating lymphocytes migrate to the tumor site to prevent tumor progression \(^10,11\). Stromal TILs reside in the stromal compartment of the tumor, while peripheral TILs occupy the invasive tumor front \(^12,13\). Stromal TILs have been reported to be more prognostic in TNBC than peripheral TILs as stromal TILs have been associated with longer OS, increased metastasis-free survival and reduced distance recurrence as well as improved pathological complete response (pCR) following neoadjuvant chemotherapy in TNBC \(^12,14,15\). In fact, for every 10% increase in stromal TILs, a 15% reduction in relapse risk and 17%
reduction in death risk has been reported. However, recently among TNBC patients not treated with neoadjuvant chemotherapy, peripheral TILs were similarly associated with longer OS and DFS. Paradoxically, an increased presence of stromal TILs have been associated with more aggressive breast tumor features such as high grade, high stage, high Ki-67, lymph node metastasis, a triple negative breast tumor phenotype and younger age at diagnosis. Thus, we were interested in evaluating if there are differences in the presence of stromal and peripheral TILs between AR-negative and AR-positive TNBCs and investigating if stromal and peripheral TILs can function as a risk-predictive and prognostic biomarker for early-stage QNBC patients. We compared TIL counts between AR-negative and AR-positive TNBC patients observed at Emory University Hospital (EH) in Atlanta, GA in a non-neoadjuvant chemotherapy setting and assessed the predictive and prognostic role of TILs among QNBC patients.

7.3 Results

7.3.1 Clinico-pathological characteristics and biomarker expression among AR-negative and AR-positive TNBC patients

We analyzed differences in associations with demographic and clinico-pathological characteristics as well as treatment status between AR-negative (N=89) and AR-positive (N=21) TNBC patients observed at EH (Table 1). We observed a significant difference in associations with nuclear grade between the groups. Among AR-negative patients, 96% presented as grade 3 as opposed to 81% among AR-positive patients (p=0.02). We also observed modest differences in associations with Nottingham grade and radiation therapy administration based on AR status. Among AR-negative patients, 85% presented with a Nottingham grade of 3 while only 71% of AR-positive patients presented with a grade of 3 (p=0.06). Among AR-negative patients, 62% were reported to have received radiation therapy while only 38% of AR-positive patients.
underwent the treatment ($p=0.05$). We also compared differences in associations with breast cancer biomarker expression between AR-negative and AR-positive TNBC patients ($p=0.05$). We observed differences in associations with HSET expression between the patient groups. Among AR-positive patients, 76% were positive for HSET expression as opposed to 82% of AR-negative patients ($p=0.01$). Similarly, modest differences in HER3 expression emerged, where 46% of AR-positive patients were positive for the marker as opposed to 43% of AR-negative patients ($p=0.07$).

**7.3.2 Early-stage AR-negative harbor more TILs than early-stage AR-positive TNBC patients**

We investigated differences in the presence of stromal and peripheral TILs between AR-negative ($N=89$) and AR-positive ($N=21$) TNBC patients (Figure 1). We observed that stromal TIL levels were higher in AR-negative compared to AR-positive TNBC patients ($p=0.001$) as well as among non-adjuvant chemotherapy-treated ($p=0.012$) and radiation-treated ($p=0.011$) TNBC patients (Fig 1A). Stromal TILs were also higher in AR-negative ($N=76$) compared to AR-positive ($N=18$) early-stage (I-II) ($N=94$) TNBC patients ($p=0.002$) as well as among non-adjuvant chemotherapy-treated ($p=0.040$) and radiation-treated ($p=0.049$) early-stage TNBC patients ($p=0.01$) (Fig 1B). We also observed significant differences in peripheral TIL count between AR-negative and positive TNBC patients. Peripheral TIL levels were higher in AR-negative compared to AR-positive TNBC patients ($p=0.017$) as well as among non-adjuvant chemotherapy-treated ($p=0.012$) and radiation-treated ($p=0.025$) TNBC patients (Fig 1C). Peripheral TILs were also higher in AR-negative compared to AR-positive early-stage TNBC patients ($p=0.018$) as well as among non-adjuvant chemotherapy-treated ($p=0.039$) and radiation-treated ($p=0.031$) early-stage TNBC patients (Fig 1D). We observed no differences in stromal or peripheral TIL count among late-stage (III-IIIC) patients.
7.3.3 **TILs are associated with more aggressive disease among AR-negative TNBC patients**

We examined correlations between stromal (Table 3A) and peripheral (Table 3B) TILs with demographic and clinico-pathological variables among AR-negative and AR-positive TNBC patients. Regarding demographic variables, stromal TILs negatively correlated with age at diagnosis among all \( (\rho=-0.325; \ p=0.002) \) and early-stage \( (\rho=-0.320; \ p=0.005) \) AR-negative patients. Stromal TILs positively correlated with tubule formation \( (\rho=0.221; \ p=0.038) \) among AR-negative patients and positively correlated with nuclear grade among early-stage AR-positive patients \( (\rho=0.499; \ p=0.035) \). Peripheral TILs also negatively correlated with age at diagnosis among all \( (\rho=-0.307; \ p=0.003) \) and early-stage \( (\rho=-0.318; \ p=0.005) \) AR-negative patients as well as among early-stage AR-positive patients \( (\rho=-0.495; \ p=0.037) \). Among AR-positive patients, peripheral TILs were associated with high nuclear \( (\rho=0.603; \ p=0.008) \) and Nottingham \( (\rho=0.589; \ p=0.010) \) grades.

To further explore the prognostic role of TILs in QNBC, we analyzed associations of stromal (Table 4A) and peripheral (Table 4B) TILs with expression of common breast cancer biomarkers among AR-negative and AR-positive TNBC patients. Interestingly, stromal TILs positively correlated with BRCA1-associated protein 1 (BAP1) and programmed death cell protein 1 (PD1) expression among all \( (\rho=0.292; \ p=0.011, \ \rho=0.469; \ p<0.0001, \text{ respectively}) \) and early-stage \( (\rho=0.282; \ p=0.024, \ \rho=0.480; \ p<0.0001, \text{ respectively}) \) AR-negative patients. Among AR-positive patients, stromal TILs positively correlated with BAP1 expression \( (\rho=0.531; \ p=0.034) \). Peripheral TILs positively correlated with PD1 among all \( (\rho=0.401; \ p<0.0001) \) and early-stage \( (\rho=0.392; \ p<0.001) \) AR-negative patients as well. However, peripheral TILs positively correlated with forkhead box protein 1 (FOXM1) expression among all AR-negative patients \( (\rho=0.253; \ p=0.018) \) but not among AR-positive patients.
Peripheral TILs also positively correlated with BAP1 expression ($\rho=0.60; \ p=0.02$) among all ($\rho=0.652; \ p=0.006$) and early-stage ($\rho=0.603; \ p=0.017$) AR-positive patients.

7.3.4 High TIL levels were associated with better survival among early-stage AR-negative TNBC patients

We investigated the prognostic power of TILs among AR-negative TNBC patients in both Kaplan-Meier analyses and Cox proportional hazard regression models. We examined associations of stromal and peripheral TILs with DFS among AR-negative patients in Kaplan-Meier analyses (Figure 2). High peripheral TIL levels were associated with significantly longer 10-year DFS among all ($p=0.037$) (Fig 2A) and moderately longer 10-year DFS among early-stage AR-negative patients ($p=0.053$) (Fig 2B). High peripheral TIL count was also associated with longer 10-year DFS among all non-adjuvant chemotherapy-treated ($p=0.001$) (Fig 2C) and early-stage non-adjuvant chemotherapy treated ($p<0.0001$) AR-negative patients (Fig 2D). Furthermore, high peripheral TIL levels were associated with better 10-year DFS among radiation-treated early-stage AR-negative patients ($p=0.044$) (Fig 2E). We did not observe associations of stromal TILs with DFS among AR-negative patients. We also observed associations with peripheral TILs and DFS in univariate and multivariate Cox proportional hazard regression models among AR-negative TNBC patients (Table 5). High peripheral TIL levels were associated with longer 10-year DFS before ($p=0.013$; HR: 0.055 95% CI: 0.005-0.542) and after ($p=0.026$; HR: 0.062 95% CI: 0.005-0.721) adjusting for age at diagnosis and Nottingham grade among non-adjuvant chemotherapy-treated AR-negative TNBC patients.

Peripheral TILs were also associated with OS among AR-negative TNBC patients in Kaplan Meier analysis (Figure 3) and in Cox proportional hazard regression models (Table 6). High peripheral TIL count was associated with longer 10-year OS among all ($p=0.001$) (Fig 3A)
and early-stage \(p<0.0001\) (Fig 3B) non-adjuvant chemotherapy-treated AR-negative patients. We observed similar results in univariate and multivariate analyses in which high peripheral TIL levels were associated with longer 10-year OS among non-adjuvant chemotherapy-treated AR-negative patients before \(p=0.013; \text{HR: 0.055 95\% CI: 0.005-0.542}\) and after \(p=0.026; \text{HR: 0.158 95\% CI: 0.027-0.930}\) adjusting for age at diagnosis and Nottingham grade.

7.3.5 **Distinctions in TIL subsets between aggressive and less aggressive TNBCs**

TILs are comprised of a heterogeneous population of immune cell phenotypes harboring pro-tumorigenic and anti-tumorigenic characteristics. To discern distinctions in the types of infiltrating immune cells present in the tumor microenvironment between AR-negative and AR-positive TNBCs, we used the CYBERSORT tool and LM22 to estimate the relative proportions of 22 immune cell phenotypes among gene expression profiles from AR-low and AR-high expressing TNBC patient tissue specimens in the TCGA dataset and analyzed for significant differences (Figure 4). We observed significantly lower proportions of naïve B cells \(p=0.04\) and resting mast cells \(p=0.048\) among AR-low \(N=16\) compared to AR-high \(N=41\) TNBCs using a p-value cut-off of \(p<0.01\) (Fig 4A). Furthermore, we observed weakly significantly lower proportions of resting natural killer (NK) cells \(p=0.05\) and weakly significantly higher proportions of activated NK cells \(p=0.097\) among AR-low \(N=37\) compared to AR-high \(N=106\) TNBCs without using a p-value cut-off (Fig 4B).

7.4 **Discussion**

TNBC patients have the least amount of targeted treatment options compared to other breast cancer patients. A subgroup of TNBCs that lack expression of AR are even more at a disadvantage as they are often exempt from receiving AR-targeted therapy as opposed to AR-expressing TNBCs. Hence, there is an urgent need for alternative pharmacologically-targetable
and/or risk-predictive biomarkers for this unique group of TNBC patients. However, studies uncovering novel therapeutic targets or biomarkers for QNBC patients remain scarce. Cancer immunotherapy or exploiting intrinsic mechanisms of the tumor-host immune system is rapidly gaining momentum as a promising alternative strategy in eradicating tumors \(^{21}\). The immune-rich TNBC landscape has identified TNBC patients as strong candidates for novel immunotherapeutic intervention \(^{21}\). However, the role of the tumor immune microenvironment in QNBC remains elusive. Xiu and colleagues recently revealed that PD-L1 is overexpressed in QNBC compared to TNBC tumors (unpublished data) and PD-L1 is associated with a high level of TILs \(^{22}\). Hence, we investigated differences in TIL levels between QNBC and TNBC patients in a non-neoadjuvant chemotherapy setting and investigated the role of TILs as a potential, novel risk-prognostic biomarker and therapeutic target for QNBC patients.

We examined differences in associations with demographic, clinico-pathological variables, treatment, and biomarker expression between AR-negative and AR-positive TNBCs. We observed that high nuclear and Nottingham grades (grade 3) was more associated with AR-negative compared to AR-positive status among TNBC patients. This finding corroborates previous studies revealing a lack of AR expression is associated with high-grade tumors. AR-negative status was also modestly more associated with receipt of radiotherapy compared to AR-positive status. Furthermore, positive HSET and HER3 expression was more associated with AR-positivity compared to AR-negativity among TNBC patients. A previous study reported HER4 mRNA expression levels were higher in AR-positive compared to AR-negative TNBCs suggesting expression of EGFR family members may be associated with AR status \(^{23}\).

We observed a significantly higher presence of stromal and peripheral TILs in AR-negatives compared to AR-positives among all and early-stage TNBC patients. This finding is
consistent with a recent study by Davis and colleagues reporting expression of CD4 and CD8 T cell markers as well as expression of key immune checkpoint inhibitors, PD-1, PD-L1, and CTLA-4, were significantly more upregulated among AR-negative compared to AR-positive TNBC patients in TCGA dataset\textsuperscript{24}. Thus, within the highly immunogenic TNBC landscape, the QNBC subgroup may exhibit increased immune signaling activity compared to AR-expressing TNBCs suggesting QNBCs to be even more of an ideal candidate for immunotherapeutic intervention. Furthermore, this finding corroborates previous reports of increased lymphocytic infiltration among more aggressive breast tumor phenotypes and parallels literature reports of TILs being more prevalent among TNBCs compared to non-TNBCs.

We also examined associations of TILs with demographic and clinico-pathological characteristics among AR-negative and AR-positive TNBCs. Stromal and peripheral TILs were associated with younger age at diagnosis and aggressive clinico-pathological characteristics such as higher tubule formation, nuclear grade, and Nottingham grade among all and early-stage AR-negative and AR-positive patients, which is consistent with previous studies, cited earlier, reporting that TILs are associated with younger age at diagnosis and more aggressive disease.

We also analyzed associations of TILs with breast clinico-pathological biomarkers among AR-negative and AR-positive TNBC patients to glean additional insight into the prognostic and mechanistic role of TILs among these patient populations. Both stromal and peripheral TILs were associated with increased PD-1 among QNBC patients. PD-1 is frequently expressed on cytotoxic CD8+ T cells and thus, is associated with high levels of TILs, which is consistent with our findings\textsuperscript{12}. Hence, TILs may serve as a predictive biomarker for response to immune checkpoint therapy for QNBC patients. Stromal TILs were also linked to high BAP1 expression among early-stage AR-negative patients suggesting increased lymphocytic infiltration may be associated with
increased DNA damage response in QNBC patients. BAP1 is a nuclear-localized, ubiquitin carboxy-terminal hydrolase, that binds to the RING finger domain of BRCA1 to facilitate BRCA1-mediated DNA damage response and arrest of breast cancer cell proliferation\textsuperscript{25}. Thus, high TIL levels may also predict positive response to platinum-based agents such as cisplatin and carboplatin among QNBC patients. Peripheral but not stromal TILs were associated with high FOXM1 expression among AR-negative patients. FOXM1 is a transcription factor that regulates cell cycle progression, specifically mitotic division, chromosome segregation and genomic stability\textsuperscript{26,27}. It has been found to be overexpressed in approximately 85\% of TNBCs and its upregulation has been suggested to play a critical role in carcinogenesis through stimulating cell proliferation and tumor metastasis\textsuperscript{28}. Thus, the increased presence of stromal TILs among AR-negative patients may reflect increased aberrant cell division and chromosome segregation errors and may predict a positive response to FOXM1 inhibitors. Among AR-positive patients, high peripheral TIL levels were also linked to increased BAP1 expression or DNA damage suggesting an increased anti-tumor inflammatory response may be associated with genomic instability among TNBC patients irrespective of AR status.

To further explore the role of TILs as a potential prognostic biomarker for QNBC patients, we investigated associations of TIL levels with clinical outcomes among AR-negative patients. We discovered that high peripheral TIL levels were associated with longer DFS and OS among early-stage QNBC patients in Kaplan-Meier and all QNBC patients multivariate analyses, which corroborate previous studies showing that a greater presence of TILs is associated with a better prognosis in TNBC. High peripheral TIL levels were also linked to longer DFS among radiation-treated early-stage AR-negative patients suggesting that TILs may be able to predict response to radiotherapy in QNBC patients.
Our *in silico* analyses discerning distinctions in the proportions of infiltrating immune cell subsets between AR-high and AR-low expressing TNBCs supports our findings of increased anti-tumor immune activity among QNBC patients. We observed significantly lower proportions of naïve B cells and resting mast cells as well as a trend of less resting NK cells and more activated NK cells in AR-low compared to AR-high TNBCs, suggesting increased anti-tumor immune activity in QNBCs compared to TNBCs. These findings corroborate our *in vivo* findings of increased lymphocytic infiltration among AR-negative TNBC patients and support previous reports of a greater presence of immune cell infiltration associated with more aggressive breast cancer. However, further investigation is necessary to unravel the composition of the tumor immune microenvironment of AR-negative TNBC tumors for precise immunotherapeutic intervention.

Our work suggest a novel, alternative risk-predictive and prognostic biomarker for QNBC patients who are insusceptible to AR-targeted treatment as opposed to AR-positive TNBCs. TILs are currently being tested in clinical trials as a therapeutic strategy for TNBC patients\(^2^9\). Adoptive T cell therapy has already shown promising results in clinical trials for melanoma patients as an approximate 50% reduction in tumor size was observed among half of the patients\(^3^0\). Thus, the administration of TIL immunotherapy to QNBC patients harboring low levels of TILs may be beneficial for this patient population and serves as a potential, alternative therapeutic option for this challenging disease. The addition of PD-1/PD-L1 checkpoint blockade therapy to TIL immunotherapy may elicit a more robust response among this patient population. Our study may also encourage the development of novel immunotherapeutic strategies designed to counteract mechanisms that restrict tumor lymphocytic infiltration, which may be advantageous for early-stage QNBC patients. Our work encourages further investigation.
of tumor-host immune interactions among AR-negative TNBCs to uncover more novel immunotherapeutic targets. Conveniently, groups such as the International TILs Working Group in 2014 and the International Immuno-Oncology Biomarker Working Group on Breast Cancer have developed standardized methodologies for evaluating stromal TILs in hematoxylin and eosin (H&E) stained tumor tissue sections as standard routine histopathological practice. These methodologies encourage the implementation of TILs in the clinic as a standard clinicopathological parameter for prediction of patient prognosis. Our study suggests evaluation of TILs should be done routinely in the clinic for AR-negative TNBC patients, who lack other prognostic markers and treatment options. However, our findings were uncovered in a non-neoadjuvant chemotherapy setting which represents a limitation to our study. Validation of our findings in additional non-neoadjuvant chemotherapy and neoadjuvant chemotherapy treated patient cohorts as well as more characterization of immune cell subsets among AR-enriched and non-enriched triple negative breast tumors will be critical, however, to establishing TILs as a robust prognostic biomarker and therapeutic strategy for QNBC patients.

7.5 Methods

7.5.1 Study Cohort

We analyzed a cohort of 121 TNBC patients observed at EH in Atlanta, GA from 2002 (initial day of diagnosis) to 2016 (last day of contact). We obtained approval and informed consent from the Emory University Institutional Review Board to gain access to patient information and samples used in this study and to perform the experiments conducted in this article. We have a human subjects assurance on file. The study was conducted in accordance with relevant guidelines/regulations. The percentage of stromal and peripheral TILs was assessed in hematoxylin- and eosin-stained patient specimens as described by Krishnamurti and colleagues.
Stromal TILs were evaluated according to the International TILs working group 2014 in which the percentage of stromal tissues occupied by infiltrating lymphocytes and plasma cells were estimated. Intratumoral lymphocytes, or lymphocytes in direct contact with tumor cells, were excluded from the evaluation as no patients in this study underwent neoadjuvant chemotherapy. Peripheral TILs were also evaluated as the percentage of stromal lymphocytes occupying the entire circumference of the invasive tumor front with a width of roughly one dozen tumor cells. The percentage of stromal and peripheral TILs were estimated in intervals including <5%, 5-10%, 11-50% and >50%. Patient demographic characteristics, clinico-pathological variables, and breast cancer biomarker status were recorded for each patient. This article referred to the 7th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM Classification and Stage groupings for breast cancer. Demographic characteristics include self-reported race and age at the time of diagnosis. Patient treatment was recorded including adjuvant chemotherapy and radiation therapy.

### 7.5.2 Immunohistochemistry and scoring

Biomarker expression was evaluated through standard immunohistochemical (IHC) staining and scored as Hscore (percentage x intensity), Nper (nuclear percentage) and Nhscore (nuclear percentage x intensity). Intensity of staining were scored as 0=none, 1=low, 2=moderate, and 3=high. No patients in this study underwent neoadjuvant chemotherapy. Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Antibody details and concentrations can be found in Supplementary Table S3. The maximum score for any given H-score is 300. Patient samples were stained, scored, and reviewed at EH. Scoring was performed by two independent pathologists blinded to clinical
annotation and individual scores were averaged. TNBC patients negative or positive for AR (Nper) were classified as AR-negative and AR-positive TNBC patients, respectively.

7.5.3 Follow Up

Initial diagnosis of patients occurred between 2002 and 2016. Median duration of follow-up for EH was approximately 6 years. Initial dates of diagnoses, treatment start and completion dates, and last dates of contact were recorded for each patient. Survival status (alive/dead) was also recorded for available patients in addition to survival time. The date of last follow-up for the last patient seen is March 3, 2016.

7.5.4 Gene Expression Dataset and Infiltrating Immune Cell Composition Determination

We queried the publically available The Cancer Genome Atlas (TCGA) breast dataset from the TCGA portal for all TNBC patients. Publically available ER/PR/HER2/AR Immunohistochemical data was used to determine TNBC/QNBC status and a log-rank test was performed to stratify TNBC patients into AR-low and AR-high expressing subgroups. We utilized the Cell type Identification By Estimating Relative Subsets of known RNA Transcripts (CYBERSORT) tool, which is a deconvolution algorithm that uses a set of reference gene expression values, to resolve intratumoral immune cell composition in gene expression data in tumor samples of mixed cell types. The Leukocyte gene signature matrix (LM22), which is comprised of 547 genes that resolves 22 human hematopoietic cell phenotypes [seven T cell types, naïve and memory B cells, plasma cells, natural killer cells, and myeloid subsets] in tumor samples, was used to identify proportions of each immune cell type in TNBC patients based on their gene expression profiles in the TCGA dataset. To avoid false positives, gene expression data was processed using 1000 permutations. As the input data was normalized, the quantile normalization option was disabled.
7.5.5 **Statistical Analysis**

The significance level for all analyses was $p<0.05$ and 95% confidence intervals estimated. SAS 9.4 program was used to generate test statistics and 2-tailed univariate $p$-values were reported. Chi-square tests were performed to analyze differences in associations with demographic characteristics, breast clinico-pathological variables and biomarkers, as well as treatment information between AR-negative and AR-positive TNBC patients. Wilcoxon rank-sum tests were performed to determine differences in the means of stromal and peripheral TILs between AR-negative and AR-positive TNBC patients. Pearson correlation coefficients ($\rho$) were computed to determine correlations between stromal and peripheral TILs with demographic and clinico-pathological variables as well as biomarker expression among AR-negative and AR-positive TNBC patients. Spearman’s rank correlation coefficients ($\rho$) were generated for categorical variables. Unadjusted and adjusted multivariate Cox proportional hazard models were computed to assess the impact of TILs on 10-year OS and disease-free survival (DFS) before and after controlling for age and Nottingham grade. The Kaplan-Meier analysis was conducted using SAS 9.4 program to estimate survival function for AR-negative TNBC patients over a 10-year period based on high and low TIL levels. A log-rank test was used to stratify stromal and peripheral TILs into high ($\geq 10$) and low (<10) groups to evaluate associations of a high and low presence of TILs with better or worse survival for Kaplan-Meier analyses. Wilcoxon rank-sum tests were performed to determine significant differences in the proportions of immune cell fractions between AR-low and AR-high expressing TNBCs after generating CIBERSORT outputs. A $p$-value cutoff of $<0.01$ was used. A log-rank test was also performed to identify the appropriate cut-off for low and high expression of AR among TNBC patients in the TCGA dataset.
### 7.6 Tables and Figures

**Table 7.1 Clinico-pathological characteristics of AR-negative and AR-positive TNBC patients observed at EH.**

<table>
<thead>
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<th>Clinical characteristic</th>
<th>AR-Negative (n=88)</th>
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*All variables were calculated using the chi-square test.*
Table 7.2 Breast cancer biomarker expression among AR-positive and AR-negative TNBC patients observed at EH.

<table>
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<tr>
<th>Biomarkers</th>
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<td>n</td>
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</tr>
<tr>
<td>PRC1 (Nper)</td>
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<td></td>
<td>0.53</td>
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<tr>
<td>Negative</td>
<td>43</td>
<td>48.81</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
<td>33.7</td>
<td>8</td>
</tr>
<tr>
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<td>17.88</td>
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<td>62.92</td>
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<tr>
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<td>5.62</td>
<td>2</td>
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<tr>
<td>Aurora A (Nscore)</td>
<td></td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
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<tr>
<td>Positive</td>
<td>40</td>
<td>44.44</td>
<td>11</td>
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<tr>
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<tr>
<td>Survivin (Nper)</td>
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<td>3</td>
<td>3.57</td>
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</tr>
<tr>
<td>Positive</td>
<td>71</td>
<td>79.78</td>
<td>18</td>
</tr>
<tr>
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<td>16.55</td>
<td>2</td>
</tr>
<tr>
<td>BAP1 (Nper)</td>
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<td></td>
<td>0.23</td>
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</tr>
<tr>
<td>Positive</td>
<td>71</td>
<td>79.78</td>
<td>16</td>
</tr>
<tr>
<td>Missing/Unknown</td>
<td>14</td>
<td>16.55</td>
<td>5</td>
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<tr>
<td>Positive</td>
<td>71</td>
<td>79.78</td>
<td>18</td>
</tr>
<tr>
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<td>15.73</td>
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<td>Positive</td>
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<td>89.27</td>
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<tr>
<td>Positive</td>
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<tr>
<td>Missing/Unknown</td>
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<td>14.61</td>
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<td>HER2 (Nscore)</td>
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</tr>
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<td>36</td>
<td>41.45</td>
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</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>42.7</td>
<td>10</td>
</tr>
<tr>
<td>Missing/Unknown</td>
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<td>16.55</td>
<td>4</td>
</tr>
<tr>
<td>HER4 (Nscore)</td>
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<td></td>
<td>0.12</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>49.44</td>
<td>7</td>
</tr>
<tr>
<td>Positive</td>
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<td>14</td>
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<tr>
<td>Missing/Unknown</td>
<td>4</td>
<td>4.49</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Abbreviations: AA, African American; EA, European American; AR, Androgen Receptor; FOXM1, Forkhead box 1 protein; PRC1, protein regulator of cytokinesis 1; RARa, Retinoic acid receptor alpha; BAP1, BRCA1-associated protein 1; PD1, Programmed cell death protein 1; TIL, Tumor infiltrating lymphocytes; Nscore, percentage x intensity; Nper, nuclear percentage; Nscore, nuclear percentage x intensity. *P values were calculated using the Chi-square test.
Table 7.3 Antibodies and dilutions used for immunohistochemical biomarker staining at EH.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Subcellular location</th>
<th>Clone</th>
<th>Source</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSET (NH-score)</td>
<td>Nuclear</td>
<td>AB72452</td>
<td>Abcam</td>
<td>polyclonal rabbit</td>
<td>1:4000</td>
</tr>
<tr>
<td>FOXM1 (NPER)</td>
<td>Nuclear</td>
<td>G-5</td>
<td>Santa Cruz</td>
<td>monoclonal mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>AR (NPER)</td>
<td>Nuclear</td>
<td>AR441</td>
<td>ThermoFisher</td>
<td>monoclonal mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>PRC1 (NPER)</td>
<td>Nuclear</td>
<td>H-70</td>
<td>Santa Cruz</td>
<td>polyclonal rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>AURORA A (NH-SCORE)</td>
<td>Nuclear</td>
<td>AB1287</td>
<td>Abcam</td>
<td>polyclonal Rabbit</td>
<td>1:20</td>
</tr>
<tr>
<td>SURVIVIN (NPER)</td>
<td>Nuclear</td>
<td>71G4b7</td>
<td>Cell Signaling</td>
<td>polyclonal Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>BAP1 (NPER)</td>
<td>Nuclear</td>
<td>BAP1(c-4)</td>
<td>Santa Cruz</td>
<td>monoclonal mouse</td>
<td>1:40</td>
</tr>
<tr>
<td>β-CATENIN (H-SCORE)</td>
<td>Nuclear</td>
<td>E-5</td>
<td>Santa Cruz</td>
<td>monoclonal mouse</td>
<td>1:160</td>
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<tr>
<td>PD1 (H-SCORE)</td>
<td>Membrane</td>
<td>NAT105</td>
<td>Abcam</td>
<td>monoclonal mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>RARα (H-SCORE)</td>
<td>Membrane</td>
<td>C-20</td>
<td>Santa Cruz</td>
<td>polyclonal rabbit</td>
<td>1:20</td>
</tr>
<tr>
<td>EGFR (H-SCORE)</td>
<td>Membrane</td>
<td>C31G7</td>
<td>Life technologies</td>
<td>monoclonal mouse</td>
<td>1:25</td>
</tr>
<tr>
<td>HER3 (H-SCORE)</td>
<td>Membrane</td>
<td>DAK-H3-IC</td>
<td>Dako</td>
<td>monoclonal mouse</td>
<td>1:25</td>
</tr>
<tr>
<td>HER4 (H-SCORE)</td>
<td>Cytoplasmic</td>
<td>HFR-1</td>
<td>Abcam</td>
<td>monoclonal mouse</td>
<td>3:1</td>
</tr>
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</table>

**Abbreviations:** FOXM1, Forkhead box 1 protein; PRC1, protein regulator of cytokinesis 1; RARα, Retinoic acid receptor α; BAP1, BRCA1 associated protein 1; PD1, Programmed cell death protein 1; H-score, percentage x intensity; Nper, nuclear percentage; Nh-score, nuclear percentage x intensity; N/A, not applicable.
Table 7.4 Correlation of stromal TILs with demographic and clinico-pathological variables among AR-negative and AR-positive TNBC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stage</th>
<th>Test Statistic</th>
<th>Clinicopathological variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Age&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AR-Neg</td>
<td>Early and Late</td>
<td>$p$</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.002</td>
<td>0.938</td>
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<tr>
<td></td>
<td>Early</td>
<td>$p$</td>
<td>0.32</td>
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<tr>
<td></td>
<td>$p$ value</td>
<td>0.905</td>
<td>0.063</td>
</tr>
<tr>
<td>AR-Pos</td>
<td>Early and Late</td>
<td>$p$</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.136</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>Early</td>
<td>$p$</td>
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<tr>
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<td>$p$ value</td>
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<td>0.249</td>
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Table 7.4B Correlation of peripheral TILs with demographic and clinico-pathological variables among AR-negative and AR-positive TNBC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stage</th>
<th>Test Statistic</th>
<th>Clinicopathological variables</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td>Age&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AR-Neg</td>
<td>Early and Late</td>
<td>$p$</td>
<td>0.307</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
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<td>0.566</td>
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<td></td>
<td>Early</td>
<td>$p$</td>
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</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.505</td>
<td>0.335</td>
</tr>
<tr>
<td>AR-Pos</td>
<td>Early and Late</td>
<td>$p$</td>
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<tr>
<td></td>
<td>$p$ value</td>
<td>0.096</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>Early</td>
<td>$p$</td>
<td>0.485</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.937</td>
<td>0.006</td>
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</table>

<sup>a</sup>Pearson correlation test ($p$).

<sup>b</sup>Spearman's rank correlation test ($p$).
**Table 7.5 Correlation of stromal TILs with clinico-pathological biomarkers among AR-negative and AR-positive TNBC patients.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stage</th>
<th>Test Statistic</th>
<th>Clono-pathological biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\rho$</td>
<td>BAP1 (NPer)</td>
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<td>AR-Neg</td>
<td>Early and Late</td>
<td>$p$ value</td>
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</tr>
<tr>
<td></td>
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<td>$p$ value</td>
<td>0.282</td>
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<tr>
<td>AR-Pos</td>
<td>Early and Late</td>
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<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Early</td>
<td>$p$ value</td>
<td>0.034</td>
</tr>
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</table>

**Table 7.5B Correlation of peripheral TILs with clinico-pathological biomarkers among AR-negative and AR-positive TNBC patients.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Stage</th>
<th>Test Statistic</th>
<th>Clinico-pathological biomarker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\rho$</td>
<td>BAP1 (Nper)</td>
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<td>0.182</td>
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<td>$p$ value</td>
<td>0.017</td>
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**Abbreviations:** $\rho$, pearson correlation coefficient; Pos, positive; Neg, negative; Hscore, percentage Intensity; Nper, nuclear percentage.
Table 7.6 Prediction of peripheral TILs for 10-year DFS among AR-negative TNBC patients.

<table>
<thead>
<tr>
<th>AR-Negative patients</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td>Overall</td>
<td>0.069; 0.156 (0.021, 1.157)</td>
<td>0.064; 0.147 (0.019, 1.121)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>0.105; 0.187 (0.025, 1.418)</td>
<td>0.061; 0.141 (0.018, 1.094)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>0.013; 0.055 (0.005, 0.542)</td>
<td>0.026; 0.062 (0.005, 0.721)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.992; (-)</td>
<td>0.996; (-)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.217; 0.438 (0.118, 1.622)</td>
<td>0.398; 0.538 (0.127, 2.268)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age at diagnosis and Nottingham grade. Peripheral TILs were treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.
Comparison of stromal and peripheral TILs between AR-negative and AR-positive TNBC patients.

Comparison of stromal TILs between all AR-negative and AR-positive TNBC patients (A) and early-stage AR-negative and AR-positive TNBC patients (B) overall and with or without adjuvant chemotherapy (chemo) or radiation (rad) treatment. Comparison of peripheral TILs between all AR-negative and AR-positive TNBC patients (C) and early-stage AR-negative and AR-positive TNBC patients (D) overall and with or without adjuvant chemotherapy (chemo) or radiation (rad) treatment. *p<0.05, **p<0.01.
Peripheral TILs were stratified into high (≥30) and low (<30) subgroups using a log-rank test. Kaplan-Meier curves were generated to estimate impact of high and low peripheral TIL levels on disease-free survival (DFS) over a 10-year period among AR-negative TNBC patients. Prediction of high and low peripherals TILs on 10-year DFS among all AR-negative patients (A), early-stage AR-negative patients (B), non-adjuvant chemotherapy-treated AR-negative patients (C), non-adjuvant chemotherapy-treated early-stage AR-negative patients (D), and radiation-treated early-stage AR-negative patients (E).

**Figure 7.2 Peripheral TILs are associated with disease-free survival among early-stage AR-negative TNBC patients.**
Peripheral TILs were stratified into high (≥15) and low (<15) subgroups using a log-rank test. Kaplan-Meier curves were generated to estimate impact of high and low peripheral TIL levels on overall survival over a 10-year period among AR-negative TNBC patients. Prediction of high and low peripherals TILs on 10-year OS among all and early-stage non-adjuvant chemotherapy-treated AR-negative patients.

Figure 7.3 Peripheral TILs are associated with overall survival among early-stage AR-negative TNBC patients.
Figure 7.4 Comparison of intratumoral immune cell fractions between AR- low and high TNBC samples.

CIBERSORT tool was used to determine the proportions of distinct infiltrating immune cells among AR- low and high TNBCs isolated from the TCGA breast dataset. The color gradient from green to red represents the increasing fractions of intratumoral immune cells. Immune cell fractions that showed statistically significant differences between AR- low and high TNBCs with lower proportions of naïve B cells and resting mast cells among AR-low compared to AR-high TNBCs with a p-value cut-off of <0.01. Immune cell fractions that showed weakly statistically significant differences (0.05>p<0.1) between the races with a smaller proportion of resting NK...
cells and greater proportion of activated NK cells among AR-low compared to AR-high TNBCs with no p-value cut-off.

7.7 References


8 RACIAL DISPARITIES IN LYMPHOCYTIC INFILTRATION AMONG EARLY-STAGE AFRICAN-AMERICAN AND EUROPEAN-AMERICAN QUADRUPLE NEGATIVE BREAST TUMORS

8.1 Abstract

The role of androgen receptor (AR) in TNBC remains controversial, however, some studies report that lack of AR expression in TNBC or quadruple negative breast cancer (QNBC) confers a more aggressive disease course. Emerging evidence suggests that QNBC is more prevalent among women of African descent. Tumor infiltrating lymphocytes (TILs) are inflammatory mediators have been associated with favorable clinical outcomes and response to neoadjuvant and adjuvant chemotherapy but more aggressive disease characteristics in TNBC. We compared stromal and peripheral TILs between AA (N=55) and EA (N=20) QNBC patients treated at Emory University Hospital in Atlanta, GA. We also performed differential gene expression analysis between AA and EA QNBC patients in a publicly available gene expression dataset.

Among early-stage (I-II) QNBC patients (N=63), we observed more stromal TILs in AA TNBCs (N=44) compared with EA TNBCs (N=19) (p=0.047). Stromal TILs negatively correlated with age at diagnosis among all (ρ=-0.272; p=0.044) and positively correlated with programmed cell death protein 1 (PD1) expression among early-stage (ρ=0.460; p=0.001) AA QNBC patients. High peripheral TIL levels predicted longer 10-year OS among early-stage AA QNBC patients in multivariate analyses (p=0.015; HR:0.155). Our gene expression analyses revealed significant upregulation of biological pathways, gene ontologies, and genes reflecting increased pro-inflammatory activity in AA compared with EA AR-low expressing TNBCs. Our findings indicate a previously unrecognized racial disparity in the tumor immune microenvironment
among early-stage QNBCs. Furthermore, peripheral TIL levels may be able to serve as a risk-prognostic biomarker for early-stage AA QNBC patients.

8.2 Introduction

Triple negative breast cancer (TNBC) is the most difficult form of breast cancer to treat owing to its lack of the pharmacologically targetable biomarkers of proliferation, estrogen receptor, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) (1) [1]. The disease disproportionately afflicts younger women as well as women of African descent(2-4). TNBC patients of African ancestry also exhibit more unfavorable clinico-pathological characteristics and acquire more aggressive TNBC subtypes such as basal-like and androgen receptor-negative TNBC or quadruple negative breast cancer (QNBC) compared with TNBC patients of European descent (3, 5-10). Furthermore, our group recently reported that AAs experience poorer survival than EAs among QNBC patients (unpublished data). Owing to variability in AR staining cut-offs, roughly 45-88% of TNBCs have been reported to lack AR nuclear expression(11). AR-targeted therapies have emerged as a promising alternative therapeutic strategy for AR-expressing TNBCs(12, 13). However, most AR-negative TNBCs are less susceptible to AR-targeted therapy. Furthermore, the prognostic role of AR in TNBC remains controversial, however, a lack of AR expression has been associated with poor differentiation, high clinical stage, high Ki-67, high mitotic index, increased lymphovascular invasion, increased risk of recurrence and distant metastasis, and worse 5-year disease-free survival and overall survival (OS) (14-19). Hence, an increased prevalence of AR-negative TNBC among AA women may be contributing to the racial disparate burden in TNBC. Thus, novel alternative biomarkers and therapies targeting AA QNBC are urgently needed to help alleviate this burden.

Tumor infiltrating lymphocytes (TILs) have emerged as key mediators of the anti-tumoral
inflammatory response. Upon neoplastic transformation, TILs migrate to the tumor site to prevent progression (20, 21). TILs that migrate to the tumor stroma are considered stromal TILs and stromal TILs that occupy the invasive tumor front or periphery of the tumor are considered peripheral TILs(22, 23). Accumulating evidence suggests that stromal TILs have a strong prognostic role in TNBC. Stromal TILs have been associated with improved overall survival (14), increased metastasis-free survival, reduced risk of relapse and reduced distance recurrence in TNBC (24). For every 10% increase in stromal TILs, a 15% reduction in relapse risk and 17% reduction in death risk has been reported(24). Furthermore, a high level of stromal TILs has been associated with improved pathological complete response following neoadjuvant chemotherapy (NAC), validating TILs as a predictive biomarker in TNBC (23-25). Recently in a non-NAC setting, peripheral TILs were associated with high Nottingham grade as well as longer OS and disease-free survival among TNBC patients in univariate and multivariate analyses(22). Paradoxically, the presence of stromal TILs has been associated with more aggressive clinico-pathological tumor characteristics in BC such as high grade, high stage, high Ki-67, lymph node metastasis, a TNBC phenotype, and young age at diagnosis (25-31). Furthermore, Davis et al. recently reported that T cell immune response and checkpoint markers were significantly more upregulated among QNBC compared to TNBC patients in the TCGA dataset and AA QNBCs were more enriched in immune gene signatures compared to EA QNBCs(32). Thus, we were interested in evaluating if there was a disparity in the presence of TILs among AA and EA QNBC tumors and if TILs could function as a risk-prognostic biomarker in QNBC patients of African descent.

8.3 Results

8.3.1 Clinico-pathological characteristics among racially-distinct QNBC patients

We compared clinico-pathological variables and treatment information between AA
(n=55) and EA (n=20) QNBC patients observed at EH (Table 2). We observed a significant difference in associations with mitotic index between AA and EA QNBC patients ($p=0.012$). We observed a significantly greater proportion of AA patients with a high mitotic score compared with EA patients, wherein 61.33% of AAs and 13.3% of EAs exhibited a mitotic count of 3. We also discovered a significant difference in associations with Nottingham grade and race. A significantly greater percentage of AA patients exhibited a high Nottingham grade compared with EA patients in which, 66.7% of AAs and 17.3% of EAs exhibited a score of 3. We also examined differences in associations with expression of established clinical biomarkers in BC between AA and EA QNBC patients to gain more insight into differences in disease pathology between these patient populations (Table 3). We only observed a significant difference in associations with β-catenin expression between the racially-distinct QNBC patients ($p=0.049$), with 62.7% of AA QNBCs positive for β-catenin compared with only 16% of EA QNBCs.

### 8.3.2 AAs harbor more TILs than EAs among early-stage QNBC patients

We investigated differences in the presence of stromal and peripheral TILs between AA ($N=55$) and EA ($N=20$) QNBC patients (Figure 1). We observed that stromal TILs were weakly higher in AAs compared with EAs among all QNBC patients ($p=0.1$) (Fig 1A). We also examined disparities in stromal and peripheral lymphocytic infiltration among racially-distinct early-stage QNBC patients to consider if distinctions in TIL count in early-stage disease could be influencing differences in prognosis between patients of African and European descent. Among early-stage (I-II) ($N=63$) QNBC patients, AAs ($N=44$) harbored significantly more stromal TILs than EAs ($N=19$) ($p=0.047$). We also investigated if racial differences persisted based on type of treatment received among early-stage patients to evaluate if treatment could be impacting racial disparities in tumor lymphocytic infiltration. Among early-stage QNBC patients treated with radiation, AAs
\(N=24\) exhibited a higher presence of stromal TILs than EAs \((N=14)\) \((p=0.05)\) but not among chemotherapy-treated patients. We did not observe significant differences in peripheral TILs between the racial groups among all QNBC patients and among the subset of early-stage QNBC patients regardless of radiation treatment (Fig 1B).

### 8.3.3 TILs are associated with more aggressive disease features among early-stage AA QNBC patients

We examined correlations of stromal (Table 4) and peripheral TILs (Table 5) with demographic and clinico-pathological variables among all and early-stage racially-distinct QNBC patients. Correlation coefficients of stromal and peripheral TILs with demographic and clinico-pathological characteristics among QNBC patients can be found in Tables 6 and 7, respectively. Among AA patients, stromal TILs negatively correlated with age at diagnosis \((\rho=-0.272; p=0.044)\). Stromal TILs also negatively correlated with age at diagnosis among early-stage EA patients \((\rho=-0.844; p=0.004)\). Moreover, stromal TILs positively correlated with lymph node involvement among all EA patients \((\rho=0.461; p=0.041)\). Regarding clinical biomarkers, stromal TILs positively correlated with programmed cell death protein 1 (PD1) expression among early-stage AA patients \((\rho=0.460; p=0.001)\) as well as chemotherapy- and radiation- treated early-stage \((\rho=0.662; p<0.0001, \rho=0.725; p<0.0001, \text{respectively})\) AA patients. Furthermore, stromal TILs positively correlated with expression of the ligand for PD1, programmed cell death ligand 1 (PD-L1), among chemotherapy- and radiation- treated early-stage \((\rho=0.513; p=0.004, \rho=0.545; p=0.006, \text{respectively})\) AA patients. Stromal TILs also significantly correlated with PD1 and PD-L1 expression among early-stage chemotherapy- and radiation- treated early-stage EA patients. However, stromal TILs also positively correlated with epidermal growth factor receptor (EGFR)
expression among early-stage EA patients ($\rho=0.867; p=0.012$) as well as with BRCA1-associated protein 1 (BAP1) among radiation-treated early-stage EA patients ($\rho=0.661; p=0.038$).

We observed similar associations with peripheral TILs among racially-distinct QNBC patients. Peripheral TILs also negatively correlated with age at diagnosis among all AA patients ($\rho=-0.289; p=0.032$) and among early-stage EA patients ($\rho=-0.784; p=0.012$). Furthermore, peripheral TILs positively correlated with lymph node involvement and among early-stage ($\rho=0.440; p=0.015$) and chemotherapy-treated early-stage ($\rho=0.381; p=0.038$) AA patients. Peripheral TILs also positively correlated with lymph node involvement ($\rho=0.481; p=0.032$) and lymphovascular invasion ($\rho=0.457; p=0.043$) among EA patients and these associations persisted among chemotherapy- and radiation treated early-stage EA patients. Peripheral TILs also positively correlated with PD1 among early-stage AA ($\rho=0.289; p=0.034$) and chemotherapy- and radiation treated ($\rho=0.390; p=0.033, \rho=0.476; p=0.019$, respectively) early stage AA patients. However, peripheral TILs positively correlated with EGFR expression among early-stage ($\rho=0.867; p=0.012$) and radiation-treated early-stage ($\rho=0.717; p=0.020$) EA patients.

### 8.3.4 High TIL levels associated with better survival among early-stage AA QNBC patients

We investigated the prognostic value of stromal and peripheral TILs among racially-distinct QNBC patients to elucidate the role of TIL count in the racially disparate burden in QNBC disease. We analyzed the impact of TILs on survival in early-stage disease and examined the influence of treatment on TILs and prognosis to gain more insight into the prognostic role of lymphocytic infiltration. Stromal and peripheral TILs were stratified into high and low subgroups using a log-rank test and Kaplan-Meier analyses were performed to determine the impact of TILs on survival over a 10-year period (Figure 2). We observed that peripheral TILs were associated with significantly with longer 10-year overall survival (14) among all AA QNBC patients.
Furthermore, peripheral TILs were associated with significantly better 10-year OS among radiation treated \((p=0.032)\) but not chemotherapy treated \((p=0.095)\) AA patients \(\text{(Fig 2A-C)}\). High peripheral TIL levels were also associated with significantly longer OS among early-stage AA QNBC patients \((p=0.011)\) \(\text{(Fig 2D)}\). Moreover, high peripheral TILs levels were associated with significantly better 10-year OS among radiation treated early-stage \((p=0.031)\) but not among chemotherapy-treated early-stage \((p=0.074)\) AA patients \(\text{(Fig 2E-F)}\). Associations of peripheral TILs with OS among EA QNBC patients were not observed \(\text{(data not shown)}\). Associations of stromal TILs with OS were also not observed among both AA and EA patients.

We also assessed unadjusted and adjusted associations of stromal and peripheral TILs with 10-year OS among AA and EA QNBC patients by computing univariate and multivariate Cox proportional hazard regression models adjusted for age, grade, and stage \(\text{(Table 8)}\). Similarly, in Kaplan-Meier analyses, high peripheral TIL levels predicted longer 10-year OS among AA QNBC patients in unadjusted \((p=0.020; \text{HR:0.290})\) and in multivariate Cox proportional hazard regression model \((p=0.027; \text{HR:0.272})\). Among radiation-treated AA QNBC patients, high peripheral TIL levels also predicted significantly longer 10-year OS in adjusted analyses \((p=0.042; \text{HR:0.025})\). Furthermore, among early-stage AA QNBC patients, high peripheral TILs predicted significantly better 10-year OS before \((p=0.020; \text{HR:0.290})\) and after adjusting for potential confounding factors \((p=0.015; \text{HR:0.15})\). Peripheral TILs were unable to predict survival among EA QNBC patients. Stromal TIL were unable to predict survival among both AA and EA QNBC patients.

**8.3.5 Increased upregulation of immune response-related genes, pathways and gene ontologies among AA compared with EA AR-low expressing TNBC patients**

We investigated differential expression of immune response-related genes, pathways, and gene ontologies between AA compared with EA AR-low expressing TNBC patients isolated from
TCGA breast dataset. We utilized the GAGE and Pathview packages to analyze differences in biological pathways or experimentally-derived differential expression sets and gene ontologies, respectively between AA and EA AR-low expressing TNBCs in TCGA breast dataset. We observed significantly more upregulation of the leukocyte transendothelial migration expression set among AA compared with EA AR-low expressing TNBC samples ($p=0.049$) (Table 9). We also observed significantly more upregulation of gene ontologies reflecting a pro-inflammatory immune response such as innate immune response, interferon-gamma-mediated signaling pathway, type I interferon-mediated signaling pathway, interleukin-1 secretion, vasodilation, and natural killer cell differentiation and activation ($p<0.05$) among AA compared with EA AR-low expressing TNBC patients (Table 10). We also observed significantly more upregulation of pro-inflammatory genes among AA compared to EA QNBC patients ($p<0.05$) (Table 11).

### 8.4 Discussion

Recent evidence suggest that women of African ancestry are also disproportionately afflicted by QNBC, indicating that AA patients are less susceptible to AR-targeted therapy than EA patients. The role of the tumor immune microenvironment in the racial disparate burden in QNBC remains elusive. Hence, we investigated the prognostic and predictive role of TIL among racially-distinct QNBC patients to determine their potential as a promising risk-prognostic biomarker and/or therapeutic target among QNBC patients of African descent.

We observed a significantly greater proportion of AA patients with a high mitotic score and Nottingham grade than EA patients suggesting QNBC patients of African descent exhibit more aggressive disease features than QNBC patients of European descent. These findings also corroborate Bhattarai et al.’s findings suggesting a poorer prognosis among AA compared with EA QNBC patients (unpublished data). We also discovered a significantly greater proportion of
AAs positive for β-catenin compared with EAs among QNBC patients. Wnt/β-catenin signaling is highly upregulated in TNBC and associated with increased risk for metastasis(39). β-catenin has been found to positively correlate with stromal lymphocytic infiltration in BC, suggesting that Wnt/β-catenin signaling may be playing a critical role in BC anti-tumoral immunity(31). Furthermore, β-catenin overexpression was recently reported to underlie an aggressive disease course among AA QNBC patients. Thus, differential expression of this marker between AA and EA QNBCs may be playing a role in the racial disparate burden in QNBC.

We discovered that the percentage of stromal TILs were significantly higher among AA compared with EA among early-stage QNBC patients as well as among early-stage QNBC patients treated with radiotherapy. Thus, a higher TIL count may be reflective of increased antitumoral immune response among AAs compared with EAs in early-stage QNBC disease. We speculate that increased inflammatory activity among AA tumors, despite their poorer clinical outcomes compared to EAs, might be reflective of an enhanced immune response as a result of their more aggressive disease. Among AA QNBC patients, both stromal and peripheral TILs were associated with younger age at diagnosis, which corroborates previous reports of increased tumor lymphocytic infiltration among TNBC patients diagnosed at a younger age. Furthermore, peripheral TILs were associated with increased lymph node involvement among early-stage AA QNBC patients as well as chemotherapy-treated early-stage AA QNBC patents, which is also similar to previous reports of increased tumor lymphocytic infiltration associated with greater lymph node involvement in TNBC. In regards to clinical biomarkers, both stromal and peripheral TILs were associated with increased PD1 expression among early-stage AA QNBC patients as well as among chemotherapy- and radiation- treated early-stage AA QNBC patients suggesting elevated levels of PD1-expressing TILs and immunosuppression among this patient population in
early-stage disease irrespective of treatment. Stromal TILs were also associated with increased PD-L1 expression among chemotherapy- and radiation- treated early-stage AA QNBC patients, further indicating increased TIL immunosuppression among AA QNBC patients in the early stages of their disease. Among EA QNBC patients, high stromal and peripheral TIL levels were also associated with younger age at diagnosis as well as increased lymph node involvement and lymphovascular invasion. Stromal TILs positively correlated with BAP1 among radiation-treated early-stage EA patients suggesting TILs may be associated with increased DNA damage response among these patients.

We observed that high peripheral TIL levels predicted significantly better 10-year OS among all and early-stage AA QNBC patients in both Kaplan-Meier and multivariate analyses. Furthermore, high peripheral TIL levels predicted significantly better 10-year OS among all and early-stage radiation treated AA QNBC patients in Kaplan-Meier analyses and among all radiation treated AA QNBC patients in multivariate analyses. Thus, these findings indicate that peripheral TIL levels may be able to risk-stratify early-stage QNBC patients of African descent for better or worse survival and that peripheral TILs may be able to predict response to radiotherapy among AA QNBC patients. Furthermore, our results suggest therapies that induce antitumoral immunity such as adoptive T cell therapy may be selectively beneficial for QNBC patients of African descent.

Our gene expression analyses revealed significantly more upregulation of pro-inflammatory genes, biological pathways, and gene ontologies among AA compared with EA AR-low expressing TNBC patients in the TCGA breast dataset. Particularly, we observed significantly more enrichment of the leukocyte transendothelial migration biological pathway among AA compared with EA AR-low expressing TNBCs suggesting AA QNBC patients may exhibit more
recruitment of pro-inflammatory mediators. Furthermore, gene ontologies reflecting increased T cell and NK cell antitumoral immunity were significantly more upregulated among AA compared with EA AR-low expressing TNBCs. Moreover, genes encoding chemokines and cytokines as well as other key mediators in the T- and NK- cell-mediated antitumoral immune response were significantly more upregulated in AA compared with EA among AR-low expressing TNBC samples. In addition, the innate immune response gene ontology was significantly more upregulated among AA compared with EA AR-low expressing TNBC samples suggesting increased innate immunity among QNBC patients of African compared with European descent.

Our study uncovers previously unrecognized racial differences in the tumor immune microenvironment among QNBCs, reveals a novel, alternative risk-prognostic biomarker and suggests early-stage AA QNBC patients harboring lower levels of TILs may be ideal candidates for TIL immunotherapy, particularly among those undergoing radiotherapy. However, robust validation of our results in additional non-NAC and NAC patient cohorts as well as characterization of TIL immune subsets in early-stage QNBC patients of African ancestry will be critical to successfully achieving this aim.

8.5 Methods

8.5.1 Study Cohort

We analyzed a cohort of 75 TNBC patients negative for AR expression (AR Nh-score) among 121 TNBC patients treated at Emory University Hospital (EH) in Atlanta, GA from 2002 (initial day of diagnosis) to 2016 (last day of contact). We obtained approval and permission from the institutional review board at EH to gain access to patient information and samples used in this study. The percentage of stromal and peripheral TILs was assessed in hematoxylin- and eosin-stained patient specimens as described by Krishnamurti and colleagues (22). TILs were evaluated
according to the International TILs working group 2014 in which the percentage of stromal tissues occupied by infiltrating lymphocytes and plasma cells were estimated (33). Intratumoral lymphocytes, or lymphocytes in direct contact with tumor cells, were excluded from the evaluation and only stromal lymphocytes were counted. Peripheral TILs were evaluated as the percentage of stromal lymphocytes occupying the entire circumference of the invasive tumor front with a width of roughly one dozen tumor cells (22). The percentage of stromal and peripheral TILs were estimated in intervals including <5%, 5-10%, 11-50% and >50%. Patient demographic characteristics, clinico-pathological variables, and breast cancer (BC) biomarker status were evaluated and recorded for each patient. Demographic characteristics include self-reported race and age at the time of diagnosis. This article referred to the 7th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) TNM Classification and Stage groupings for BC (34). Information on patient treatment was also recorded including adjuvant chemotherapy and radiation therapy. No patients in this study underwent NAC.

8.5.2 Immunohistochemistry and scoring

Expression of all BC biomarkers investigated in this study was determined through immunohistochemical (IHC) staining and scored as H-score (percentage x intensity), Nper (nuclear percentage) and Nh-score (nuclear percentage x intensity). Intensity of staining were scored as 0=none, 1=low, 2=moderate, and 3=high. Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Antibody details and concentrations can be found in Table 1. The maximum score for any given H-score is 300. Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Patient samples were stained, scored, and reviewed at EH. Scoring was performed by two independent pathologists blinded to clinical annotation and individual scores.
were averaged.

8.5.3 **Follow Up**

Initial diagnosis of patients occurred between 2002 and 2016. Median duration of follow-up for EH was approximately 6 years. Initial dates of diagnoses, treatment start and completion dates, and last dates of contact were recorded for each patient. Survival status (alive/dead) was also recorded for each patient in addition to survival time. The date of last follow-up for the last patient seen is March 3, 2016.

8.5.4 **Differential Gene Expression Determination**

We queried the publically available The Cancer Genome Atlas (TCGA) breast dataset from the TCGA portal for all TNBC patients(35). Publically-available ER/PR/HER2/AR IHC data was used to determine TNBC and QNBC status. We performed a log-rank test to identify the optimal cut-off to stratify the TNBC patients into high- (≥160.43) and low- (<160.43) AR expressing patients. According to the race data, we selected AA and EA samples out of the AR-low expressing TNBC samples. We performed differential gene expression analysis for 15,942 genes between the ethnic groups using the DESeq2 software tool(36). Differential expression analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontologies between the ethnic groups were predicted by GAGE and Pathview packages, respectively(37, 38).

8.5.5 **Statistical Analysis**

The significance level for all analyses was $p<0.05$ with 95% confidence intervals (CIs). Chi-square tests were performed to analyze differences in demographic characteristics, breast clinico-pathological variables and biomarkers, and treatment information between AA and EA QNBC patients. SAS 9.4 program was used to generate test statistics and 2-tailed univariate $p$-values were reported. Wilcoxon rank-sum tests were performed to determine differences in the
means of stromal and peripheral TILs between AA and EA patient populations. Pearson correlation coefficients (ρ) were computed to determine associations between stromal and peripheral TILs with demographic and breast clinico-pathological variables and biomarkers among AA and EA QNBC patients. Spearman’s rank correlation coefficients (ρ) were computed for categorical covariates. Unadjusted and adjusted multivariate Cox proportional hazard regression models were computed to assess the impact of TILs on 10-year OS before and after controlling for age, Nottingham grade, and stage. The Kaplan-Meier analysis was conducted using SAS 9.4 program to estimate survival function for AA and EA QNBC patients over a 5- or 10- year period based on high and low TIL levels. A log-rank test was used to stratify stromal and peripheral TILs into high and low groups to evaluate associations of a high and low presence of TILs with better or worse survival among racially-distinct QNBC patients. The Wald test was used to test for significance among differentially expressed genes between the racial-distinct patient populations using the DESeq2 software tool.

8.6 Tables and Figures

*Table 8.1 Antibodies and dilutions used for immunohistochemical biomarker staining at EH.*
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Subcellular location</th>
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<th>Source</th>
<th>Species</th>
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**Abbreviations:** FOXM1, Forkhead box 1 protein; AR, androgen receptor; PRC1, protein regulator of cytokinesis 1; RARα, Retinoic acid receptor α; BAP1, BRCA1 associated protein 1; PD1, Programmed cell death protein 1; PD-L1, Programmed cell death ligand 1, H-score, percentage x intensity; Nper, nuclear percentage; Nh-score, nuclear percentage x intensity; N/A, not applicable.

**Table 8.2 Clinico-pathological and treatment characteristics of AA and EA QNBC patients observed at EH.**
Table 8.3 Breast cancer biomarker expression among AA and EA QNBC patients observed at EH.

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<td><strong>PD-1 (H-score)</strong></td>
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Table 8.4 Correlation of stromal TILs with clinico-pathological variables and biomarkers among early-stage AA and EA QNBC patients.

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<th>Patients</th>
<th>Clinico-pathological variables and biomarkers</th>
<th>Age(^a)</th>
<th>LN total(^b)</th>
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<th>PD-L1 (H-score)(^a)</th>
<th>EGFR (H-score)(^a)</th>
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\(^a\)Pearson correlation test (ρ)  
\(^b\)Spearman’s rank correlation test (ρ)  
Abbreviations: Chemo, adjuvant chemotherapy; Rad, radiation; LN, lymph node.
Table 8.5 Correlation of peripheral TILs with clinico-pathological variables and biomarkers among early-stage AA and EA QNBC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Clinico-pathological variables and biomarkers</th>
<th>Age(^a)</th>
<th>Nottingham score(^b)</th>
<th>LN total(^b)</th>
<th>LVI(^b)</th>
<th>EGFR (H-score)(^b)</th>
<th>PD-1 (H-score)(^b)</th>
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\(^a\)Pearson correlation test (ρ)

\(^b\)Spearman’s rank correlation test (ρ)

Abbreviations: Chemo, adjuvant chemotherapy; Rad, radiation; LN, lymph node; LVI, lymphovascular invasion.
**Table 8.6 Correlation of stromal TILs with clinico-pathological variables and biomarkers among early-stage QNBC patients.**

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<th>Patients</th>
<th>Clinico-pathological variables and biomarkers</th>
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<tr>
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<td>p value</td>
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<tr>
<td>Early</td>
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<td>p value</td>
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<td>ρ 0.077</td>
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<td>p value</td>
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<td>p value</td>
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<td>n 38.000</td>
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<sup>a</sup>Pearson correlation test (ρ)

<sup>b</sup>Spearman’s rank correlation test (ρ)

**Abbreviations:** Chemo, adjuvant chemotherapy; Rad, radiation; LN, lymph node.
### Table 8.7 Correlation of peripheral TILs with clinico-pathological variables and biomarkers among early-stage QNBC patients.

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<td>(n)</td>
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\(^a\)Pearson correlation test (\(\rho\))  
\(^b\)Spearman’s rank correlation test (\(\rho\))  
Abbreviations: Early, early-stage (I-II); Chemo, adjuvant chemotherapy; Rad, radiation; LN, lymph node.
Table 8.8 Prediction of peripheral TILs for 10-year OS among AA QNBC patients.

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<th>$p$ value; HR (95% CI)</th>
<th>$p$ value; HR (95% CI)</th>
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<td>Unadjusted model</td>
<td>Adjusted model</td>
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<td>Early and late stage</td>
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<tr>
<td>Overall</td>
<td>0.020; 0.290 (0.102, 0.290)</td>
<td>0.027; 0.272 (0.086,0.864)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.153; 0.377 (0.099,1.438)</td>
<td>0.162; 0.338 (0.074,1.547)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.074; 0.231 (0.046, 1.155)</td>
<td><strong>0.042; 0.025 (0.001,0.879)</strong></td>
</tr>
<tr>
<td>Early-stage</td>
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</tr>
<tr>
<td>Overall</td>
<td><strong>0.023; 0.208 (0.054, 0.804)</strong></td>
<td><strong>0.015; 0.155 (0.034, 0.694)</strong></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.761 ;0.71 (0.078, 6.455)</td>
<td>0.0617; 0.548 (0.052,5.815)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.079; 0.081 (0.005,1.339)</td>
<td>0.997; 0.000 (0,000,0.000)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. Peripheral TILs were treated as a categorical variable.
Table 8.9 Immune response related-pathways significantly upregulated in AAs compared to EAs among AR-low TNBC patients in TCGA dataset.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Statistical mean</th>
<th>p value</th>
<th>q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04512 ECM-receptor interaction</td>
<td>2.554</td>
<td>0.006</td>
<td>0.933</td>
</tr>
<tr>
<td>hsa04510 Focal adhesion</td>
<td>2.078</td>
<td>0.019</td>
<td>0.933</td>
</tr>
<tr>
<td>hsa04962 Vasopressin-regulated water reabsorption</td>
<td>1.97</td>
<td>0.027</td>
<td>0.933</td>
</tr>
<tr>
<td>hsa04720 Long-term potentiation</td>
<td>1.711</td>
<td>0.045</td>
<td>0.933</td>
</tr>
<tr>
<td>hsa04670 Leukocyte transendothelial migration</td>
<td>1.661</td>
<td>0.049</td>
<td>0.933</td>
</tr>
</tbody>
</table>
Table 8.10 Immune response-related gene ontologies more upregulated among AA compared to EA AR-low samples in TCGA database.

<table>
<thead>
<tr>
<th>Gene ontology</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0045087 innate immune response</td>
<td>0.004</td>
</tr>
<tr>
<td>GO:0071347 cellular response to interleukin-1</td>
<td>0.010</td>
</tr>
<tr>
<td>GO:0034341 response to interferon-gamma</td>
<td>0.010</td>
</tr>
<tr>
<td>GO:0060333 interferon-gamma-mediated signaling pathway</td>
<td>0.013</td>
</tr>
<tr>
<td>GO:0071346 cellular response to interferon-gamma</td>
<td>0.014</td>
</tr>
<tr>
<td>GO:0034340 response to type I interferon</td>
<td>0.019</td>
</tr>
<tr>
<td>GO:0070555 response to interleukin-1</td>
<td>0.020</td>
</tr>
<tr>
<td>GO:0060337 type I interferon-mediated signaling pathway</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0071357 cellular response to type I interferon</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0050701 interleukin-1 secretion</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:0050704 regulation of interleukin-1 secretion</td>
<td>0.028</td>
</tr>
<tr>
<td>GO:0042311 vasodilation</td>
<td>0.030</td>
</tr>
<tr>
<td>GO:0032088 negative regulation of NF-kappaB transcription factor activity</td>
<td>0.033</td>
</tr>
<tr>
<td>GO:0001779 natural killer cell differentiation</td>
<td>0.035</td>
</tr>
<tr>
<td>GO:0002526 acute inflammatory response</td>
<td>0.036</td>
</tr>
<tr>
<td>GO:0042089 cytokine biosynthetic process</td>
<td>0.038</td>
</tr>
<tr>
<td>GO:0032732 positive regulation of interleukin-1 production</td>
<td>0.041</td>
</tr>
<tr>
<td>GO:0050716 positive regulation of interleukin-1 secretion</td>
<td>0.041</td>
</tr>
<tr>
<td>GO:0031331 positive regulation of cellular catabolic process</td>
<td>0.042</td>
</tr>
<tr>
<td>GO:0032649 regulation of interferon-gamma production</td>
<td>0.044</td>
</tr>
<tr>
<td>GO:0030101 natural killer cell activation</td>
<td>0.046</td>
</tr>
<tr>
<td>GO:0042035 regulation of cytokine biosynthetic process</td>
<td>0.049</td>
</tr>
</tbody>
</table>
Table 8.11 Immune-related genes significantly upregulated in AAs compared to EAs among AR-low TNBC patients in the TCGA database.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Name</th>
<th>Base mean</th>
<th>log2Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSG00000203710</td>
<td>CR1</td>
<td>complement C3b/C4b receptor 1 (Knops blood group)</td>
<td>32.382</td>
<td>1.529</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENSG00000152672</td>
<td>CLEC4F</td>
<td>C-type lectin domain family 4 member F</td>
<td>37.483</td>
<td>1.762</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENSG00000121966</td>
<td>CXCR4</td>
<td>C-X-C motif chemokine receptor 4</td>
<td>2758.089</td>
<td>1.322</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENSG00000189377</td>
<td>CXCL17</td>
<td>C-X-C motif chemokine ligand 17</td>
<td>260.445</td>
<td>1.649</td>
<td>0.001</td>
</tr>
<tr>
<td>ENSG00000276409</td>
<td>CCL14</td>
<td>C-C motif chemokine ligand 14</td>
<td>86.378</td>
<td>1.51</td>
<td>0.002</td>
</tr>
<tr>
<td>ENSG00000150630</td>
<td>VEGFC</td>
<td>vascular endothelial growth factor C</td>
<td>109.034</td>
<td>0.921</td>
<td>0.002</td>
</tr>
<tr>
<td>ENSG00000185201</td>
<td>IFITM2</td>
<td>interferon induced transmembrane protein 2</td>
<td>2450.015</td>
<td>1.232</td>
<td>0.002</td>
</tr>
<tr>
<td>ENSG00000081985</td>
<td>IL12RB2</td>
<td>interleukin 12 receptor subunit beta 2</td>
<td>435.675</td>
<td>1.317</td>
<td>0.003</td>
</tr>
<tr>
<td>ENSG00000164330</td>
<td>EBF1</td>
<td>early B-cell factor 1</td>
<td>94.082</td>
<td>1.063</td>
<td>0.003</td>
</tr>
<tr>
<td>ENSG00000112936</td>
<td>C7</td>
<td>complement C7</td>
<td>42.535</td>
<td>1.424</td>
<td>0.004</td>
</tr>
<tr>
<td>ENSG00000066056</td>
<td>TIE1</td>
<td>tyrosine kinase with immunoglobulin like and EGF like domains 1</td>
<td>312.695</td>
<td>0.958</td>
<td>0.005</td>
</tr>
<tr>
<td>ENSG00000176435</td>
<td>CLEC14A</td>
<td>C-type lectin domain family 14 member A</td>
<td>240.377</td>
<td>0.904</td>
<td>0.006</td>
</tr>
<tr>
<td>ENSG00000138795</td>
<td>LEF1</td>
<td>lymphoid enhancer binding factor 1</td>
<td>285.862</td>
<td>0.955</td>
<td>0.008</td>
</tr>
<tr>
<td>ENSG00000186047</td>
<td>DLEU7</td>
<td>deleted in lymphocytic leukemia, 7</td>
<td>5.205</td>
<td>1.004</td>
<td>0.012</td>
</tr>
<tr>
<td>ENSG0000006016</td>
<td>CRLF1</td>
<td>cytokine receptor like factor 1</td>
<td>346.321</td>
<td>1.192</td>
<td>0.012</td>
</tr>
<tr>
<td>ENSG00000173757</td>
<td>STAT5B</td>
<td>signal transducer and activator of transcription 5B</td>
<td>1414.081</td>
<td>0.553</td>
<td>0.012</td>
</tr>
<tr>
<td>ENSG00000072736</td>
<td>NFATC3</td>
<td>nuclear factor of activated T-cells 3</td>
<td>728.145</td>
<td>0.49</td>
<td>0.013</td>
</tr>
<tr>
<td>ENSG00000188211</td>
<td>NCR3L</td>
<td>natural killer cell cytotoxicity receptor 3 ligand 1</td>
<td>22.919</td>
<td>0.966</td>
<td>0.021</td>
</tr>
</tbody>
</table>
Figure 8.1 Comparison of stromal and peripheral TILs among racially-distinct QNBC patients.

Comparison of stromal and peripheral TILs between all (overall), early-stage (I-II) (early), early-stage chemotherapy- and radiation- treated AA and EA QNBC patients. Abbreviations: Chemo refers to chemotherapy and Rad refers to radiation treatment. *p<0.05
Peripheral TILs were stratified into high (≥10) and low (<10) subgroups using a log-rank test. Kaplan-Meier curves were generated to estimate impact of high and low peripheral TIL levels on overall survival over a 10-year period among AA QNBC patients. Prediction of high and low peripherals TILs on 10-year OS among all (A), chemotherapy-treated (B), radiation-treated (C), early-stage (I-II) (D), chemotherapy treated early-stage (E) and radiation treated early-stage (F) AA QNBC patients

8.7 References


22. Krishnamurti U, Wetherilt CS, Yang J, Peng L, Li X. Tumor-infiltrating lymphocytes are significantly associated with better overall survival and disease-free survival in triple-negative but not estrogen receptor-positive breast cancers. Hum Pathol. 2017;64:7-12.
9 LACK OF HER4 SIGNALING PREDICTS POOR PROGNOSIS AMONG BIOGEOGRAPHICALLY-DISTINCT TRIPLE NEGATIVE BREAST CANCER PATIENTS: A MULTI-INSTITUTIONAL STUDY

9.1 Abstract

Triple negative breast cancer (TNBC) disproportionately afflicts African-American (AA) women compared to European-American (EA) women. West African (WA) women exhibit higher TNBC incidence and mortality rates than AA women. Identification of biomarkers underlying this global burden are urgently needed. We analyzed differences in expression of breast cancer (BC)-related immunohistochemical biomarkers between self-reported European, EA, AA, and WA TNBC patients treated at Nottingham University in Nottingham, UK, Emory University in Atlanta, GA, and Olabisi Onabanjo University Teaching Hospital in Sagamu, Nigeria, respectively. We discovered differences in expression of the human epidermal growth factor receptor (HER) family; HER1/EGFR, HER3 and HER4 between the racial groups (p<0.0001). HER4 cytoplasmic H-score and a summation of EGFR membrane and HER4 cytoplasmic H-scores (EGFR-HER4) decreased with increasing self-reported African ancestry. Differential gene expression analysis revealed that EGFR (ERBB1) and HER4 (ERBB4) genes were expressed lower among AA compared to EA TNBC samples (p<0.05). A lack of HER4 expression was associated with higher Nottingham grade (p=0.03) and mitotic index (p=0.03) among AA patients and higher Ki67 (p=0.04) among early-stage AA patients. In multivariate models, low EGFR-HER4 score predicted shorter 10-year overall (p=0.03; HR: 33.33) and disease-free (p=0.04; HR: 33.33) survival among non-chemotherapy treated AA patients. Furthermore, peroxisome proliferator-activated receptor (PPAR) signaling was downregulated more among TNBC patients with low compared to high EGFR-HER4 scores (p=0.04). These
results suggest that HER4 signaling differs among biogeographically-distinct TNBC patients and that lack of HER4 and EGFR-HER4 expression may predict more aggressive disease among AA and indigenous African TNBC patient populations. Our findings also suggest that the PPAR signaling pathway has potential therapeutic implications among patients with low HER4 and EGFR-HER4 scores.

9.2 Introduction

Triple negative breast cancer (TNBC) comprises a fifth of breast cancer (BC) cases worldwide but remains the most fatal subgroup of BC owing to its more aggressive clinical disease course. TNBC is defined by a lack of estrogen receptor (ER) and progesterone receptor (PR) expression, and human epidermal growth factor receptor 2 (HER2) amplification underscore the urgent need for identification of treatment targets for this patient population. Studies suggest that biogeographic ancestry may be a critical driver of aggressive TNBC. African-American (AA) women are disproportionately afflicted with TNBC \(^1\), tend to be diagnosed at a younger age and show more aggressive clinicopathological features at presentation than European-American TNBC patients. Furthermore, West African (WA) women experience significantly higher TNBC incidence and mortality rates than AA women\(^2\)\(^-\)\(^7\). WAs also present with TNBC at a younger age and with higher grade compared to AAs among TNBC patients. Moreover, TNBC is more prevalent among WA women compared to women residing in Europe and other parts of the world.

The human epidermal growth factor receptor (EGFR) family consists of four tyrosine kinase receptors (EGFR/HER1, HER2, HER3, and HER4) that stimulate growth signaling pathways involved in cell proliferation, growth, survival, and differentiation. EGFR is overexpressed in at least 50% of TNBCs, which is notably higher than in other BC subtypes\(^8\).
High EGFR copy number, immunoreactivity and membrane expression have been shown to be independent prognostic indicators of poorer overall survival (OS) and disease-free survival (DFS) in TNBC, suggesting EGFR to be a potentially targetable and risk-predictive biomarker in TNBC\textsuperscript{9-12}. HER3 is the only receptor in the family that is catalytically inactive and requires dimerization with other members in order to be activated\textsuperscript{13}. HER3 overexpression has been reported in approximately 20-30\% of invasive breast carcinomas\textsuperscript{14}. Reports on the prognostic role of HER3 in BC remain conflicted however, it has been associated with poorer overall survival (OS) and DFS in TNBC\textsuperscript{13,15}.

HER4 is reported to be downregulated in 18-75\% of BCs and upregulated in 7-29\% of cases\textsuperscript{16}. In comparison to non-TNBCs, its expression has been reported to be significantly lower among TNBCs\textsuperscript{17}. It can exist in four isoforms due to alternative mRNA splicing and one of these isoforms can be cleaved and released into the cytoplasm as a soluble HER4 intracellular domain (4ICD) that can translocate to the nucleus\textsuperscript{18-20}. 4ICD displays a diverse range of roles including modulating cell proliferation and pro-apoptotic pathways\textsuperscript{21,22}. 4ICD has been reported to impede breast tumor progression by activating genes that promote cellular differentiation and inhibit proliferation\textsuperscript{23}. Thus, 4ICD is often associated with a favorable prognosis such as longer DFS in BC and TNBCs\textsuperscript{13,24-26}. 4ICD has also been associated with luminal and well-differentiated histology, ER and PR positivity, low histological grade, and low Ki67\textsuperscript{27}. However, reports on the prognostic value of HER4 in TNBC remain conflicted\textsuperscript{17,28}.

We conducted a multi-institutional study in which we screened European, US (EA and AA), and Nigerian for differences in immunohistochemical (IHC) expression of BC-related biomarkers. Interestingly, we only observed significant differences in EGFR, HER3, and HER4
expression between all four ethnic groups. We also investigated the prognostic role of these markers among these ethnically-distinct patient populations.

9.3 Results

9.3.1 Distinctions in clinico-pathological characteristics and survival among biogeographically-distinct TNBC patient populations

We assessed differences in associations with demographic characteristics, clinico-pathological variables and treatment information between UK, EA, AA and Nigerian TNBC patients (Table 1). The Nigerian cohort had the highest percentage of patients diagnosed with TNBC before 40 and the lowest percentage of postmenopausal patients. The Nigerian cohort had the largest percentage of patients with tubule formation score of 3 and the AA cohort had the highest percentage of patients with a mitotic score of 3, nuclear grade of 3, clinical stage of 3, and highest Ki-67 scores. Stage information was unavailable for the Nigerian cohort. We also compared associations with IHC expression of 11 BC-related markers between all four cohorts (Table 2).

We compared OS in Kaplan-Meier analyses between all four cohorts (Figure 1). We observed significant differences in OS between all 4 cohorts (p<0.0001). Nigerian patients exhibited shorter 5-year OS than UK patients and AA patients exhibited shorter 5-year OS than EA patients (Fig 1A). Nigerian patients also exhibited shorter 10-year OS than UK patients and AA patients exhibited shorter 10-year OS than EA patients (Fig 1B).

9.3.2 TNBC patients of African descent show low HER4 expression

We screened all four ethnic groups for differences in expression of 11 common BC-related IHC biomarkers in Table S2 (data not shown). We discovered significant differences in only the EGFR family (EGFR, HER3 and HER4) between all four patient populations suggesting
a role for this family in driving distinct tumor biology among the biogeographically-distinct cohorts \((p<0.0001)\) (Figure 2). Interestingly, HER4 expression directly decreased with increasing presumed African ancestry as expression was highest among European and EA patients but lowest among Nigerian and AA patients \((p<0.0001)\) (Fig 2C). Since, the receptors often heterodimerize to initiate signaling activity, we assessed differences in the combined scores of EGFR and HER3 (Fig 2D), EGFR and HER4 (Fig 2E) and HER3 and HER4 (Fig 2F) between the study cohorts. Interestingly, we also observed that EGFR-HER4 score was highest among European and EA patients but lowest among Nigerian and AA patients \((p<0.0001)\) (Fig 2D-E). Mean values of EGFR family expression levels among each racial group can be found in Table 3. Owing to a different HER4 antibody used among the Nigerian cohort, we excluded Nigerian patients from our comparison analyses involving HER4 and EGFR-HER4 scores and yielded similar results (Figure 3). Representative images of membrane EGFR and cytoplasmic HER4 staining can be found in Fig 3Ai and ii, respectively. HER4 (Fig 3B) and EGFR-HER4 (Fig 3C) scores directly decreased with increasing presumed African ancestry \((p<0.0001)\).

9.3.3 Low EGFR-HER4 expression is associated with more aggressive disease features among patients of African descent

Since HER4 and EGFR-HER4 scores decreased with increasing presumed African ancestry, we determined associations of HER4 and EGFR-HER4 scores with clinicopathological parameters among all (Table 4) and early-stage (I-II) (Table 5) TNBC patients for each ethnic group. HER4 expression negatively correlated with Ki67 percentage among EA patients \((p=0.04)\). Furthermore, EGFR-HER4 negatively correlated with pT of TNM staging \((p=0.04)\) among the UK cohort. Among early-stage UK patients, EGFR-HER4 negatively correlated with tubule formation \((p=0.03)\). Among AA patients, HER4 negatively correlated with Nottingham
grade ($p=0.03$) and mitotic index ($p=0.03$). Furthermore, among early-stage AA patients, HER4 expression negatively correlated with Ki67 percentage ($p=0.04$).

We also analyzed correlations of HER4 and EGFR-HER4 scores with IHC biomarkers associated with BC tumorigenesis/progression among all (Table 6) and early-stage (Table 7) TNBC patients from the UK and US (AA and EA) cohorts. The biomarkers mentioned in this section were not assessed among Nigerian patients. We observed that cytoplasmic HER4 expression correlated positively with nuclear AR ($p=0.02$) and RARα ($p=0.02$) expression among European patients. Furthermore, EGFR-HER4 correlated positively with stromal tumor infiltrating lymphocytes (TILs) among EA patients ($p=0.04$). We observed similar results among early-stage European and EA patients. Among AA patients, EGFR-HER4 also correlated positively with nuclear AR expression ($p=0.03$). However, in contrast to European patients, HER4 correlated negatively with nuclear RARα ($p=0.03$) expression among AA TNBC. Furthermore, HER4 expression correlated negatively with PD1 expression among AA patients ($p=0.03$). We also observed similar results among early-stage AA patients with the exception of PD1.

9.3.4 Low HER4 and EGFR-HER4 expression predict worse prognosis among TNBC patients of African ancestry

We also assessed the prognostic role of cytoplasmic HER4 and EGFR-HER4 among ethnically-distinct TNBC patients by investigating the impact of their expression on survival. The IHC expression of cytoplasmic HER4 and EGFR-HER4 was stratified into high and low subgroups among each ethnic cohort and the impact of high and low expression of HER4 and EGFR-HER4 scores on 5- and 10- year survival was estimated through Kaplan-Meier analysis (Figure 4). Low HER4 and EGFR-HER4 predicted worse survival among patients of both
African and European descent. Low EGFR-HER4 predicted significantly shorter 10-year OS among non-chemotherapy-treated AA patients ($p=0.040$) (Fig 4A). Low HER4 (Fig 4B) and EGFR-HER4 (Fig 4C) predicted significantly shorter 5-year OS among UK patients ($p=0.008$ and $p=0.023$, respectively). Furthermore, among early-stage UK patients, low HER4 (Fig 4D) and EGFR-HER4 (Fig 4E) predicted significantly shorter 5-year OS ($p=0.001$ and $p=0.006$, respectively). Moreover, among chemotherapy-treated early-stage UK patients, low HER4 was associated with significantly poorer survival ($p=0.007$) (Fig 4F). We also investigated the impact of HER4 and EGFR-HER4 on DFS among ethnically-distinct TNBC patients using Kaplan-Meier analyses and yielded similar results (Figure 5).

We also investigated the prognostic role of HER4 and EGFR-HER4 among the ethnically-distinct TNBC patient populations by computing Cox proportional hazard regression models adjusted for age, grade, and stage. Similar to Kaplan-Meier analyses, low HER4 and EGFR-HER4 predicted poorer survival among patients of European and African descent. Low HER4 expression predicted significantly shorter 5-year OS among all ($p=0.04$; HR: 1.59) and early-stage ($p=0.004$; HR: 2.00) UK patients (Table 8). Low EGFR-HER4 also predicted shorter 5-year OS among early-stage ($p=0.011$; HR: 2.08) and early-stage radiation treated UK patients ($p=0.006$; HR: 2.78) in adjusted models (Table 9). Furthermore, we observed that low HER4 (Table 10) and EGFR-HER4 (Table 11) predicted shorter 5-year DFS among all radiation treated and early-stage radiation treated UK patients. Low EGFR-HER4 predicted significantly shorter 5-year OS in adjusted model among non-radiation treated AA patients ($p=0.036$; HR: 20) (Table 4). Low EGFR-HER4 also predicted shorter 10-year OS in multivariate analyses among non-chemotherapy and non-radiation treated AA patients as well as among early-stage non-radiation treated AA patients (Table 12). Similar to OS results, low EGFR-HER4 was associated
with shorter 5-year (Table 4) and 10-year DFS among non-radiation treated AA patients as well as shorter 10-year DFS among non-chemotherapy treated AA patients (Table 13).

9.3.5 **EGFR and EGFR signaling-related genes are underexpressed among AA TNBC patients compared to EA TNBC patients**

We also investigated differential expression of EGFR and EGFR signaling-related genes between AA (n=41) and EA (n=87) TNBC patients in the TCGA RNA sequenced dataset to confirm our protein expression results. Utilizing the DESeq2 software tool, we analyzed differences in expression of 15,942 genes between AA and EA TNBC patients in TCGA breast dataset. Log2fold changes can be found in Table 14. In line with our protein expression findings, the genes encoding EGFR (ERBB1) and HER4 (ERBB4) were significantly more downregulated in AA compared to EA samples ($p<0.05$). We also observed significant underexpression of the EGFR pathway receptor substrate 8 (EPS8) and 15 (EPS15) genes in AA compared to EA patients ($p<0.05$). Furthermore, we observed significant underexpression of genes encoding the ligands for EGFR and HER4 such as neuregulin 3 (NRG3), neuregulin 4 (NRG4), and heparin binding EGF like growth factor (HBEGF) ($p<0.05$) in AA compared to EA patients. Moreover, we also discovered underexpression of genes encoding key molecules in downstream pathways of EGFR and HER4 in AA compared to EA samples ($p<0.05$).

9.3.6 **Peroxisome proliferator-activated receptor signaling more upregulated among high compared to low EGFR-HER4 gene-expressing TNBC patients**

We also identified differentially-expressed biological pathways (Table 15) and gene ontologies (Table 16) between high (n=85) and low (n=58) EGFR-HER4 gene-expressing subgroups among TNBC patients in TCGA dataset. Interestingly, we discovered significant upregulation of the peroxisome proliferator-activated receptor (PPAR) signaling pathway among
high compared to low EGFR-HER4 expressing patients \((p=0.036)\). We also observed significant upregulation of pathways regulated by PPAR signaling among high compared to low EGFR-HER4 \((p<0.05)\). We also discovered significant upregulation of gene ontologies reflecting metabolic and catabolic processes regulated by PPAR signaling \((p<0.05)\) as well as other gene ontologies indicative PPAR signaling \((p<0.05)\). Furthermore, we found significant upregulation of gene ontologies reflecting inflammatory activity, which is modulated by PPAR signaling \((p<0.05)\).

### 9.4 Discussion

The absence of conventional BC markers, ER, PR, and HER2, in triple negative breast tumors has prompted the investigation into alternative actionable targets. We screened all four ethnic groups (Europeans, EAs, AAs, and West Africans) for differences in expression levels of 11 common BC biomarkers. TNBC patients in the UK cohort are comprised of predominantly Europeans who harbor low levels of African ancestry. EAs have been reported to harbor anywhere between 1-2% African ancestry depending on the region of the US they reside\(^2\). AAs represent a highly admixed population with a reported average of 24% of European ancestry, which also varies according to the region of the US they reside\(^2\). AAs harbor a reported average of 73.2% of African ancestry, most of which can be traced back to western Africa according to genetic studies. Genetic ancestry typing was not performed in our study.

Interestingly, the EGFR family members were the only biomarkers that exhibited significant differences in expression levels between all four biogeographically-distinct patient populations. Furthermore, expression of HER4 and EGFR-HER4 showed lowest expression in Nigerian patients and highest expression in UK patients. Our gene expression analyses corroborated our protein expression findings as we observed significant downregulation of
ERBB1 and ERBB4 among AA compared to EA TNBC samples. As consistent with the literature mentioned earlier, cytoplasmic HER4 and EGFR-HER4 was associated with more favorable disease features among TNBC patients of both European and African descent. Our findings of low HER4 expression and EGFR-HER4 score among AA and Nigerian patients compared to their European counterparts may rationalize their increased presentation of poor prognostic disease features among TNBC patients of African compared to European descent. We discovered cytoplasmic HER4 and EGFR-HER4 expression was prognostic among biogeographically-distinct patients in both Kaplan-Meier and multivariate analyses as high expression of these markers predicted better survival.

PPAR signaling was the key pathway that was discovered to be significantly upregulated among high compared to low EGFR-HER4 score patients. PPAR signaling has been reported to regulate glucose homeostasis, steroid hormone biosynthesis, lipid metabolism, hydrogen peroxide metabolism, insulin signaling, inflammation, and cell cycle regulation\(^{33-37}\). The role of PPAR signaling in cancer remains controversial, however the pathway has been reported to exhibit an antitumor effect in BC by reducing cell growth and proliferation and inducing differentiation and apoptosis. Hence, a lack of EGFR-HER4 signaling may be associated with a poorer prognosis among TNBC patients of African descent owing to a deficiency in PPAR signaling. PPAR agonists may be promising therapeutic strategies for TNBC patients of African descent lacking EGFR-HER4 signaling.

Our study further corroborates previous findings revealing that patients of African ancestry tend to lack expression of common BC targets such as ER, PR, HER2, AR and now HER4 expression. Our findings present additional biomarkers that can risk-stratify patients of African descent during the early stages of the disease to improve their clinical management.
9.5 Methods

9.5.1 Patient Cohorts

We procured TNBC patient datasets from 3 different institutions: (1) Nottingham University Hospital (NH) in Nottingham, UK, (2) Emory University Hospital (EH) in Atlanta, GA, and (3) Olabisi Onabanjo University Teaching Hospital (OOTH) in Sagamu, Nigeria. NH, EH and OOTH cohorts were comprised of 322, 121 and 307 TNBC patients, respectively. NH patients were diagnosed between 2007-2013, EH patients were diagnosed between 2002-2016 and the OOTH patients were diagnosed between 2011-2016. We obtained consent and permission from the institutional review boards at each institution to obtain access to deidentified patient information used in this study. None of the patients treated at EH received neoadjuvant chemotherapy.

9.5.2 Immunohistochemistry and scoring

Biomarker expression was determined through standard IHC staining and scored as H-score (percentage x intensity), Nper (nuclear percentage) and NH-score (nuclear percentage x intensity). Intensity of staining were scored as 0=none, 1=low, 2=moderate, and 3=high. Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Antibody details and concentrations can be found in Table 17. The maximum score for any given H-score is 300 and for combined (summed) H-scores is 600. Combined biomarker scores were determined by summing the scores for each biomarker to yield a single score. NH and OOTH patient samples were centrally stained, scored, and reviewed at NH. EH patient samples were stained, scored, and reviewed at EH. Scoring was performed by two independent pathologists blinded to clinical annotation and individual scores were averaged.
9.5.3 Follow-Up

Median duration of follow-up for each cohort was approximately 6 years for EH, 5 years for NH, and 1 year for OOTH patients. Survival status (alive/dead) was recorded for each patient at last follow-up in addition to survival time.

9.5.4 Statistical Analyses

The significance level for all analyses was set to $p<0.05$ with 95% confidence intervals (CIs). Chi-square tests were performed to analyze differences in frequency of demographic characteristics, breast clinicopathological variables and biomarkers, as well as treatment information between UK, AA, EA, and Nigerian TNBC patients. SAS 9.4 program was used to generate test statistics and 2-tailed univariate $p$-values were reported. One-way ANOVA tests were performed to determine differences in mean expression of BC biomarkers between all four patient groups. Associations between HER4 H-score and combined EGFR and HER4 H-scores (EGFR-HER4) with demographic and breast clinico-pathological variables and biomarkers among each ethnic group were determined by computing Spearman’s rank correlation coefficients ($\rho$) for categorical covariates and Pearson correlation coefficients for continuous covariates. Unadjusted and adjusted multivariate Cox proportional hazard regression models were computed to assess the impact of HER4 and EGFR-HER4 scores on 5- and 10-year OS and disease-free survival (DFS) before and after controlling for age, Nottingham grade and stage. The Kaplan-Meier analysis was conducted using SAS 9.4 program to estimate survival function for each patient group based on high and low expression levels of HER4 and EGFR-HER4 score. A log-rank test was used to stratify HER4 and EGFR-HER4 scores into high and low subgroups.
9.5.5 **Differential Gene Expression Analysis**

We queried The Cancer Genome Atlas (TCGA) breast dataset for TNBC patients. Publically-available ER/PR/HER2 IHC data was used to determine TNBC status. We performed differential gene expression analysis between high and low EGFR-HER4 gene-expressing subgroups using the DESeq2 software tool\textsuperscript{29}. EGFR-HER4 scores were determined by summing the normalized gene expression values for each biomarker. Differential expression analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontologies between high and low EGFR-HER4-expressing TNBC patients were predicted by GAGE and Pathview packages, respectively\textsuperscript{30,31}. High and low EGFR-HER4-expressing TNBC patients were stratified according to the median.

9.6 **Tables and Figures**

*Table 9.1 Clinico-pathological characteristics and treatment information of biogeographically-distinct TNBC patient populations.*
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**Abbreviations:** AA, African American; EA, European American; T, tumor size; N, lymph node metastasis; M, distant metastasis; "P" values were calculated using the chi-square test.

Table 9.2 Breast cancer biomarker expression among biogeographically-distinct TNBC patient populations.
### Table 9.3 Differences in HER family member expression between ethnically-distinct TNBC patient populations.

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</tr>
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<td>16.57</td>
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<td>16.57</td>
<td>19</td>
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</tr>
</tbody>
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**Abbreviations:** AA, African-American; EA, European-American; FOXM1, Forkhead box 1 protein; PRC1, protein regulator of cytkinesis 1; RARα, Retinoic acid receptor α; RAP1, BRCA1 associated protein 1; PD1, Programmed cell death protein 1; H-score, percentage x intensity; Nperf, nuclear percentage; Nh-score, nuclear percentage x intensity; *P values were calculated using the Chi-square test.
Table 9.4 Correlation of HER4 and EGFR-HER4 scores with clinicopathological variables among biogeographically-distinct TNBC patients.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>EGFR family member</th>
<th>Test Statistic</th>
<th>Tubule form</th>
<th>Nott. grade</th>
<th>MI</th>
<th>Ki67%</th>
<th>Tumor size (cm)</th>
<th>LN-pos</th>
<th>pT</th>
<th>pN</th>
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<td>0.04</td>
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</tbody>
</table>

* Spearman’s rank correlation test (p)*
* Pearson correlation test (r) *

**Abbreviations:** Nig: Nigeria; Nott.: Nottingham; MI: mitotic index; LN-pos, lymph node positive.

Table 9.5 Correlation of HER4 and EGFR-HER4 scores with clinicopathological variables among biogeographically-distinct early-stage TNBC patients.
Table 9.6 Correlation of HER4 and EGFR-HER4 scores with clinical BC biomarkers among biogeographically-distinct TNBC patients.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>EGFR family member</th>
<th>Test Statistic</th>
<th>AR (N=per)</th>
<th>RARα (N=per)</th>
<th>PD1 (Hscore)</th>
<th>Stromal TILs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>HER4</td>
<td>ρ</td>
<td>0.15</td>
<td>0.15</td>
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</table>

Pearson correlation test (p)

Abbreviations: H-score, percentage x intensity; N=per, nuclear percentage; M=per, membrane percentage; NH-score, nuclear percentage x intensity; AR, androgen receptor; RARα, retinoic acid receptor alpha; PD-1, programmed cell death protein 1; TILs, tumor infiltrating lymphocytes.

Table 9.7 Correlation of HER4 and EGFR-HER4 scores with clinical BC biomarkers among biogeographically-distinct early-stage TNBC patients.
<table>
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<th>Cohort</th>
<th>EGFR family member(s)</th>
<th>Test Statistic</th>
<th>AR (Nper)</th>
<th>RARα (Nper)</th>
<th>BAP1 (Nper)</th>
<th>Stromal TILs (%)</th>
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<td></td>
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<td>AA</td>
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</table>

Pearson correlation test (ρ)

Abbreviations: H-score, percentage x intensity; Nper, nuclear percentage; Mper, membrane percentage; NH-score, nuclear percentage x intensity; AR, androgen receptor; RARα, retinoic acid receptor alpha; BAP1, BRCA1-associated protein 1; TILs, tumor infiltrating lymphocytes.

Table 9.8 Impact of cytoplasmic HER4 on 5-year survival among biogeographically-distinct TNBC patients.
<table>
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<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
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<td>Adjusted model</td>
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<td></td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.928; 1.048 (0.890, 0.360)</td>
<td>0.966; 1.067 (3.154, 0.036)</td>
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<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.372; 0.532 (2.128, 0.133)</td>
<td>0.256; 0.396 (1.861, 0.081)</td>
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</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.579; 1.842 (15.873, 0.213)</td>
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</tr>
<tr>
<td>Radiation</td>
<td>0.958; 1.038 (4.150, 0.260)</td>
<td>0.714; 0.718 (4.201, 0.123)</td>
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<tr>
<td>Non-Radiation</td>
<td>0.951; 0.954 (4.292, 0.212)</td>
<td>0.291; 4.673 (83.333, 0.534)</td>
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<tr>
<td><strong>Early-stage</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.494; 1.773 (9.174, 0.344)</td>
<td>0.546; 1.658 (8.621, 0.321)</td>
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<tr>
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<td>-</td>
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</tr>
<tr>
<td>Radiation</td>
<td>0.733; 1.517 (16.667, 0.138)</td>
<td>0.870; 1.225 (14.084, 0.107)</td>
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<tr>
<td>Non-Radiation</td>
<td>0.634; 1.733 (16.667, 0.180)</td>
<td>0.591; 1.862 (7.857, 0.193)</td>
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</tr>
<tr>
<td><strong>EA</strong></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
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<tr>
<td><strong>Early and late stage</strong></td>
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</tr>
<tr>
<td>Overall</td>
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<tr>
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<tr>
<td><strong>Early-stage</strong></td>
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<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.746; 0.632 (10.101, 0.040)</td>
<td>-</td>
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</tr>
<tr>
<td><strong>UK</strong></td>
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<td>Unadjusted model</td>
<td>Adjusted model</td>
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</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.009; 1.748 (2.666, 1.150)</td>
<td>0.641; 1.587 (2.469, 1.020)</td>
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<tr>
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<td>0.070; 2.227 (5.283, 0.939)</td>
<td>0.123; 2.087 (5.318, 0.818)</td>
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<tr>
<td>Non-chemotherapy</td>
<td>0.257; 1.321 (2.141, 1.633)</td>
<td>0.309; 1.303 (2.174, 0.782)</td>
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<td></td>
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<tr>
<td>Radiation</td>
<td>0.003; 2.330 (4.167, 1.316)</td>
<td>0.645; 1.812 (3.268, 1.095)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.669; 0.885 (0.620, 2.060)</td>
<td>0.445; 0.775 (1.515, 0.402)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.001; 2.058 (3.195, 1.323)</td>
<td>0.0037; 2.008 (3.215, 1.254)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.012; 3.155 (7.634, 1.305)</td>
<td>0.0262; 2.809 (6.944, 1.131)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.104; 1.439 (2.396, 0.862)</td>
<td>0.209; 1.425 (2.475, 0.820)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>&lt;0.001; 3.125 (5.882, 1.667)</td>
<td>0.001; 2.857 (5.556, 1.493)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.558; 0.950 (1.818, 0.538)</td>
<td>0.752; 0.900 (1.754, 0.467)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nigeria</strong></td>
<td></td>
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<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
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</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.235; 1.401 (2.439, 0.803)</td>
<td>0.547; 1.188 (2.075, 0.679)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.235; 1.401 (2.439, 0.803)</td>
<td>0.547; 1.188 (2.075, 0.679)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.957; 0.977 (2.095, 0.413)</td>
<td>0.694; 1.195 (2.097, 0.422)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.199; 1.618 (3.556, 0.780)</td>
<td>0.727; 1.140 (2.387, 0.545)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Multivariable Cox proportional hazard model adjusted for age, Nottingham grade, and stage. HRs are for low vs. high expression. HER4 was treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.
Table 9.9 Prediction of EGFR-HER4 scores on 5-year overall survival among biogeographically-distinct TNBC patients.

<table>
<thead>
<tr>
<th>Location</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.946; 0.963 (2.833, 0.328)</td>
<td>0.804; 1.168 (3.968, 0.343)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.168; 4.651 (41.167, 0.524)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.209; 0.260 (2.123, 0.032)</td>
<td>0.133; 0.162 (1.742, 0.015)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.176; 2.857 (13.158, 0.624)</td>
<td>0.036; 2.000 (333.333, 1.121)</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.655; 1.412 (6.410, 0.311)</td>
<td>0.531; 1.675 (8.475, 0.333)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.172; 4.854 (47.619, 0.503)</td>
<td>0.104; 6.849 (71.425, 0.671)</td>
</tr>
<tr>
<td><strong>EA</strong></td>
<td>p value; HR (95% CI)</td>
<td>p value; HR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.783; 0.703 (8.621, 0.057)</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.428; 3.164 (55.556, 0.184)</td>
<td>-</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.971; 1.053 (18.182, 0.061)</td>
<td>-</td>
</tr>
<tr>
<td><strong>UK</strong></td>
<td>p value; HR (95% CI)</td>
<td>p value; HR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.025; 1.736 (2.817, 1.071)</td>
<td>0.130; 1.468 (2.494, 0.889)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.556; 1.420 (4.587, 0.439)</td>
<td>0.901; 1.086 (3.922, 0.300)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.114; 1.543 (2.646, 0.901)</td>
<td>0.280; 1.379 (2.469, 0.770)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.008; 2.273 (4.348, 1.235)</td>
<td>0.151; 1.640 (3.125, 0.840)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.4151; 0.718 (1.590, 0.325)</td>
<td>0.342; 0.658 (1.587, 0.272)</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.007; 2.028 (3.401, 1.212)</td>
<td>0.011; 2.070 (3.623, 1.182)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.219; 2.101 (6.897, 0.643)</td>
<td>0.417; 1.672 (5.780, 0.483)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.084; 1.667 (2.976, 0.934)</td>
<td>0.155; 1.603 (3.077, 0.836)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.001; 3.630 (6.250, 1.538)</td>
<td>0.006; 2.778 (5.882, 1.316)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.641; 0.633 (1.652, 0.373)</td>
<td>0.662; 0.826 (2.000, 0.346)</td>
</tr>
<tr>
<td><strong>Nigeria</strong></td>
<td>p value; HR (95% CI)</td>
<td>p value; HR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
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<tr>
<td>Early and late stage</td>
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<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.195; 1.254 (1.802, 0.887)</td>
<td>0.212; 1.254 (1.789, 0.879)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.195; 1.264 (1.802, 0.887)</td>
<td>0.212; 1.254 (1.789, 0.879)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.590; 1.171 (2.083, 0.658)</td>
<td>0.276; 1.397 (2.545, 0.766)</td>
</tr>
<tr>
<td>Non-Radiation</td>
<td>0.225; 1.322 (3.406, 0.842)</td>
<td>0.523; 1.912 (1.852, 0.731)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. EGFR-HER4 was treated as a categorical variable. HRs are for low vs. high expression. (-), indicates that 95% CIs were unable to be generated.
Table 9.10 Prediction of HER4 with 5-year disease-free survival among biogeographically-distinct TNBC patients.

<table>
<thead>
<tr>
<th></th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td><strong>AA</strong></td>
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<tr>
<td>Early and late stage</td>
<td></td>
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</tr>
<tr>
<td>Overall</td>
<td>0.485; 1.377 (3.378, 0.581)</td>
<td>0.690; 1.215 (3.165, 0.467)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.974; 1.018 (2.924, 0.354)</td>
<td>0.586; 0.725 (2.294, 0.230)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.570; 1.889 (16.129, 0.218)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.437; 1.563 (4.868, 0.508)</td>
<td>0.791; 1.239 (4.902, 0.313)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.970; 1.029 (4.673, 0.227)</td>
<td>0.325; 4.187 (71.429, 0.243)</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.298; 2.033 (7.752, 0.534)</td>
<td>0.364; 1.832 (7.143, 0.473)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.630; 1.427 (6.061, 0.335)</td>
<td>0.755; 1.267 (5.616, 0.286)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.404; 2.024 (10.638, 0.386)</td>
<td>0.458; 1.916 (10.638, 0.344)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.606; 1.825 (17.857, 0.186)</td>
<td>0.008; 1.876 (20.833, 0.169)</td>
</tr>
<tr>
<td><strong>EA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.729; 0.708 (6.025, 0.029)</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.501; 2.320 (27.027, 0.200)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.406; 0.359 (4.016, 0.032)</td>
<td>-</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.381; 0.341 (3.773, 0.031)</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.810; 1.414 (23.810, 0.085)</td>
<td>-</td>
</tr>
<tr>
<td><strong>UK</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.164; 1.332 (1.986, 0.690)</td>
<td>0.142; 1.364 (2.056, 0.901)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.212; 1.730 (4.062, 0.733)</td>
<td>0.275; 1.653 (4.092, 0.686)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.713; 1.089 (1.721, 0.690)</td>
<td>0.358; 1.252 (2.024, 0.775)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.009; 2.174 (3.846, 1.220)</td>
<td>0.010; 2.174 (4.000, 1.205)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.657; 1.167 (2.273, 0.602)</td>
<td>0.259; 1.493 (3.033, 0.746)</td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.073; 1.471 (2.242, 0.964)</td>
<td>0.076; 1.493 (2.320, 0.958)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.049; 2.415 (5.814, 1.004)</td>
<td>0.077; 2.232 (10.870, 0.916)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.654; 1.119 (1.860, 0.689)</td>
<td>0.523; 1.162 (1.908, 0.709)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.002; 2.703 (5.090, 1.408)</td>
<td>0.003; 2.673 (5.051, 1.414)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.502; 1.205 (2.326, 0.513)</td>
<td>0.276; 1.471 (2.941, 0.735)</td>
</tr>
<tr>
<td><strong>Nigeria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.683; 1.119 (1.916, 0.654)</td>
<td>0.771; 1.083 (1.862, 2.492)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.683; 1.119 (1.916, 0.654)</td>
<td>0.771; 1.083 (1.862, 0.630)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.755; 1.147 (2.703, 0.486)</td>
<td>0.292; 1.595 (3.811, 0.684)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.986; 0.595 (1.986, 0.498)</td>
<td>0.300; 3.692 (1.387, 0.343)</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval. Cox proportional hazard model adjusted for age, Nottingham grade, and stage. HRs are for low vs. high expression. HER4 was treated as a categorical variable. (-) indicates that 95% CIs were unable to be generated.
Table 9.11 Prediction of EGFR-HER4 score on 5-year disease-free survival among biogeographically-distinct TNBC patients.

<table>
<thead>
<tr>
<th></th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td><strong>AA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.346; 1.553 (3.876, 6.622)</td>
<td>0.249; 1.534 (5.952, 6.582)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.680; 0.761 (2.778, 2.020)</td>
<td>0.572; 7.196 (3.322, 3.113)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.178; 4.505 (40.000, 0.505)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.887; 0.916 (3.058, 0.274)</td>
<td>0.847; 0.855 (4.292, 0.171)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.120; 3.268 (14.520, 0.717)</td>
<td><strong>0.015; 19.23 (333.333, 1.233)</strong></td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.192; 2.320 (8.197, 6.655)</td>
<td>0.107; 3.425 (15.385, 0.767)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.851; 1.172 (6.135, 0.224)</td>
<td>0.752; 1.361 (9.174, 0.202)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.974; 0.972 (5.435, 0.174)</td>
<td>0.627; 1.271 (10.732, 0.150)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.131; 5.747 (55.556, 0.533)</td>
<td>0.120; 6.250 (62.5, 0.621)</td>
</tr>
<tr>
<td><strong>EA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.558; 0.499 (5.154, 0.048)</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.542; 1.792 (20.833, 0.154)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.714; 0.630 (7.407, 0.053)</td>
<td>-</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.428; 3.165 (55.556, 0.184)</td>
<td>-</td>
</tr>
<tr>
<td><strong>UK</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.346; 0.799 (0.530, 1.275)</td>
<td>0.403; 1.229 (1.988, 0.758)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.817; 0.871 (0.270, 2.805)</td>
<td>0.828; 0.943 (3.300, 0.270)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.636; 0.873 (0.522, 1.461)</td>
<td>0.444; 1.238 (2.132, 0.718)</td>
</tr>
<tr>
<td>Radiation</td>
<td><strong>0.008; 2.328 (4.345, 1.235)</strong></td>
<td><strong>0.015; 2.174 (4.008, 1.163)</strong></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.603; 1.235 (2.630, 0.571)</td>
<td>0.459; 1.389 (3.226, 0.355)</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.235; 1.351 (2.217, 0.822)</td>
<td>0.237; 1.306 (2.264, 0.615)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.380; 0.589 (5.524, 0.521)</td>
<td>0.546; 1.456 (4.926, 0.430)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.643; 1.139 (1.972, 0.657)</td>
<td>0.507; 1.166 (2.088, 1.292)</td>
</tr>
<tr>
<td>Radiation</td>
<td><strong>0.003; 2.778 (5.556, 1.408)</strong></td>
<td><strong>0.003; 0.35 (9.17, 0.70)</strong></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.561; 1.203 (6.038, 0.585)</td>
<td>0.498; 1.351 (3.125, 0.531)</td>
</tr>
<tr>
<td><strong>Nigeria</strong></td>
<td></td>
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</tr>
<tr>
<td>Overall</td>
<td>0.980; 1.009 (1.416, 0.717)</td>
<td>0.973; 1.006 (1.414, 0.715)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.985; 1.008 (1.416, 0.717)</td>
<td>0.973; 1.006 (0.707, 0.715)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.789; 1.079 (1.896, 0.615)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.716; 0.923 (1.422, 0.598)</td>
<td>0.194; 1.466 (2.604, 0.428)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade; end stage. HRs are for low vs. high expression. EGFR-HER4 were treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.
Table 9.12 Prediction of EGFR-HER4 score on 10-year overall survival among racially-distinct TNBC patients.

<table>
<thead>
<tr>
<th>AA</th>
<th></th>
<th>Unadjusted model</th>
<th>Adjusted model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p value; HR (95% CI)</td>
<td>p value; HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.496; 1.383 (3.521, 0.543)</td>
<td>0.324; 1.704 (4.902, 0.591)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.223; 0.279 (2.179, 0.036)</td>
<td>0.092; 0.119 (1.418, 0.010)</td>
<td></td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.061; 4.739 (23.809, 0.933)</td>
<td><strong>0.033; 34.482 (1000, 1.323)</strong></td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.356; 0.372 (3.030, 0.046)</td>
<td>0.152; 0.164 (1.942, 0.014)</td>
<td></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.160; 2.347 (10.100, 0.713)</td>
<td><strong>0.022; 9.901 (71.429, 1.401)</strong></td>
<td></td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.225; 2.092 (6.897, 0.635)</td>
<td>0.065; 3.717 (14.925, 0.922)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.643; 0.602 (5.155, 0.070)</td>
<td>0.791; 0.743 (6.667, 0.083)</td>
<td></td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.093; 6.757 (62.5, 0.727)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.117; 3.155 (13.333, 0.749)</td>
<td><strong>0.039; 8.000 (58.824, 1.111)</strong></td>
<td></td>
</tr>
<tr>
<td>EA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early and late stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.997; 0.995 (9.709, 0.102)</td>
<td>0.471; 2.469 (28.571, 0.211)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.682; 1.656 (18.182, 0.149)</td>
<td>0.170; 10.526 (3333.333, 0.363)</td>
<td></td>
</tr>
<tr>
<td>Early-stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.814; 1.339 (16.490, 0.119)</td>
<td>0.506; 2.375 (30.303, 0.186)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.477; 0.365 (2.740, 0.171)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. HRs are for low vs. high expression. EGFR-HER4 were treated as a categorical variable. (-), indicates that 95% CIs were unable to be generated.
Table 9.13 Prediction of EGFR-HER4 score on 10-year disease-free survival among racially-distinct TNBC patients.

<table>
<thead>
<tr>
<th>AA</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.327; 1.485 (3.279, 0.673)</td>
<td>0.182; 1.842 (4.505, 0.751)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.656; 0.777 (2.364, 0.256)</td>
<td>0.470; 0.626 (2.232, 0.175)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.072; 4.386 (22.222, 0.876)</td>
<td><strong>0.036; 33.333 (1000, 1.248)</strong></td>
</tr>
<tr>
<td>Radiation</td>
<td>0.893; 1.082 (3.413, 0.343)</td>
<td>0.879; 0.896 (3.663, 0.220)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.222; 2.096 (6.897, 0.639)</td>
<td><strong>0.029; 8.264 (55.556, 1.237)</strong></td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.268; 1.727 (4.545, 0.656)</td>
<td>0.078; 2.710 (8.197, 0.894)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.935; 0.947 (3.508, 0.256)</td>
<td>0.747; 1.252 (4.878, 0.321)</td>
</tr>
<tr>
<td>Non-chemotherapy</td>
<td>0.110; 6.061 (55.556, 0.666)</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.962; 1.040 (6.051, 0.214)</td>
<td>0.857; 1.172 (6.667, 0.206)</td>
</tr>
<tr>
<td>Non-radiation</td>
<td>0.166; 2.755 (11.494, 0.657)</td>
<td>0.0510; 6.897 (47.619, 0.993)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EA</th>
<th>p value; HR (95% CI)</th>
<th>p value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model</td>
</tr>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.926; 0.898 (8.696, 0.093)</td>
<td>0.360; 3.623 (55.556, 0.230)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.682; 1.656 (18.182, 0.149)</td>
<td>0.487; 2.695 (43.478, 0.165)</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.881; 1.182 (13.158, 0.107)</td>
<td>0.404; 3.236 (50.000, 0.205)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.460; 2.849 (45.455, 0.177)</td>
<td>0.537; 2.410 (40.000, 0.147)</td>
</tr>
</tbody>
</table>

**Abbreviations:** HR, hazard ratio; CI, confidence interval. Cox proportional hazard model adjusted for age, Nottingham grade, and stage. HRs are for low vs. high expression. EGFR-HER4 were treated as a categorical variable. (+), indicates that 95% CIs were unable to be generated.
### Table 9.14 Genes significantly upregulated among AA compared to EA TNBC patients in TCGA dataset.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Ensembl</th>
<th>Name</th>
<th>Base Mean</th>
<th>log2FoldChange</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENS000000108244</td>
<td>SPZ2</td>
<td>2251</td>
<td>insulin-like growth factor 2</td>
<td>2918.300</td>
<td>-1.233</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000108264</td>
<td>BRF5</td>
<td>3172</td>
<td>B-raf proto-oncogene, v-raf murine leukemia virus oncoprotein</td>
<td>234.988</td>
<td>0.992</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109105</td>
<td>RSADF</td>
<td>158.158</td>
<td>RSAD and EF-hand domain containing</td>
<td>162.000</td>
<td>-0.959</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109416</td>
<td>JAK2</td>
<td>3714</td>
<td>JAK2</td>
<td>836.724</td>
<td>-3.653</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>PDECD7B</td>
<td>271.75</td>
<td>phosphatidylase 7B</td>
<td>75.863</td>
<td>-3.667</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000107376</td>
<td>PCALM</td>
<td>8301</td>
<td>phosphatidylinositol binding clathrin assembly protein</td>
<td>4418.07</td>
<td>-3.328</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109737</td>
<td>NRG3</td>
<td>10718</td>
<td>neuregulin 3</td>
<td>19.24</td>
<td>-1.094</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109409</td>
<td>PRSS2</td>
<td>62243</td>
<td>phosphatidylinositol-34 5-phosphatase</td>
<td>30.374</td>
<td>-0.965</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109598</td>
<td>NPIP1</td>
<td>3628</td>
<td>insulin-like growth factor 1 phosphatase</td>
<td>2157.030</td>
<td>-0.644</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109371</td>
<td>PPM1</td>
<td>253439</td>
<td>insulin-like growth factor 1 phosphatase</td>
<td>66.016</td>
<td>-0.677</td>
<td>0.001</td>
</tr>
<tr>
<td>ENS000000109624</td>
<td>RASSF9A</td>
<td>156.924</td>
<td>RAS association domain family member 9</td>
<td>67.670</td>
<td>-0.732</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000010923</td>
<td>RASSF2</td>
<td>98.14</td>
<td>RASSF2, member RAS oncogene family</td>
<td>97.493</td>
<td>-0.735</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000107350</td>
<td>FHLH1D1</td>
<td>400224</td>
<td>FHLH1D1, homolog and coiled-coil domain containing D1</td>
<td>3.552</td>
<td>-0.905</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109220</td>
<td>MRGFRG1</td>
<td>10796</td>
<td>regulator of G-protein signaling 14</td>
<td>396.438</td>
<td>-0.494</td>
<td>0.001</td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>PDECD7B</td>
<td>5137</td>
<td>phosphatidylase 7B</td>
<td>63.212</td>
<td>-0.858</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>ENS000000109141</td>
<td>5137</td>
<td>phosphatidylase 7B</td>
<td>63.212</td>
<td>-0.858</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP59</td>
<td>2059</td>
<td>epidermal growth factor receptor pathway substrate 6</td>
<td>695.525</td>
<td>-0.525</td>
<td>0.002</td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP60</td>
<td>1239.79</td>
<td>phosphatidylserine 3 5-phosphatase type A</td>
<td>-0.985</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP61</td>
<td>152.86</td>
<td>phosphatidylserine 3 5-phosphatase type B</td>
<td>200.285</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP62</td>
<td>186.61</td>
<td>phosphatidylserine 3 5-phosphatase type C</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP63</td>
<td>841.41</td>
<td>phosphatidylserine 3 5-phosphatase type D</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP64</td>
<td>152.86</td>
<td>phosphatidylserine 3 5-phosphatase type E</td>
<td>200.285</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP65</td>
<td>186.61</td>
<td>phosphatidylserine 3 5-phosphatase type F</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP66</td>
<td>841.41</td>
<td>phosphatidylserine 3 5-phosphatase type G</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP67</td>
<td>152.86</td>
<td>phosphatidylserine 3 5-phosphatase type H</td>
<td>200.285</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP68</td>
<td>186.61</td>
<td>phosphatidylserine 3 5-phosphatase type I</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>ENS000000109740</td>
<td>EP69</td>
<td>841.41</td>
<td>phosphatidylserine 3 5-phosphatase type J</td>
<td>-0.987</td>
<td>0.032</td>
<td></td>
</tr>
</tbody>
</table>
Table 9.15 Biological pathways upregulated or downregulated among high compared to low EGFR-HER4 expressing TNBC patients in TCGA dataset.

<table>
<thead>
<tr>
<th>Upregulated pathways</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa00340 Histidine metabolism</td>
<td>0.003</td>
</tr>
<tr>
<td>hsa00350 Tyrosine metabolism</td>
<td>0.005</td>
</tr>
<tr>
<td>hsa00360 Phenylalanine metabolism</td>
<td>0.006</td>
</tr>
<tr>
<td>hsa04610 Complement and coagulation cascades</td>
<td>0.012</td>
</tr>
<tr>
<td>hsa00140 Steroid hormone biosynthesis</td>
<td>0.026</td>
</tr>
<tr>
<td>hsa04910 Insulin signaling pathway</td>
<td>0.034</td>
</tr>
<tr>
<td>hsa03050 Proteasome</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>hsa03320 PPAR signaling pathway</strong></td>
<td><strong>0.030</strong></td>
</tr>
<tr>
<td>hsa00071 Fatty acid metabolism</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa00660 Glutamate metabolism</td>
<td>0.048</td>
</tr>
<tr>
<td>hsa00380 Tryptophan metabolism</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Downregulated pathways</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa03010 Ribosome</td>
<td>0.002</td>
</tr>
<tr>
<td>hsa00534 Glycosaminoglycan biosynthesis - heparan sulfate</td>
<td>0.034</td>
</tr>
</tbody>
</table>
Table 9.16 Gene ontologies upregulated or downregulated among high compared to low EGFR-HER4 expressing TNBC patients in TCGA dataset.
<table>
<thead>
<tr>
<th>Upregulated gene ontologies</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0009022 steroid metabolic process</td>
<td>0.001</td>
</tr>
<tr>
<td>GO:0043648 dicarboxylic acid metabolic process</td>
<td>0.003</td>
</tr>
<tr>
<td>GO:0034754 cellular hormone metabolic process</td>
<td>0.002</td>
</tr>
<tr>
<td>GO:0042572 retinol metabolic process</td>
<td>0.003</td>
</tr>
<tr>
<td>GO:0044262 small molecule catalytic process</td>
<td>0.006</td>
</tr>
<tr>
<td>GO:0044712 single-organism catalytic process</td>
<td>0.006</td>
</tr>
<tr>
<td>GO:0051185 cofactor metabolic process</td>
<td>0.001</td>
</tr>
<tr>
<td>GO:0006520 cellular amino acid metabolic process</td>
<td>0.002</td>
</tr>
<tr>
<td>GO:0042743 hydrogen peroxide metabolic process</td>
<td>0.007</td>
</tr>
<tr>
<td>GO:0034599 cellular response to oxidative stress</td>
<td>0.008</td>
</tr>
<tr>
<td>GO:0008209 androgen metabolic process</td>
<td>0.009</td>
</tr>
<tr>
<td>GO:0019193 hormone biosynthetic process</td>
<td>0.009</td>
</tr>
<tr>
<td>GO:0046364 monoaccharide biosynthetic process</td>
<td>0.01</td>
</tr>
<tr>
<td>GO:0042180 cellular ketone metabolic process</td>
<td>0.01</td>
</tr>
<tr>
<td>GO:0046667 response to antibiotic</td>
<td>0.01</td>
</tr>
<tr>
<td>GO:0042448 progestosterone metabolic process</td>
<td>0.011</td>
</tr>
<tr>
<td>GO:0016101 serotonin metabolic process</td>
<td>0.012</td>
</tr>
<tr>
<td>GO:0042744 hydrogen peroxide catalytic process</td>
<td>0.012</td>
</tr>
<tr>
<td>GO:0055114 oxidation-reduction process</td>
<td>0.013</td>
</tr>
<tr>
<td>GO:0060944 glucoseoneogenesis</td>
<td>0.014</td>
</tr>
<tr>
<td>GO:002449 lymphocyte mediated immunity</td>
<td>0.015</td>
</tr>
<tr>
<td>GO:0061603 2-oxoglutarate metabolic process</td>
<td>0.016</td>
</tr>
<tr>
<td>GO:0060681 cellular aldehyde metabolic process</td>
<td>0.016</td>
</tr>
<tr>
<td>GO:0036106 response to cytokine stimulus</td>
<td>0.016</td>
</tr>
<tr>
<td>GO:0042445 hormone metabolic process</td>
<td>0.017</td>
</tr>
<tr>
<td>GO:0045348 negative regulation of myeloid cell differentiation</td>
<td>0.017</td>
</tr>
<tr>
<td>GO:0051581 response to cAMP</td>
<td>0.017</td>
</tr>
<tr>
<td>GO:0008772 porphyrin-containing compound metabolic process</td>
<td>0.017</td>
</tr>
<tr>
<td>GO:0043649 dicarboxylic acid catalytic process</td>
<td>0.019</td>
</tr>
<tr>
<td>GO:0006649 regulation of sodium ion transmembrane transporter activity</td>
<td>0.019</td>
</tr>
<tr>
<td>GO:1901605 alpha-amino acid metabolic process</td>
<td>0.019</td>
</tr>
<tr>
<td>GO:0033013 histone CRE metabolic process</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:0017248 B cell mediated immunity</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:0051185 cofactor catalytic process</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:0016523 retinol metabolic process</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:0016554 organic acid catalytic process</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0046305 carnobyle acid catalytic process</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0010863 regulation of lipid storage</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0002625 immune effector process</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:001915 lipolipid storage</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0006563 alcohol metabolic process</td>
<td>0.021</td>
</tr>
<tr>
<td>GO:0016604 immunoglobulin mediated immune response</td>
<td>0.022</td>
</tr>
<tr>
<td>GO:0008203 cholesterol metabolic process</td>
<td>0.022</td>
</tr>
<tr>
<td>GO:0072663 reductase oxygen species metabolic process</td>
<td>0.022</td>
</tr>
<tr>
<td>GO:0009308 amine metabolic process</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:0051607 defence responses to virus</td>
<td>0.023</td>
</tr>
<tr>
<td>GO:0001189 activation of MAPK activity</td>
<td>0.025</td>
</tr>
<tr>
<td>GO:0006721 terpenoid metabolic process</td>
<td>0.025</td>
</tr>
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**Downregulated gene ontologies**

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| GO:0045047 | protein targeting to ER                    | &lt;0.001 |
| GO:0072599 | establishment of protein localization to endoplasmic reticulum | &lt;0.001 |
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| GO:0048667 | cell morphogenesis involved in neuron differentiation | 0.001 |
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| GO:0031344 | regulation of cell projection organization  | 0.001 |
| GO:0060284 | regulation of cell development             | 0.002 |
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Table 9.17 Antibodies and dilutions used for immunohistochemical biomarker staining.

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**Abbreviations:** FOXM1, Forkhead box 1 protein; PRC1, protein regulator of cytokinesis 1; RARa, Retinoic acid receptor α; BAP1, BRCA1 associated protein 1; PD1, Programmed cell death protein 1; H-score, percentage x intensity; Nper, nuclear percentage; NH-score, nuclear percentage x intensity; N/A, not applicable.
Figure 9.1 Overall survival among ethnically-distinct TNBC patients.
Comparison of 5-year (A) and 10-year (B) overall survival between UK, EA, AA and Nigerian TNBC patient cohorts treated at NH, EH and OOTH respectively, using Kaplan-Meier survival analyses. A log-rank test was used to determine statistical differences in survival between the patient groups. \( p < 0.05 \) was set as the level of significance.
Figure 9.2 Comparison of EGFR family members between ethnically-distinct TNBC patients. Box and whisker plots comparing IHC expression of EGFR (p<0.0001) (A), HER3 (p<0.0001) (B), HER4 (p<0.0001) (C), combined EGFR-HER3 (p<0.0001) (D), combined EGFR-HER4 (p<0.0001) (E) and combined HER3-HER4 (p<0.0001) (F) scores between European (UK), EA, AA, and Nigerian TNBC patients. One-way ANOVA test was performed to determine significant differences between the racial groups. p<0.05 was considered significant.
Figure 9.3 Comparison of HER4 and combined EGFR-HER4 scores between biogeographically-distinct TNBC patients.

Representative microscopic images of membrane EGFR (Ai) and cytoplasmic HER4 (Aii) IHC staining. Box and whisker plots comparing IHC expression of cytoplasmic HER4 (B) and combined EGFR-HER4 (C) scores between European (UK), EA, and AA TNBC patients. One-way ANOVA test was performed to determine significant differences in expression of the markers between the biogeographically-distinct patient populations. Images acquired at 200x magnification. $p$$<$0.05 was considered significant.
HER4 and combined EGFR-HER4 scores were stratified into high (≥4 for HER4; ≥5 for EGFR-HER4) and low (<4 for HER4; <5 for EGFR-HER4) subgroups using a log-rank test. The impact of high and low expression of HER4 and combined EGFR-HER4 score on 5- and 10-year overall survival among biogeographically-distinct TNBC patients.
survival among treated TNBC patients of African and European descent was estimated through Kaplan-Meier analyses. Survival curves (10-year OS) of non-chemotherapy-treated AA patients stratified based on high vs. low expression of EGFR-HER4 (A), survival curves (5-year OS) of UK TNBC patients stratified based on high vs. low HER4 score (B), survival curves (5-year OS) of UK TNBC patients stratified based on high vs. low EGFR-HER4 score (C), survival curves (5-year OS) of early-stage UK TNBC patients stratified based on high vs. low HER4 expression (D), survival curves (5-year OS) of early-stage UK TNBC patients stratified based on high vs. low EGFR-HER4 expression (E), and survival curves (5-year OS) of chemotherapy-treated early-stage UK TNBC patients stratified based on high vs. low HER4 expression (F). $p<0.05$ was considered significant.
HER4 and combined EGFR-HER4 scores were stratified into high (≥4 for HER4 and ≥5 for EGFR-HER4) and low (<4 for HER4 and <5 for EGFR-HER4) subgroups using a log-rank test. The impact of high and low expression of HER4 and combined EGFR-HER4 score on 5- and 10-
year disease-free survival (DFS) among treated TNBC patients of African and European descent estimated through Kaplan-Meier analyses. Survival curves (10-year DFS) of non-chemotherapy-treated AA patients stratified based on high vs. low expression of EGFR-HER4 (A), survival curves (5-year DFS) of radiation-treated UK patients stratified based on high vs. low expression of HER4 (B) and EGFR-HER4 (C), survival curves (5-year DFS) of radiation-treated early-stage UK patients stratified based on high vs. low expression HER4 (D) and combined EGFR-HER4 scores (E), and survival curves (5-year DFS) of chemotherapy-treated early-stage UK patients stratified based on high vs. low expression of HER4 (F). p<0.05 was considered significant.

9.7 References


34. Lehrke M, Lazar MA: The many faces of PPARgamma. Cell 123:993-9, 2005


10 LACK OF COMBINED IMMUNOHISTOCHEMICAL HER3-HER4 SCORE PREDICTS POOR PROGNOSIS AMONG EARLY-STAGE AFRICAN-AMERICAN QUADRUPLE NEGATIVE BREAST CANCER PATIENTS

10.1 Abstract

Quadruple negative breast cancer (QNBC) has recently emerged as a subgroup of triple negative breast cancer (TNBC) and is defined by the absence of androgen receptor (AR), which differentiates them from TNBC. Although controversial, a lack of AR expression in TNBC has been reported to confer an aggressive disease course. Recent evidence suggest that QNBC disproportionately affects women of African descent. The role of the other three members of the epidermal growth factor receptor (EGFR) family (EGFR/HER1, HER3, HER4) in AA QNBC remains underexplored. We compared individual and combined immunohistochemical (IHC) expression scores of these EGFR family members between formalin-fixed, paraffin-embedded resection AR-negative and AR-positive AA TNBC patient samples from Emory Hospital in Atlanta, GA. We performed differential gene expression analyses to determine differences in mRNA expression of the EGFR family members and downstream signaling between AR-low and AR-high expressing AA TNBC patients in a publically-available dataset. Combined IHC HER3 and HER4 expression (HER3-HER4 score) was significantly lower among QNBC (n=48) compared to TNBC (n=14) early-stage (I-II) AA patients (p=0.04). Gene expression analyses revealed lower HER4 expression and downstream signaling among AR-low compared to AR-high expressing AA TNBC patients. Lack of HER3-HER4 score was associated with high Ki-67 percentage (p=0.03) and Nottingham score (p=0.04) among early-stage AA QNBC patients. Moreover, low HER3-HER4 score predicted shorter 10-year overall survival among early-stage AA QNBC patients in Kaplan-Meier analysis (p=0.047). In silico analyses revealed more
downregulation of pro-apoptotic genes as well as gene ontologies reflecting cell death, loss of cell-cell contact, and pro-inflammatory pathways among AR-low compared to AR-high AA TNBC samples ($p<0.05$). However, we observed more upregulation of biological pathways and gene ontologies reflecting cell cycle progression, cell proliferation, and DNA damage repair response among AR-low compared to AR-high AA TNBC patients ($p<0.05$). Our findings suggest that a low HER3-HER4 score may be associated with early-stage QNBC disease among AAs and may serve as a valuable risk-prognostic biomarker for this patient population. Our results also indicate that early-stage AA QNBC patients lacking HER3-HER4 expression may exhibit sensitivity to therapies targeting cell cycle progression and DNA damage response.

10.2 Introduction

Triple negative breast cancer (TNBC) is the most aggressive form of breast cancer that continues to confound researchers and clinicians today. TNBC is characterized by a lack of expression of the pharmacologically-targetable biomarkers, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) expression rendering this subgroup of breast cancer patients refractory to traditional endocrine and HER2-targeted systemic therapies (1). Anthracycline- and taxane-based chemotherapy remain the cornerstone of TNBC treatment however, about 30-40% of TNBC patients achieve pCR and experience improved disease-free survival (DFS)(2, 3). The disease is further typified by more aggressive tumor characteristics such as higher grade, clinical stage, and proliferation, a more aggressive clinical course characterized by increased lymph node, cerebral, and visceral metastasis, and a poorer prognosis defined by higher mortality rates compared to non-TNBCs(4-9). Furthermore, TNBC disproportionately afflicts younger and African-American (AA) women(10). Moreover, within the inherently aggressive subtype, AAs exhibit more aggressive disease features, acquire
more aggressive TNBC subtypes, and suffer poorer clinical outcomes compared to European-Americans (EAs) patients (11-17). Androgen receptor (AR) has recently emerged as a promising therapeutic target for TNBC patients, however AAs have been reported to lack expression of AR or harbor quadruple negative breast cancer (QNBC) disease significantly more than EAs among TNBC patients rendering them more refractory to AR-targeted therapies(17). Furthermore, although the prognostic role of AR in TNBC remains controversial, a lack of AR expression has been reported to be associated with poor differentiation, high clinical stage, high Ki-67, high mitotic index, increased lymphovascular invasion, increased risk of recurrence and distant metastasis, and worse 5-year disease-free survival (DFS) and overall survival (OS) suggesting the high absence of AR expression among AAs may be contributing to the racial disparate burden in TNBC (18-22). Furthermore, our group recently reported that AA’s exhibit poorer survival than EAs among QNBC patients. Thus, novel alternative risk-prognostic biomarkers and therapeutic strategies are urgently needed to target QNBC disease in AA women.

The human epidermal growth factor receptor (EGFR) family plays a critical role in cell proliferation, growth, survival, and differentiation through stimulating key growth signaling pathways such as PI3 kinase, Ras-Raf-MAPK, JNK, and PLCγ(23-25). The family is comprised of four structurally related tyrosine kinase receptors ((EGFR/HER1, HER2, HER3, and HER4) that are activated upon ligand binding, hetero- or homo- dimerization of these receptors, and subsequent phosphorylation of tyrosine residues on the intracellular tyrosine kinase domain(26). Aberrant expression of these receptors can stimulate carcinogenesis by promoting loss of cell adhesion, polarity, and initiating invasion and angiogenesis(27, 28). EGFR is overexpressed in approximately 50% of TNBCs, which is significantly higher compared to other BC subtypes(17, 24). High EGFR copy number, immunoreactivity, and expression have been reported to
independently predict poorer overall survival (OS) and disease-free survival (DFS) in TNBC providing evidence for EGFR as a potentially targetable and risk-predictive biomarker in TNBC(29-32). HER3 is the only receptor in the family that is catalytically inactive and can only be activated if it dimerizes with other members in the family(33). The receptor has been reported to be overexpressed in roughly 20-30% of invasive breast carcinomas. Evidence on the prognostic role of HER3 in breast cancer are conflicted however, the receptor has been linked to poorer OS and DFS in TNBC. HER4 is the only family member to exist in four isoforms as a result of alternative mRNA splicing(34). One of these isoforms can be cleaved as a soluble HER4 intracellular (4ICD) in the cytoplasm and translocate to the nucleus(35-37). HER4 has been consistently reported to modulate cell proliferation and pro-apoptotic pathways and thus has been linked to a favorable prognosis in BC and specifically among TNBCs(33, 38-41). Furthermore, elevated HER4 gene expression levels during chemotherapy has been shown to predict response to neoadjuvant chemotherapy in breast cancer(42).

It may be worthy to investigate the role of the other three EGFR family members as alternative risk-predictive and/or therapeutic targets in QNBC owing to its inherent lack of ER, PR, HER2, and AR expression. The role of the EGFR growth factor receptor family in QNBC and in the racial disparate burden remains elusive, however recent evidence suggests that the EGFR family members may be critical drivers in QNBC disease etiology and progression. AR has been reported to regulate expression of members of the EGFR family and vice versa in multiple cancer types(43-51). Researchers at Caris Research Institute recently observed significantly higher EGFR expression among AR-negative compared to AR-positive TNBCs. In contrast, HER4 mRNA expression levels have been reported to be significantly lower among AR-negative compared to AR-positive TNBC patients(17). Thus, a lack of HER4 expression
may be associated with QNBC disease and serve as a novel prognostic biomarker for QNBC patients. Hence, we examined the prognostic and predictive role of the EGFR family members in AA QNBC disease to explore the potential of these receptors to improve risk-prognostication and/or be exploited for therapeutic intervention among this patient population. We immunostained specimens from AA TNBC patients observed at Emory University Hospital (EH) for AR and the EGFR family receptors and examined differences in their immunohistochemistry (IHC) expression levels between AR-negative and AR-positive patients, as well as investigated the prognostic role of this receptor family in AA QNBC.

10.3 Results

10.3.1 Clinico-pathological characteristics and biomarker expression among AR-negative and AR-positive AA TNBC patients

We analyzed differences in associations with demographic and clinico-pathological characteristics as well as treatment status between AR-negative (n=61) and AR-positive (n=16) AA TNBC patients observed at EH (Table 1) We did not observe significant differences in associations with demographic and clinico-pathological characteristics between AA QNBC and TNBC patients. However, we did observe moderate significant differences associations with nuclear grade, Nottingham grade, and lymph node status between the groups. A higher percentage of AA QNBCs (76.6%) presented with a nuclear grade of 3 compared to AA TNBCs (18.2%) \( (p=0.139) \). A higher proportion of AA QNBCs (72.7%) also presented with a Nottingham grade of 3 compared to AA TNBC patients (15.6%) \( (p=0.062) \). Furthermore, a greater percentage of AA QNBC patients (26%) exhibited lymph node involvement compared to AA TNBC patients (2.6%) \( (p=0.110) \).
10.3.2 **AR-negatives exhibit lower combined immunohistochemical HER3-HER4 score than AR-positives among AA TNBC patients**

We examined differences in the IHC expression of the EGFR family members [EGFR (membranous), HER3 (membranous), and HER4 (cytoplasmic)] between AR-negative and AR-positive AA TNBC patients. We also compared combinations of the scores between the groups to determine differences in dimerization and cooperative signaling. We only observed significant differences in combined IHC HER3 and HER4 expression (HER3-HER4 score) between QNBC and TNBC AA patients, in which HER3-HER4 score was significantly lower in AR-negative (n=61) compared to AR-positive (n=16) TNBC patients (p=0.014) (Figure 1). We further investigated differences in HER3-HER4 score between the groups by comparing the scores between chemotherapy treated and non-treated AA QNBC and TNBC patients, as well as among early-stage treated patients (Figure 2). Representative microscopic images of HER3 (membranous) and HER4 (cytoplasmic) IHC staining can be found in Fig 2A. Interestingly, we also observed significantly lower HER3-HER4 score among all (p=0.014) and chemotherapy-treated (p=0.014) AR-negative (n=42) and AR-positive (n=12) AA TNBC patients (Fig 2B). Furthermore, HER3-HER4 score was significantly lower among QNBCs (n=48) compared to TNBCs (n=14) among early-stage AA patients (p=0.04) as well as among early-stage AA patients treated with chemotherapy (p=0.03) (Fig 2C).

10.3.3 **Lack of HER3-HER4 score is associated with more aggressive disease features among early-stage AA QNBC patients**

We investigated associations of HER3-HER4 score with clinico-pathological variables (Table 2) and biomarkers (Table 3) among AA QNBC patients. Regarding clinico-pathological characteristics, HER3-HER4 score negatively correlated with aggressive tumor features among
AA patients. Among all AA patients, HER3-HER4 score negatively correlated with Ki67 percentage ($\rho=-0.294; p=0.023$) and Nottingham score ($\rho=-0.275; p=0.033$). Moreover, HER3-HER4 score negatively correlated with Ki67 percentage among early-stage AA patients ($\rho=-0.307; p=0.034$) and negatively correlated with Nottingham score among early-stage AA patients administered chemotherapy ($\rho=-0.366; p=0.040$). However, among AA patients administered chemotherapy, HER3-HER4 score positively correlated with sentinel lymph node (SLN) involvement ($\rho=0.325; p=0.036$). Regarding clinical biomarkers, HER3-HER4 score also negatively correlated with biomarkers associated with aggressive disease among AA QNBC patients. Among all ($\rho=-0.322; p=0.020$) and early-stage ($\rho=-0.315; p=0.040$) AA patients, HER3-HER4 score negatively correlated with nuclear forkhead box protein M1 (FOXM1) expression. HER3-HER4 score also negatively correlated with nuclear retinoic acid receptor alpha (RAR$\alpha$) expression among all ($\rho=-0.326; p=0.014$) and early-stage ($\rho=-0.361; p=0.014$) AA patients. Furthermore, among early-stage AA patients, HER3-HER4 score negatively correlated with BRCA1-associated protein 1 (BAP1) expression ($\rho=-0.294; p=0.043$).

**10.3.4 Lack of combined IHC HER3-HER4 score predicts poorer survival among early-stage AA QNBC patients**

We investigated the prognostic and predictive power of HER3-HER4 score among AA QNBC patients. HER3-HER4 scores were stratified into high ($\geq 6$) and low ($<6$) subgroups using a log-rank test among AA QNBC patients and Kaplan-Meier analyses were performed to estimate the impact of HER3-HER4 score on survival over a 5- and 10-year period (Figure 2). We observed that low HER3-HER4 score moderately predicted poorer 10-year ($p=0.136$) and 5-year ($p=0.0137$) OS among all AA QNBC patients (Fig 2A-B). However, low HER3-HER4 score significantly predicted poorer 10-year OS among early-stage AA QNBC patients ($p=0.047$).
(Fig 2C). Furthermore, low HER3-HER4 score weakly predicted poorer 10-year \( (p=0.108) \) OS among early-stage radiation treated AA patients (Fig 2D). We also computed Cox proportional hazard regression models to assess unadjusted and adjusted associations of HER3-HER4 score with 5- and 10-year OS among AA QNBC patients (Table 4). Low HER3-HER4 score moderately predicted shorter 10-year OS in adjusted analyses among all \( (p=0.057; \text{HR: 0.310;} \) 95% CI: 0.093-1.035) and early-stage \( (p=0.054; \text{HR: 0.251;} \) 95% CI: 0.062-1.025) AA QNBC patients.

**Downregulation of genes, pathways, and gene ontologies involved in HER4 signaling among AA QNBC patients**

We investigated differential expression of genes, pathways, and gene ontologies between AR-low and AR-high AA patients isolated from TCGA breast dataset. We utilized the GAGE package to analyze differences in biological pathways or experimentally-derived differential expression sets between AR-low and AR-high AA TNBCs in TCGA breast dataset (Table 5). We observed significantly more upregulation of cell proliferation and DNA damage repair expression sets such as cell cycle, DNA replication, base-excision repair, mismatch repair, and nucleotide excision repair \( (p<0.05) \) and significantly more downregulation of downstream pathways of HER4 signaling such as MAPK, JAK/STAT, calcium signaling pathways, and adenylate cyclase-modulating G-protein coupled receptor signaling pathway \( (p<0.05) \) among AR-low compared to AR-high AA TNBCs. Furthermore, we discovered significantly more downregulation of pro-inflammatory expression sets such as leukocyte transendothelial migration and intestinal immune network for IgA production as well as chemokine, T cell, and B cell signaling pathways among AR-low compared to AR-high AA patients \( (p<0.05) \). Moreover,
we also found significantly more downregulation of cell-cell contact such as focal adhesion, cell adhesion molecules (CAMs), and gap junction among AR-low compared to AR-high AAs (p<0.05).

We utilized the Pathview package to analyze differences in gene ontologies between AR-low and AR-high AA TNBCs in TCGA breast dataset (Table 6). We also observed significantly more upregulation of gene ontologies associated with cell cycle progression such as interphase, S phase, mitosis, DNA replication, cell cycle checkpoint, M/G1 transition of mitotic cell cycle, and G1/S transition of mitotic cell cycle as well as DNA damage response such as DNA repair, response to DNA damage stimulus, DNA integrity checkpoint, and DNA damage checkpoint (p<0.05). Gene ontologies associated with downstream signaling of HER4 such as MAPK cascade, ERK1 and ERK2 cascade, phospholipase C activity, cAMP metabolic process, phosphatidylinositol 3-kinase cascade, second-messenger-mediated signaling, and platelet derived growth factor receptor signaling pathway were significantly more downregulated in AR-low compared to AR-high AA TNBC samples (p<0.05). Gene ontologies associated with regulation of cell death, differentiation, and proliferation such as cell type specific apoptotic processes, negative regulation of cell death, negative regulation of programmed cell death, negative regulation of cell proliferation, and negative regulation of cell differentiation were also significantly more downregulated among AR-low compared to AR-high AA samples (p<0.05). In addition, gene ontologies indicative of cell-cell contact and tissue homeostasis such as cell-cell adhesion, actin cytoskeleton organization, extracellular matrix organization, cell-substrate adhesion, cell-matrix adhesion, cell adhesion mediated by integrin, positive regulation of cell migration, and positive regulation of cell motility were significantly more downregulated among AR-low compared to AR-high AA samples (p<0.05). Furthermore, gene ontologies reflective of
a pro-inflammatory response such as T cell activation, lymphocyte activation, lymphocyte differentiation, inflammatory response, B cell activation, leukocyte proliferation, leukocyte migration, adaptive immune response, and humor immune response were significantly more downregulated in AR-low compared to AR-high AA TNBC samples \( (p<0.05) \).

Utilizing the DESeq2 software tool, we analyzed differences in expression of 15,942 genes between AR-low and AR-high AA TNBC patients in the TCGA breast dataset. Log\(_2\) fold changes can be found in (Table 7). We observed the gene encoding HER4, ERBB4, was significantly more downregulated among AR-low compared to AR-high AA TNBCs \( (p<0.05) \). Furthermore, we discovered that the gene encoding the HER4 ligand that induces its proteolytic cleavage, heparin-binding epidermal growth factor (HB-EGF), was significantly more downregulated in AR-low compared to AR-high AA TNBC samples \( (p<0.05) \). We also observed significantly more downregulation of genes encoding key signaling molecules in the downstream pathways of HER4 signaling such as phosphodiesterase 1A (PTGER3), phosphatidylinositol-3,4,5-triphosphate dependent Rac exchange factor 2 (PREX2), inositol-3-phosphate synthase 1 (ISYNA1), phospholipase C like 1 (PLCL1), Ras guanyl releasing protein 2 (RASGRP2), mitogen-activated protein kinase binding protein 1 (MAPKBP1), Janus kinase 3 (JAK3), and signal transducer and activator of transcription 5B (STAT5B) among AR-low compared to AR-high AA patients \( (p<0.05) \). We also observed significantly more downregulation of genes encoding G-protein signaling such as G-protein-coupled receptor 4 (GPR4) and regulator of G-protein signaling 22 \( (p<0.05) \). Furthermore, we found that pro-apoptotic caspases such as caspase 10 (CASP10) and 12 (CASP12) were significantly more downregulated among AR-low compared to AR-high AA TNBCs \( (p<0.05) \).
10.4 Discussion

TNBC patients of African descent suffer the most aggressive clinical disease course, the poorest prognosis, and have the least amount of targeted treatment options in comparison to TNBC patients of other ethnicities. Thus, novel risk-predictive biomarkers and therapeutic targets are urgently needed to circumvent a poor prognosis among this patient population. AR has recently emerged as a promising alternative therapeutic target for TNBC patients, however, AA TNBC patients have been reported to lack AR expression significantly more than EA TNBC patients, leaving AAs less susceptible to AR-targeted therapy than EAs among TNBC patients. Furthermore, a lack of AR expression has been associated with a poor prognosis among TNBC patients of African descent. Thus, alternative actionable targets are needed for AA TNBC patients lacking AR expression. The other EGFR family members (EGFR/HER1, HER3, and HER4) have also emerged as promising alternative prognostic and/or pharmacologically-targetable biomarkers in TNBC. Hence, in this study we investigated the potential of these EGFR family members as novel, alternative biomarkers for AA TNBC patients absent of AR expression.

We compared IHC expression of the three EGFR family members as well as combined variations of the receptors between AR-negative and AR-positive AA TNBC patients and observed that only HER3-HER4 score significantly differed between the patient groups. Specifically, HER3-HER4 score was significantly lower among all and early-stage QNBC compared to TNBC AA patients suggesting that a lack of HER3-HER4 signaling may be underlying more aggressive TNBC among patients of African ancestry. Furthermore, HER3-HER4 score was significantly lower among all and early-stage chemotherapy-treated QNBC
compared to TNBC AA patients. As previously discussed, HER3 is catalytically inactive unless it dimerizes with another EGFR family member and HER4 expression is the only family member that is associated with a better prognosis in TNBC owing to its pro-apoptotic and antiproliferative activity. Hence, lower HER3-HER4 expression among AR-absent compared to AR-expressing AA TNBC patients may reflect reduced cell death and increased proliferation among AA QNBC patients. Furthermore, these findings suggest less dimerization of HER3 with HER4 and downstream signaling among AA QNBC compared to AA TNBC patients.

We also discovered that a lack of HER3-HER4 expression was associated with more aggressive disease features among AA QNBC patients. Low IHC HER3-HER4 score was associated with high Ki-67 percentage and Nottingham score among all and early-stage AA QNBC patients. Low IHC HER3-HER4 score was also associated with high Ki-67 index among early-stage AA QNBC patients administered chemotherapy. Thus, our observation of low IHC HER3-HER4 score associated with high Ki-67 proliferation may be reflective of low HER4 signaling and consequently reduced modulation of cell proliferation among AA QNBC patients. Furthermore, low HER3-HER4 score was associated with high FOXM1, RARα, and BAP1 expression among all and early-stage AA QNBC patients. FOXM1 is a transcription factor that regulates cell cycle progression, particularly mitotic division, chromosome segregation and genomic stability (57, 58). It has been found to be overexpressed in approximately 85% of TNBCs and its upregulation has been suggested to play a critical role in carcinogenesis through stimulating cell proliferation and tumor metastasis (59). Thus, our observation of low HER3-HER4 score associated with high FOXM1 expression may be indicative of increased deregulation of cell cycle progression, aberrant mitotic division, missegregation of chromosomes, and genomic instability and subsequently tumorigenesis and progression among
AA QNBC patients. Hence, FOXM1 may serve as a viable, alternative therapeutic target for AA QNBC patients lacking HER3-HER4 expression. RARα is known to stimulate growth inhibition of ER-positive but not, ER-negative BC cell lines as they tend to express RARα at lower levels(60). The role of RARα in TNBC remains elusive however, our finding of low HER3-HER4 score associated with increased RARα expression suggest that AA QNBC patients with low HER3-HER4 signaling may also be sensitive to retinoid therapy. BAP1 acts a tumor suppressor and plays a key role in the DNA damage response pathway. Hence, upregulation of BAP1 among low HER3-HER4-expressing AA QNBC patients may be indicative of increased DNA damage among this patient population suggesting they may be susceptible to DNA repair targeted therapy.

We also discovered that low HER3-HER4 score significantly predicted poorer 10-year OS among early-stage AA QNBC patients in Kaplan-Meier analysis. Furthermore, low HER3-HER4 score predicted shorter 10-year OS among early-stage AA QNBC patients in multivariate analyses. Thus, these findings suggest that a lack of HER3-HER4 expression may be associated with more aggressive disease among early-stage AA TNBC patients lacking AR expression and that HER3-HER4 score may serve as an alternative risk-prognostic biomarker for this patient population.

Our gene expression analyses revealed that HER4 gene expression was significantly more downregulated among AR-low compared to AR-high AA TNBC samples in TCGA dataset, which corroborates our protein expression results. Furthermore, we observed significantly more downregulation of signaling pathways and gene ontologies reflecting downstream signaling of HER4 as well as genes encoding key downstream signaling molecules in the HER4 pathway among AR-low compared to AR-high AA TNBC samples. We also
observed significantly more downregulation of gene ontologies and genes encoding key
downstream proteins in processes modulated by HER4 signaling such as apoptosis, cell
proliferation, and cell differentiation among AR-low compared to AR-high AA TNBC samples.
This finding suggest that more downregulation of HER4 signaling may reflect less cell death as
well as more aberrant cell proliferation and differentiation among AR-low compared to AR-high
AA TNBC patients with low HER4 signaling. In addition, we observed significantly more
downregulation of biological pathways and gene ontologies reflecting pro-inflammatory activity
and cell-cell contact among AR-low compared to AR-high AA TNBC samples suggesting
suppressed antitumoral immune response and increased metastatic potential among AR-low AA
patients with low HER4 signaling. However, we discovered significantly more upregulation of
biological pathways and gene ontologies indicating increased cell cycle progression, cell
proliferation, and DNA damage response among AR-low compared to AR-high AA TNBC
patients. This finding also reflects our protein expression correlation results indicating that lack
of combined HER3-HER4 score was associated with high FOXM1 and BAP1 expression further
emphasizing that therapies targeting cell cycle progression and DNA damage response may be
selectively beneficial for AA QNBC patients with a low HER3-HER4 score.

Our study uncovers a valuable risk-prognostic biomarker for AA QNBC patients in early-
stage disease. Our findings suggest that HER3-HER4 score should be assessed among AA
TNBC patients lacking AR expression in the clinic to guide clinical decision-making and
segment patients into optimal treatment paths. Furthermore, our results suggest alternative
therapeutic strategies for AA QNBC patients with low HER4 signaling such as FOXM1
inhibition, cytotoxic chemotherapy, and other cell-cycle progression-targeting therapies as well
as DNA damage response inhibition such as platinum-based agents. Thus, stratifying AA QNBC
patients into high and low HER3-HER4 score may notably improve risk-prognostication and prognosis among this patient population and subsequently help reduce the racial disparate burden in TNBC. However, robust validation of our findings in additional cohorts is necessary in order to successfully achieve these aims.

10.5 Methods

10.5.1 Study Cohort

We analyzed a cohort of 121 TNBC patients observed at EH in Atlanta, GA from 2002 (initial day of diagnosis) to 2016 (last day of contact). We isolated 77 AA TNBC patients from this cohort and stratified them into AR-negative and AR-positive subgroups based on IHC AR nuclear percentage. Negativity was determined as $<1\%$ expression and positivity was determined as $\geq 1\%$ expression. We obtained approval and permission from the institutional review board at EH to gain access to patient information and samples used in this study. Patient demographic characteristics, clinico-pathological variables, and breast cancer biomarker status were recorded for each patient. This article referred to the 7th edition of the American Joint Committee on Cancer)/Union for International Cancer Control TNM Classification and Stage groupings for breast cancer (52). Demographic characteristics include self-reported race and age at the time of diagnosis. Adjuvant chemotherapy treatment was recorded. No patients in this study underwent neoadjuvant chemotherapy. Regarding adjuvant chemotherapy, 54 patients underwent treatment and 9 patients did not.

10.5.2 Immunohistochemistry and scoring

Biomarker expression was evaluated through standard IHC staining and scored as H-score (percentage x intensity), NH-score (nuclear percentage x intensity), and Nper (nuclear percentage). Intensity of staining were scored as 0=none, 1=low, 2=moderate, and 3=high.
Negativity was determined as <1% expression and positivity was determined as ≥1% expression for all biomarkers. Antibody details and concentrations can be found in Table 8. The maximum score for any given H-score is 300 and for combined (summed) H-scores is 600. Combined biomarker scores were determined by summing the scores for each biomarker to yield a single score. EH patient samples were stained, scored, and reviewed at EH. Scoring was performed by two independent pathologists blinded to clinical annotation and individual scores were averaged.

10.5.3 Follow Up

Initial diagnosis and follow-up of patients occurred between 2002 and 2016. Initial dates of diagnoses, treatment start and completion dates, and last dates of contact were recorded for each patient. Survival status (alive/dead) was also recorded for each patient in addition to survival time. The date of last follow-up for the last patient seen is March 3, 2016.

10.5.4 Gene Expression Dataset

We queried the publically available The Cancer Genome Atlas (TCGA) breast dataset from the TCGA portal for all TNBC patients. Publically available ER/PR/HER2/AR IHC data was used to determine TNBC and QNBC status. According to the available race data, we selected AA patients out of the TNBC samples. We performed a log-rank test to identify the optimal cut-off to stratify the AA TNBC patients into high- and low- AR expressing subgroups. We performed differential gene expression analysis between AR-low and AR-high expressing AA TNBC samples using the DESeq2 software tool. Differentially expressed genes with a \( p \) value of <0.05 and log2fold change of above +1 and below -1 were selected in this study. Differential expression analyses of Kyoto Encyclopedia of Genes and Genomes pathways and gene ontologies between the patient groups were predicted by GAGE and Pathview packages,
respectively(55, 56). A heat map was generated to depict the differentially expressed genes among the patient groups of interest.

10.5.5 Statistical Analysis

The significance level for all analyses was p<0.05 with 95% confidence intervals (CIs). SAS 9.4 program was used to generate test statistics and 2-tailed univariate p-values were reported. Chi-square tests were performed to analyze differences in demographic characteristics, breast clinico-pathological variables and biomarkers, as well as treatment information between AR-negative and AR-positive AA TNBC patients. Wilcoxon rank-sum tests were performed to determine differences in the means in expression of the EGFR family members between AR-negative and AR-positive AA TNBC patients. Spearman’s rank correlation coefficients (ρ) were computed to determine associations of the EGFR family members with demographic and clinico-pathological variables as well as biomarker expression among AR-negative AA TNBC patients. Unadjusted and adjusted multivariate Cox proportional hazard models were computed to assess the impact of combined IHC HER3-HER4 score on 10-year OS before and after controlling for age, Nottingham grade, and stage. The Kaplan-Meier analysis was conducted using SAS 9.4 program to estimate survival function for AR-negative AA TNBC patients over a 10- year period based on high and low combined IHC HER3-HER4 score. A log-rank test was used to stratify combined IHC HER3-HER4 expression with better or worse survival in Kaplan-Meier analyses. A log-rank test was also performed to identify the appropriate cut-off for low and high expression of AR among AA TNBC patients in the TCGA dataset. The Wald test was used to test for significance among differentially expressed genes between the patient groups using the DESeq2 software tool.
10.6 Tables and Figures
Table 10.1 Clinico-pathological characteristics of AA QNBC and TNBC patients observed at EH.

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*p* values were calculated using the chi-square test.
Table 10.2 Correlation of combined HER3-HER4 score with clinico-pathological variables among AA QNBC patients.

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<td>&lt;i&gt;n&lt;/i&gt;</td>
<td>48.000</td>
<td>48.000</td>
<td>48.000</td>
</tr>
<tr>
<td>Early Chemo</td>
<td></td>
<td>-0.316</td>
<td>-0.366</td>
<td>0.238</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;p&lt;/i&gt; value</td>
<td>0.078</td>
<td>0.040</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;n&lt;/i&gt;</td>
<td>32.000</td>
<td>32.000</td>
<td>32.000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pearson correlation test (p)

<sup>b</sup>Pearson correlation test (p)

Abbreviations: Chemo, adjuvant chemotherapy; SLN, sentinel lymph node.
Table 10.3 Correlation of combined HER3-HER4 score with clinico-pathological biomarkers among AA QNBC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Clinico-pathological biomarkers</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BAP1 (Nhscore)$^b$</td>
<td>FOXM1 (Nper)$^a$</td>
<td>RARα (Nper)$^b$</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>0.004</td>
<td>-0.322</td>
<td>-0.326</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.975</td>
<td>0.020</td>
<td>0.014</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>52.000</td>
<td>52.000</td>
<td>56.000</td>
</tr>
<tr>
<td>Chemo</td>
<td></td>
<td>-0.227</td>
<td>-0.315</td>
<td>-0.275</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.149</td>
<td>0.069</td>
<td>0.095</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>42.000</td>
<td>34.000</td>
<td>38.000</td>
</tr>
<tr>
<td>Early</td>
<td></td>
<td>-0.294</td>
<td>-0.315</td>
<td>-0.361</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.043</td>
<td>0.040</td>
<td>0.014</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>48.000</td>
<td>43.000</td>
<td>46.000</td>
</tr>
<tr>
<td>Early Chemo</td>
<td></td>
<td>0.014</td>
<td>-0.330</td>
<td>-0.297</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.946</td>
<td>0.093</td>
<td>0.111</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>27.000</td>
<td>27.000</td>
<td>30.000</td>
</tr>
</tbody>
</table>

$^a$Pearson correlation test (p)
$^b$Pearson correlation test (p)

Abbreviations: Chemo, adjuvant chemotherapy; Nh-score, nuclear percentage x intensity; Nper, nuclear percentage.
Table 10.4 Prediction of combined HER3-HER4 score for 5-year OS among AA QNBC patients.

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted model</th>
<th>Adjusted model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early and late stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.130; 0.456 (0.165, 1.261)</td>
<td>0.057; 0.31 (0.093, 1.035)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.590; 0.682 (0.169, 2.746)</td>
<td>0.932; 0.932 (0.186, 4.684)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.240; 0.383 (0.077, 1.901)</td>
<td>0.934; 1.092 (0.135, 8.810)</td>
</tr>
<tr>
<td><strong>Early-stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>0.064; 0.269 (0.067, 1.080)</td>
<td>0.054; 0.251 (0.062, 1.025)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0.476; 0.490 (0.501, 3.563)</td>
<td>0.616; 0.554 (0.055, 5.588)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.155; 0.175 (0.016, 1.933)</td>
<td>0.229; 0.212 (0.017, 2.652)</td>
</tr>
</tbody>
</table>

**Abbreviations**: HR, hazard ratio; CI, confidence interval; Cox proportional hazard model adjusted for age, Nottingham grade, and stage. Peripheral TILs were treated as a categorical variable.
Table 10.5 KEGG pathways upregulated and downregulated among AR-low compared to AR-high expressing AA TNBC patients in TCGA breast dataset.

<table>
<thead>
<tr>
<th>Upregulated KEGG pathway</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa03010 Ribosome</td>
<td>0.0000000004438</td>
</tr>
<tr>
<td>hsa00190 Oxidative phosphorylation</td>
<td>0.0000000096371</td>
</tr>
<tr>
<td>hsa03040 Spliceosome</td>
<td>0.0000004781674</td>
</tr>
<tr>
<td>hsa03013 RNA transport</td>
<td>0.0001140021000</td>
</tr>
<tr>
<td>hsa03008 Ribosome biogenesis in eukaryotes</td>
<td>0.0001432472000</td>
</tr>
<tr>
<td>hsa03050 Proteasome</td>
<td>0.0003684588000</td>
</tr>
<tr>
<td>hsa00240 Pyrimidine metabolism</td>
<td>0.0008063210000</td>
</tr>
<tr>
<td>hsa03020 RNA polymerase</td>
<td>0.0032212900000</td>
</tr>
<tr>
<td>hsa00460 Base excision repair</td>
<td>0.0041222170000</td>
</tr>
<tr>
<td>hsa03030 DNA replication</td>
<td>0.0042567460000</td>
</tr>
<tr>
<td>hsa04110 Cell cycle</td>
<td>0.0054582490000</td>
</tr>
<tr>
<td>hsa03015 mRNA surveillance pathway</td>
<td>0.0154743000000</td>
</tr>
<tr>
<td>hsa04141 Protein processing in endoplasmic reticulum</td>
<td>0.0165161000000</td>
</tr>
<tr>
<td>hsa03018 RNA degradation</td>
<td>0.0169616200000</td>
</tr>
<tr>
<td>hsa04330 Mismatch repair</td>
<td>0.0170214100000</td>
</tr>
<tr>
<td>hsa03060 Protein export</td>
<td>0.0223094700000</td>
</tr>
<tr>
<td>hsa00270 Cysteine and methionine metabolism</td>
<td>0.0247532400000</td>
</tr>
<tr>
<td>hsa03420 Nucleotide excision repair</td>
<td>0.0249236000000</td>
</tr>
<tr>
<td>hsa00970 Aminoacyl-RNA biosynthesis</td>
<td>0.0345843000000</td>
</tr>
<tr>
<td>hsa04512 ECM-receptor interaction</td>
<td>0.00000000000000</td>
</tr>
<tr>
<td>hsa04510 Focal adhesion</td>
<td>0.0000116052400</td>
</tr>
<tr>
<td>hsa04514 Cell adhesion molecules (CAMs)</td>
<td>0.0000233545700</td>
</tr>
<tr>
<td>hsa04640 Hematopoietic cell lineage</td>
<td>0.0001187330000</td>
</tr>
<tr>
<td>hsa04020 Calcium signaling pathway</td>
<td>0.0003178624000</td>
</tr>
<tr>
<td>hsa04670 Leukocyte transendothelial migration</td>
<td>0.0012202528000</td>
</tr>
<tr>
<td>hsa04610 Complement and coagulation cascades</td>
<td>0.0015654000000</td>
</tr>
<tr>
<td>hsa04974 Protein digestion and absorption</td>
<td>0.0024462100000</td>
</tr>
<tr>
<td>hsa02010 ABC transporters</td>
<td>0.0025390000000</td>
</tr>
<tr>
<td>hsa04380 Osteoblast differentiation</td>
<td>0.0030851330000</td>
</tr>
<tr>
<td>hsa03260 Tyrosine metabolism</td>
<td>0.0034737900000</td>
</tr>
<tr>
<td>hsa04062 Chemokine signaling pathway</td>
<td>0.0048938370000</td>
</tr>
<tr>
<td>hsa04672 Intestinal immune network for IgA production</td>
<td>0.0127061000000</td>
</tr>
<tr>
<td>hsa04270 Vascular smooth muscle contraction</td>
<td>0.0131620400000</td>
</tr>
<tr>
<td>hsa04540 Gap junction</td>
<td>0.0145879000000</td>
</tr>
<tr>
<td>hsa04010 MAPK signaling pathway</td>
<td>0.0166225000000</td>
</tr>
<tr>
<td>hsa04810 Regulation of actin cytoskeleton</td>
<td>0.0192974200000</td>
</tr>
<tr>
<td>hsa00830 Retinol metabolism</td>
<td>0.0221452100000</td>
</tr>
<tr>
<td>hsa04630 Jnk-STAT signaling pathway</td>
<td>0.0227415200000</td>
</tr>
<tr>
<td>hsa04660 T cell receptor signaling pathway</td>
<td>0.0352655000000</td>
</tr>
<tr>
<td>hsa04662 B cell receptor signaling pathway</td>
<td>0.0376589500000</td>
</tr>
<tr>
<td>hsa00062 Drug metabolism - cytochrome P450</td>
<td>0.0446042800000</td>
</tr>
<tr>
<td>hsa04972 Pancreatic secretion</td>
<td>0.0453019000000</td>
</tr>
</tbody>
</table>
### Table 10.6 Gene ontologies upregulated among AR-low compared to AR-high AA TNBC patients.

<table>
<thead>
<tr>
<th>Upregulated gene ontologies</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0000067 M phase of mitotic cell cycle</td>
<td>3.03E-07</td>
</tr>
<tr>
<td>GO:0007006 ribosome</td>
<td>4.20E-07</td>
</tr>
<tr>
<td>GO:0046281 DNA repair</td>
<td>2.74E-08</td>
</tr>
<tr>
<td>GO:0000075 cell cycle checkpoint</td>
<td>1.02E-05</td>
</tr>
<tr>
<td>GO:0006744 response to DNA damage stimulus</td>
<td>5.95E-05</td>
</tr>
<tr>
<td>GO:0031529 interphase of mitotic cell cycle</td>
<td>4.03E-05</td>
</tr>
<tr>
<td>GO:0002168 G1/S transition of mitotic cell cycle</td>
<td>6.80E-05</td>
</tr>
<tr>
<td>GO:0007050 cell cycle arrest</td>
<td>8.07E-05</td>
</tr>
<tr>
<td>GO:0002797 M phase</td>
<td>1.03E-05</td>
</tr>
<tr>
<td>GO:0000844 S phase of mitotic cell cycle</td>
<td>1.20E-04</td>
</tr>
<tr>
<td>GO:0070093 mitotic cell cycle checkpoint</td>
<td>2.02E-04</td>
</tr>
<tr>
<td>GO:0051520 S phase</td>
<td>2.02E-04</td>
</tr>
<tr>
<td>GO:0000777 DNA damage checkpoint</td>
<td>1.97E-04</td>
</tr>
<tr>
<td>GO:0031571 mitotic cell cycle G1/S transition DNA damage checkpoint</td>
<td>1.32E-03</td>
</tr>
<tr>
<td>GO:0031575 mitotic cell cycle G1-S transition checkpoint</td>
<td>1.50E-03</td>
</tr>
<tr>
<td>GO:0060917 DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest</td>
<td>1.33E-03</td>
</tr>
<tr>
<td>GO:0030002 G1/S transition of endotic cell cycle</td>
<td>7.17E-03</td>
</tr>
<tr>
<td>GO:0041184 nucleotide-excision repair</td>
<td>2.62E-03</td>
</tr>
<tr>
<td>GO:0040153 transcription-coupled nucleotide-excision repair</td>
<td>3.14E-03</td>
</tr>
<tr>
<td>GO:0051501 cell division</td>
<td>1.34E-03</td>
</tr>
<tr>
<td>GO:0071779 G1/S transition checkpoint</td>
<td>4.55E-03</td>
</tr>
<tr>
<td>GO:0030056 DNA damage response, signal transduction by p53 class mediator</td>
<td>8.61E-03</td>
</tr>
<tr>
<td>GO:0040447 base excision repair</td>
<td>2.91E-02</td>
</tr>
<tr>
<td>GO:0000096 G2/M transition of mitotic cell cycle</td>
<td>1.87E-02</td>
</tr>
<tr>
<td>GO:0071174 regulation of mitotic cell cycle</td>
<td>3.04E-02</td>
</tr>
<tr>
<td>GO:0030070 G2 phase of mitotic cell cycle</td>
<td>2.61E-02</td>
</tr>
<tr>
<td>GO:0001567 centrosome organization</td>
<td>2.84E-02</td>
</tr>
<tr>
<td>GO:0070017 microtubule-based process</td>
<td>7.23E-02</td>
</tr>
<tr>
<td>GO:0060902 double-strand break repair</td>
<td>3.31E-02</td>
</tr>
<tr>
<td>GO:0054218 G2 phase</td>
<td>8.38E-02</td>
</tr>
<tr>
<td>GO:0000070 mitotic sister chromatid segregation</td>
<td>3.46E-02</td>
</tr>
<tr>
<td>GO:0071173 spindle assembly checkpoint</td>
<td>4.43E-02</td>
</tr>
<tr>
<td>GO:0071171 centrosome cycle</td>
<td>3.46E-02</td>
</tr>
<tr>
<td>GO:0070098 centrosome cycle</td>
<td>4.51E-02</td>
</tr>
<tr>
<td>GO:0070095 mitotic metaphase/aphase transition</td>
<td>4.88E-02</td>
</tr>
</tbody>
</table>

### Downregulated gene ontologies

<table>
<thead>
<tr>
<th>Downregulated gene ontologies</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0030334 regulation of cell migration</td>
<td>6.67E-09</td>
</tr>
<tr>
<td>GO:0154274 leukocyte activation</td>
<td>2.47E-09</td>
</tr>
<tr>
<td>GO:0030145 regulation of cell motility</td>
<td>2.68E-08</td>
</tr>
<tr>
<td>GO:0016337 cell-cell adhesion</td>
<td>1.50E-09</td>
</tr>
<tr>
<td>GO:0040948 lymphocyte activation</td>
<td>9.03E-09</td>
</tr>
<tr>
<td>GO:0060204 positive regulation of immune system process</td>
<td>6.75E-08</td>
</tr>
<tr>
<td>GO:0040060 wound healing</td>
<td>1.71E-07</td>
</tr>
<tr>
<td>GO:0071170 T cell activation</td>
<td>3.04E-07</td>
</tr>
<tr>
<td>GO:0030092 regulation of chemotaxis</td>
<td>6.05E-07</td>
</tr>
<tr>
<td>GO:0040534 hematopoietic or lymphoid organ development</td>
<td>7.19E-07</td>
</tr>
<tr>
<td>GO:0020564 regulation of leukocyte activation</td>
<td>8.06E-06</td>
</tr>
<tr>
<td>GO:0060586 chemotaxis</td>
<td>9.20E-06</td>
</tr>
<tr>
<td>GO:0031675 immune system development</td>
<td>2.00E-06</td>
</tr>
<tr>
<td>GO:0031706 G protein coupled receptor signaling pathway</td>
<td>2.18E-06</td>
</tr>
<tr>
<td>GO:0455097 positive regulation of cell differentiation</td>
<td>2.63E-06</td>
</tr>
<tr>
<td>GO:0030285 positive regulation of cell migration</td>
<td>2.71E-06</td>
</tr>
<tr>
<td>GO:0032251 leukocyte differentiation</td>
<td>3.50E-06</td>
</tr>
<tr>
<td>GO:0031281 positive regulation of lymphocyte activation</td>
<td>6.71E-06</td>
</tr>
<tr>
<td>GO:0031284 regulation of lymphocyte activation</td>
<td>9.88E-06</td>
</tr>
<tr>
<td>GO:0031675 regulation of cell adhesion</td>
<td>1.66E-05</td>
</tr>
<tr>
<td>GO:0030081 lymphocyte differentiation</td>
<td>1.34E-05</td>
</tr>
<tr>
<td>GO:0030129 extracellular matrix organization</td>
<td>1.44E-05</td>
</tr>
<tr>
<td>GO:0030129 extracellular matrix organization</td>
<td>1.44E-05</td>
</tr>
<tr>
<td>GO:0031589 cell-substrate adhesion</td>
<td>1.49E-05</td>
</tr>
<tr>
<td>GO:0007048 response to growth factor stimulus</td>
<td>2.42E-05</td>
</tr>
<tr>
<td>GO:0071160 cell-matrix adhesion</td>
<td>2.89E-05</td>
</tr>
<tr>
<td>GO:0060545 inflammatory response</td>
<td>1.29E-05</td>
</tr>
<tr>
<td>GO:0000542 regulation of purines nucleotide metabolic process</td>
<td>3.34E-05</td>
</tr>
<tr>
<td>GO:0013633 cellular response to growth factor stimulus</td>
<td>3.35E-05</td>
</tr>
<tr>
<td>GO:0031748 positive regulation of cell projection</td>
<td>3.05E-05</td>
</tr>
<tr>
<td>GO:0042113 B cell activation</td>
<td>4.12E-05</td>
</tr>
<tr>
<td>GO:0032217 T cell differentiation</td>
<td>5.35E-05</td>
</tr>
<tr>
<td>GO:0050870 positive regulation of T cell activation</td>
<td>5.35E-05</td>
</tr>
<tr>
<td>GO:0070681 leukocyte proliferation</td>
<td>9.90E-05</td>
</tr>
<tr>
<td>GO:0050000 leukocyte migration</td>
<td>1.16E-04</td>
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<tr>
<td>GO:0049591 lymphocyte proliferation</td>
<td>1.16E-04</td>
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<tr>
<td>GO:0001605 MAPK cascade</td>
<td>1.42E-04</td>
</tr>
<tr>
<td>GO:0022906 adaptive immune response</td>
<td>1.48E-04</td>
</tr>
<tr>
<td>GO:0058633 B cell receptor signaling pathway</td>
<td>2.97E-04</td>
</tr>
<tr>
<td>GO:0000295 chemokine-mediated signaling pathway</td>
<td>3.14E-04</td>
</tr>
<tr>
<td>GO:0077169 transmembrane receptor protein tyrosine kinase signaling pathway</td>
<td>3.53E-04</td>
</tr>
<tr>
<td>GO:001811 positive regulation of cell-substrate adhesion</td>
<td>4.97E-04</td>
</tr>
<tr>
<td>GO:0018603 positive regulation of phosphatidylinositol C activity</td>
<td>5.02E-04</td>
</tr>
<tr>
<td>GO:0024229 immune response-activating cell surface receptor signaling pathway</td>
<td>5.04E-04</td>
</tr>
<tr>
<td>GO:0048798 vascular endothelial growth factor receptor signaling pathway</td>
<td>5.06E-04</td>
</tr>
<tr>
<td>GO:0030687 regulation of leukocyte proliferation</td>
<td>5.76E-04</td>
</tr>
<tr>
<td>GO:0050967 positive regulation of phosphate metabolites</td>
<td>5.97E-04</td>
</tr>
<tr>
<td>GO:0046508 cAMP metabolic process</td>
<td>6.11E-04</td>
</tr>
<tr>
<td>GO:0070797 ERK1 and ERK2 cascade</td>
<td>7.95E-04</td>
</tr>
<tr>
<td>GO:0070787 G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger</td>
<td>8.87E-04</td>
</tr>
</tbody>
</table>
Table 10.7 HER4 signaling-related differentially-expressed genes between AR-low and AR-high expression among AA TNBC patients in TCGA breast dataset.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Entrez</th>
<th>Symbol</th>
<th>Name</th>
<th>Base Mean</th>
<th>logFoldChange</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSG00000115252</td>
<td>5138</td>
<td>PDOR1A</td>
<td>phosphodiesterase 1A</td>
<td>31.52415067</td>
<td>-2.07573177</td>
<td>1.678-09</td>
</tr>
<tr>
<td>ENSG00000156889</td>
<td>8243</td>
<td>PHEK2</td>
<td>phosphatidylinositol-3,5-triphosphate-dependent exchange factor 2</td>
<td>17.59517515</td>
<td>-2.52633287</td>
<td>9.151-03</td>
</tr>
<tr>
<td>ENSG00000123800</td>
<td>10994</td>
<td>LYYET</td>
<td>lymphoid enhancer-independent nuclear receptor 1</td>
<td>51.16982087</td>
<td>-3.81116492</td>
<td>4.65E-06</td>
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<tr>
<td>ENSG00000177643</td>
<td>6516</td>
<td>ITGA8</td>
<td>integrin subunit alpha 8</td>
<td>10.68912928</td>
<td>-2.35837258</td>
<td>2.02E-08</td>
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<tr>
<td>ENSG00000150628</td>
<td>5733</td>
<td>PTGER3</td>
<td>prostaglandin E receptor 3</td>
<td>13.33342339</td>
<td>-1.69734272</td>
<td>1.865-07</td>
</tr>
<tr>
<td>ENSG00000184613</td>
<td>4753</td>
<td>NELL2</td>
<td>neural EGF-like 2</td>
<td>136.1770202</td>
<td>-2.00511173</td>
<td>2.68E-07</td>
</tr>
<tr>
<td>ENSG00000660596</td>
<td>7075</td>
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Table 10.8 Antibodies and dilutions used for immunohistochemical biomarker staining at EH.

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<td>Santa Cruz</td>
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Abbreviations: AR, androgen receptor; EGFR, epidermal growth factor receptor; HER3, human epidermal growth factor receptor 3; HER4, human epidermal growth factor receptor 4; FOXM1, forkhead box protein M1; BAP1, BRCA1-associated protein 1; RARα, retinoic acid receptor alpha; H-score, percentage x intensity; Nper, nuclear percentage; NH-score, nuclear percentage x intensity.
Figure 10.1 IHC expression of the EGFR family members among AR-negative and AR-positive AA TNBC patients.

IHC expression of EGFR (membranous), HER3 (membranous), and HER4 (cytoplasmic) as well as combinations of the receptors combined were compared between AR-negative and AR-positive AA TNBC patients. H-score represent expression percentage x intensity. *p<0.05.
Figure 10.2 Combined IHC HER3-HER4 scores among AA QNBC and TNBC patients.

(A) Representative IHC staining of HER3 (membranous) and HER4 (cytoplasmic) images. (B) IHC expression of combined HER3-HER4 scores were compared between all (overall), chemotherapy treated (chemo), and non-chemotherapy treated (non-chemo) AR-negative and AR-positive AA TNBC patients. (C) Combined HER3-HER4 scores were also compared between early-stage (I-II) (early overall) and early-stage chemotherapy-treated (early chemo) AR-negative
and AR-positive AA TNBC patients. Sample size was too small to analyze early-stage non-chemotherapy treated patients. H-score represents expression percentage x intensity. *p<0.05. Images taken at 200x magnification.

Figure 10.3 Combined IHC HER3-HER4 score predicts OS among early-stage AA QNBC patients.

Combined IHC HER3-HER4 H-scores (percentage x intensity) were stratified into high (≥6) and low (<6) subgroups based on log-rank test. Kaplan-Meier curves were generated to estimate the effect of high and low IHC HER3-HER4 score on survival over a 5- and 10- year period among
AA QNBC patients. Prediction of high and low combined IHC HER3-HER4 score on 10-year OS among (A) all, (B) early-stage, and (C) radiation treated early-stage AA QNBC patients. Prediction of high and low combined IHC HER3-HER4 score on 5-year OS among radiation treated early-stage AA QNBC patients (D). *p*<0.05 was set as the level of significance.

### 10.7 References

23. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16(7):1647-55.
11 RETINOIC ACID RECEPTOR ALPHA AS A POSITIVE PROGNOSTIC BIOMARKER IN TRIPLE NEGATIVE BREAST CANCER

11.1 Abstract

Triple negative breast cancers (TNBCs) lack conventional breast cancer targets, thus lack risk-predictive biomarkers and targeted treatment options. RARα stimulates antiproliferative activity in breast cancer by inhibiting cell growth, cell differentiation, and promoting apoptosis. However, the role of RARα signaling in TNBC remains underexplored. We analyzed differences in RARα gene expression between TNBC and non-TNBC patients in two independent publicly-available breast datasets and investigated the prognostic role of nuclear RARα immunohistochemical (IHC) expression, which represents the transcriptionally active form of the biomarker, in TNBC patients treated at Nottingham Hospital (NH) (n=166) and Emory University Hospital (EH) (n=106). TNBCs expressed lower levels of RARα mRNA than non-TNBCs in the METABRIC (n=1,975) and TCGA (n=1,098) breast datasets (p<0.0001). High nuclear RARα IHC expression was an independent predictor of good prognosis in multivariable models in NH (HR=0.501, p<0.05 for BCSS and HR=0.394, p<0.01 for DMFS) and EH (HR=0.223 for BCSS p<0.05) patients. Nuclear RARα IHC expression may provide valuable risk-prognostic information and guide clinical decision making, specifically revealing a subset of TNBC patients with good prognosis. Future studies are needed to evaluate whether this high nuclear RARα group could benefit from RARα agonists or alternative therapeutic strategies and be spared harsher chemotherapeutic regimens.

11.2 Introduction

Triple negative breast cancer (TNBC), a subtype of breast cancer (BC), remains a formidable disease fraught with many challenges that elude successful clinical intervention. The
disease accounts for approximately 20% of all reported BC cases and is defined by a lack of the actionable targets, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2)\(^1\). Thus, this subgroup of BCs is excluded from receiving traditional endocrine and HER2-targeted systemic therapies and lacks risk-predictive biomarkers. Furthermore, TNBC is characterized by a more aggressive disease course typified by higher nuclear grade, clinical stage, proliferation, and increased lymph node, cerebral, and visceral metastases that all collude to result in poorer clinical outcomes compared with non-TNBCs\(^1-6\). Chemotherapy and radiation remain the standard of care for TNBC patients; however, TNBC patients often relapse within the first 5 years after diagnosis, underscoring an unmet need for alternative risk-predictive biomarkers and alternative therapeutic targets for this patient population\(^3\). Younger women and women of West African descent are disproportionately diagnosed with TNBC and also exhibit more aggressive disease features, acquire more aggressive TNBC subtypes, and experience poorer clinical outcomes compared to women of European descent among TNBC patients\(^7-13,14-17\).

Steroid or nuclear hormone receptors constitute a superfamily of transcription regulators that play critical roles in embryonic development, cell growth, cell differentiation, and homeostasis. These unique receptors dimerize in the nuclei to modulate transcription of target genes upon ligand stimulation\(^18,19\). They possess a C-terminal domain that binds to their ligand as well as a highly-conserved N-terminal zinc-finger that facilitates specificity in target DNA sequence or ligand-response element binding. The RAR and retinoic X receptor (RXR) families represent subgroups of the steroid/nuclear receptor superfamily that also comprises receptors that bind to steroid and thyroid hormones and harbor highly-conserved domains that mediate DNA and ligand-binding activities\(^20\). RAR isoforms have been identified including RAR\(\alpha\), RAR\(\beta\), and
RARγ as well as the RXR isoforms including RXRα, RXRβ, and RXRγ based on alternative mRNA splicing. Retinoids are natural and synthetic vitamin A analogues that modulate cell proliferation, differentiation, morphogenesis, metabolism, and apoptosis via RARs and RXRs\textsuperscript{21}. In the presence of retinoids, RAR heterodimerizes with RXR and the RAR/RXR dimer binds to hormone response elements on DNA such as retinoic acid or retinoid X response elements (RAREs and RXREs) to regulate transcription of target genes\textsuperscript{22,23}.

Retinoid signaling via RARs and RXRs have been shown to stimulate antiproliferative activity, unlike ER, by inhibiting cell growth, cell differentiation, and promoting apoptosis in mammary carcinoma cells\textsuperscript{24,25}. Thus, loss of RARβ has been suggested to be an early event in breast carcinogenesis\textsuperscript{21}. High levels of RARα have been observed in ER-positive BC cells, as estrogens upregulate RARα, but not in ER-negative cells\textsuperscript{24,26}. Retinoid signaling via RARα has been demonstrated to mediate growth inhibition of ER-positive BC cell lines but not in ER-negative cell lines owing to low levels of RARα which are thus, considered to be retinoid resistant\textsuperscript{25}. However, Fitzgerald et. al showed that ER-negative BC cells with RARα expression were sensitive to retinoid-stimulated growth inhibition suggesting that RARα expression correlates with retinoid-induced growth inhibition irrespective of ER status\textsuperscript{27}. However, the role of RARα in TNBC remains elusive.

Given its seeming role in suppressing breast tumorigenesis, RARα may serve as a positive prognostic biomarker in TNBC. While RARα levels are lower in TNBC than non-TNBC cells, it is possible that a subset of TNBC patients have higher levels and a more favorable prognosis, given how molecularly heterogeneous TNBCs are\textsuperscript{28,29}. Towards this end, we explored the effect of nuclear RARα levels on survival outcomes in TNBC patients. Nuclear levels were tested because they represent the transcriptionally active form of RARα\textsuperscript{30}. Ultimately, we found
that high mRNA and protein nuclear RARα levels are associated with better survival among TNBC patients in multivariate analyses. We also observed a racial disparity in nuclear RARα levels among BC and TNBC patients. Furthermore, we identified differentially-expressed genes, pathways, and gene ontologies between high and low nuclear RARα-expressing TNBC patients which may be exploited therapeutically. Thus, a subset of TNBC patients with high intratumoral RARα could potentially be spared harsher chemotherapy regimens and may benefit from RARα agonism, whereas TNBC patients with low intratumoral RARα may require more aggressive treatment.

11.3 Results

11.3.1 Nuclear RARα expression levels lower among TNBCs compared to non-TNBCs

Accumulating studies consistently suggest that RARα expression levels are lower among hormone receptor (HR)-negative compared to HR-positive breast tumor phenotypes suggesting that low levels of the protein could be conferring a more aggressive disease course. Hence, we wanted to further explore differences in nuclear RARα expression levels between TNBC and non-TNBC patients. We analyzed the METABRIC (discovery) and TCGA (validation) BC datasets to compare normalized RARα expression levels between TNBC and non-TNBC patients. As consistent with the literature, we observed significantly lower RARα expression among TNBC compared to non-TNBC patients in both the discovery and validation cohorts (Figure 1). In both the METABRIC (Fig 1A) and TCGA (Fig 1B) datasets, RARα expression levels were approximately one third lower among TNBCs than non-TNBCs (p<0.0001 and p<0.0001, respectively). Thus, our findings confirm literature evidence supporting reduced RARα signaling among TNBCs compared to non-TNBCs in two independent breast cancer
datasets and warrant further exploration of the role of RARα signaling in conferring aggressive BC.

11.3.2 Nuclear RARα expression levels are lower among TNBC patients of African compared to European descent

As previously mentioned, AA BC patients are significantly more likely to present with TNBC compared to EA BC patients. Hence, we investigated whether racial disparities exist in RARα signaling among BC and TNBC patients. Race information was unavailable for the METABRIC dataset, so we compared RARα gene expression levels between AA and EA patients only in TCGA dataset. We discovered significant differences in RARα expression among AA compared to EA BC patients in the TCGA dataset (Figure 2). RARα gene expression was significantly lower among AA (n=192) compared to EA (n=770) BC patients (p=0.003) (Fig 2A). Mean RARα gene expression was also lower in AAs (n=41) compared to EAs (n=87) among TNBC patients but did not reach statistical significance (p=0.620) (Fig 2B).

We also compared nuclear RARα protein expression levels between racially distinct TNBC patients in clinical datasets. We screened three ethnically-distinct patient populations [European (n=166), EA (n=29), and AA (n=71)] from the NH and EH datasets, respectively, for differences in expression of nuclear RARα IHC expression by performing a one-way ANOVA test. We discovered significant differences in the marker between all three patient populations suggesting a significant role for this marker in the racially disparate burden in TNBC (Figure 3) (p<0.0001). Specifically, nuclear RARα IHC expression decreased with decreasing self-reported European ancestry from European to AA samples in which Europeans exhibited the highest and AA’s exhibited the lowest expression levels overall and among chemotherapy- and radiation-treated patients. Hence, our findings indicate that racial disparities in RARα signaling exists and
reduce RARα signaling among patients of African descent could be contributing to the racial disparate burden in BC and TNBC.

11.3.3 *High nuclear RARα expression levels predict better prognosis among TNBC patients*

Based on our observed significant differences in RARα gene and nuclear protein expression levels between TNBC and non-TNBC patients, we investigated the impact of nuclear RARα IHC expression on survival among TNBC patients in two independent clinical datasets from NH (n=166) and EH (n=106). We assessed the influence of categorical nuclear RARα (low vs. high) on overall survival (OS), breast cancer specific survival (BCSS), disease-free survival (DFS) and distant metastasis-free survival (DMFS) using the Kaplan-Meier method and Cox proportional hazards regression in TNBC patients from both cohorts. High nuclear RARα H-score was associated with significantly better OS (p<0.001) (Fig 4A), 5-, 10-, and 15-year BCSS (p=0.006, p<0.001, and p=0.005, respectively) (Fig 4B-D), DFS (p<0.001) (Fig 4E), and DMFS (p<0.0001) (Fig 4F) among TNBC patients treated at NH according to Kaplan-Meier analyses (Figure 4). Representative micrograph of nuclear IHC staining can be found in Fig 5A. High nuclear RARα percentage was also associated with better OS (p=0.040) (Fig 5B) and moderately longer BCSS (p=0.090) (Fig 5C) among TNBC patients treated at EH according to the Kaplan-Meier method (Figure 5). We observed similar results using Cox proportional hazard regression models. Nuclear RARα H-score was found to be an independent predictor of better OS, BCSS, DFS, and DMFS among TNBC patients observed at NH in multivariable analysis, where the covariates adjusted for included age at diagnosis, tumor size, Nottingham grade, lymph node stage, and Ki67-labeling index (HR=0.483, p=0.001 for OS, HR=0.464, p=0.040 for 5-year BCSS, HR=0.501, p=0.043 for 10-year BCSS, HR=0.045, p=0.006 for DFS and HR=0.394, p=0.003 for DMFS) (Table 1). Furthermore, nuclear RARα H-score predicted better OS, DFS,
and DMFS irrespective of the same covariates in addition to receipt of adjuvant chemotherapy (HR=0.484, p=0.010 for OS, HR=0.457, p=0.008 for DFS and HR=0.412, p=0.005 for DMFS). Nuclear RARα percentage was also found to be an independent predictor of better OS among TNBC patients observed at EH in multivariate analysis, where the covariates adjusted for included age at diagnosis, Nottingham grade, and Ki67-labeling index (HR=0.223, p=0.0498) (Table 2). We also assessed associations of nuclear RARα expression levels with clinicopathological characteristics among both cohorts. Interestingly, we found that nuclear RARα H-score negatively correlated with lymphovascular invasion (p=-0.192; p=0.015) among TNBC patients observed at NH (Table 3).

11.4 Methods

Publicly-available datasets

We queried the publicly available Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (n=1975; patient characteristics described in 39) and The Cancer Genome Atlas (TCGA) (n=1098; patient characteristics described in 31) gene expression breast datasets from Oncomine for BC and TNBC patients 40. Publicly-available ER/PR/HER2 IHC data was used to determine TNBC status. We selected 167 and 145 TNBC samples from the METABRIC and TCGA datasets, respectively.

Clinical datasets

We procured TNBC patient databases from Nottingham University Hospital (NH) in Nottingham, UK and Emory University Hospital (EH) in Atlanta, GA. NH and EH cohorts were comprised of 166 and 106 TNBC patients, respectively. NH patients were observed from 2007-2013 and EH patients were observed from 2002-2016. We obtained consent and permission from the institutional review boards at each institution to obtain access to patient information used in
this study. Patient demographic characteristics, clinicopathological variables, and treatment information were recorded for NH (Table 4) and EH (Table 5) patients. This article referred to the 7th edition of the American Joint Committee on Cancer/Union for International Cancer Control TNM Classification and Stage groupings for BC. However, only lymph node staging was available in the NH cohort and referred to in this study. Demographic characteristics include self-reported race and age at the time of diagnosis. No EH patients received neoadjuvant chemotherapy.

**Immunohistochemistry and scoring**

Nuclear RARα expression in TNBC patients observed at NH and EH was determined through standard immunohistochemistry (IHC) staining. We immunolabeled primary TNBC specimens for RARα and calculated H-scores as the product of the nuclear staining intensity (0-3) and the percentage of cells with any nuclear staining. Antibody details and concentration can be found in Table 6. NH patient samples were stained, scored, and reviewed at NH. EH patient samples were stained, scored, and reviewed at EH. Scoring was performed by two independent pathologists blinded to clinical annotation and individual scores were averaged.

**Follow up**

Initial diagnosis occurred between 2002 and 2016 for EH patients and between 2007-2013 for NH patients. Median duration of follow-up for each cohort was approximately 6 and 5 years for EH and NH patients, respectively. Initial dates of diagnoses, treatment start and completion dates, and last dates of contact were recorded for each patient. Survival status (alive/dead) was also recorded for each patient in addition to survival time. The date of last follow-up for the last patient seen at EH is March 3, 2016 and NH is August 22, 2011.

**Statistical analyses**
The significance level for all analyses was set to \( p<0.05 \) with 95% confidence intervals. Chi-square tests were performed to analyze differences association of demographic characteristics, breast clinicopathological variables and treatment information with UK, EA, and AA TNBC patients in the NH and EH datasets, respectively. SAS 9.4 program was used to generate test statistics and 2-tailed univariate \( p \)-values were reported. Mann-Whitney U tests was performed to determine differences in nuclear RAR\( \alpha \) normalized gene expression levels between TNBC and non-TNBC patients in the METABRIC and TCGA dataset and between AA and EA BC patients in TCGA dataset. One-way ANOVA tests were performed to determine differences in mean IHC expression of nuclear RAR\( \alpha \) between UK, EA, and AA TNBC patients in the NH and EH datasets, respectively. Unadjusted and adjusted multivariate Cox proportional hazard regression models were also computed to assess the impact of nuclear RAR\( \alpha \) on survival among TNBC patients in the clinical datasets. The Kaplan-Meier analysis was also conducted using SAS 9.4 program to estimate survival function for TNBC patients observed at NH and EH based on high and low expression levels of nuclear RAR\( \alpha \). A log-rank test was used to stratify RAR\( \alpha \) into high and low subgroups to evaluate associations of a high and low expression of nuclear RAR\( \alpha \) with better or worse survival among TNBC patients. In the NH dataset, the optimal, most-significant cut point based on breast cancer specific survival (BCSS) was found using X-tile. The proportional hazards assumption was found to be satisfied because plots of partial residuals against rank time had nearly zero slope or y-intercept. Multivariable Cox models were fit using backward stepwise elimination of covariates with a \( p \)-value >0.10.

11.5 Tables and Figures
Table 11.1 Prediction of nuclear RARα Hscore on clinical outcomes among NH TNBC patients.

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<td>Adjusted model†</td>
<td>Adjusted model‡</td>
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<td>~0.001; 0.434 (0.274, 0.687)</td>
<td>0.001; 0.483 (0.283, 0.824)</td>
<td>0.010; 0.484 (0.279, 0.840)</td>
</tr>
<tr>
<td>5-year BCSS</td>
<td>0.007; 0.429 (0.231, 0.796)</td>
<td>0.040; 0.464 (0.222, 0.966)</td>
<td>0.070; 0.492 (0.234, 1.059)</td>
</tr>
<tr>
<td>10-year BCSS</td>
<td>0.001; 0.396 (0.229, 0.684)</td>
<td>0.043; 0.501 (0.257, 0.979)</td>
<td>0.166; 0.606 (0.299, 1.230)</td>
</tr>
<tr>
<td>15-year BCSS</td>
<td>0.006; 0.490 (0.295, 0.814)</td>
<td>0.181; 0.665 (0.301, 1.225)</td>
<td>0.576; 0.830 (0.430, 1.601)</td>
</tr>
<tr>
<td>DFS</td>
<td>~0.001; 0.406 (0.248, 0.667)</td>
<td>0.006; 0.445 (0.251, 0.790)</td>
<td>0.008; 0.457 (0.257, 0.812)</td>
</tr>
<tr>
<td>DMFS</td>
<td>~0.001; 0.363 (0.215, 0.611)</td>
<td>0.063; 0.394 (0.212, 0.730)</td>
<td>0.095; 0.412 (0.222, 0.764)</td>
</tr>
</tbody>
</table>

†Cox proportional hazard regression model adjusted for age at diagnosis, tumor size, Nottingham grade, lymph node stage, and Ki-67 labeling index.  
‡Cox proportional hazard regression model adjusted for age at diagnosis, tumor size, Nottingham grade, lymph node stage, Ki-67 labeling index.  
Nuclear RARα Hscore treated as a categorical variable.  

Abbreviations: HR, hazard ratio; CI, confidence interval; OS, overall survival; BCSS, breast cancer-specific survival; DFS, disease-free survival; DMFS, distant metastasis-free survival.

Table 11.2 Prediction of nuclear RARα percentage on OS among EH TNBC patients.

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>p-value; HR (95% CI)</th>
<th>p-value; HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted model</td>
<td>Adjusted model†</td>
</tr>
<tr>
<td>OS</td>
<td>0.059; 0.242 (0.056, 1.052)</td>
<td>0.0498; 0.223 (0.050, 0.998)</td>
</tr>
</tbody>
</table>

†Cox proportional hazard regression model adjusted for age at diagnosis, Nottingham grade. Nuclear RARα percentage treated as a categorical variable.  

Abbreviations: HR, hazard ratio; CI, confidence interval; OS, overall survival.

Table 11.3 Correlation of nuclear RARα with clinico-pathological variables among NH patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>p</th>
<th>p value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVI</td>
<td>-0.192</td>
<td>0.015</td>
<td>159</td>
</tr>
</tbody>
</table>

Abbreviations: LVI, lymphovascular invasion.
Table 11.4 Clinico-pathological and treatment characteristics among NH TNBC patients.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White/White British</td>
<td>49</td>
<td>28.91</td>
</tr>
<tr>
<td>Black Caribbean</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Indian</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>116</td>
<td>69.88</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-40</td>
<td>42</td>
<td>25.30</td>
</tr>
<tr>
<td>41-60</td>
<td>101</td>
<td>60.64</td>
</tr>
<tr>
<td>61-80</td>
<td>23</td>
<td>13.66</td>
</tr>
<tr>
<td><strong>Pleomorphism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.84</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>98.16</td>
</tr>
<tr>
<td><strong>Tubule formation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>13.41</td>
</tr>
<tr>
<td>3</td>
<td>142</td>
<td>86.59</td>
</tr>
<tr>
<td><strong>Mitotic index</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>3.05</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>6.1</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>90.85</td>
</tr>
<tr>
<td><strong>Nottingham grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>5.45</td>
</tr>
<tr>
<td>3</td>
<td>156</td>
<td>94.55</td>
</tr>
<tr>
<td><strong>LN Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>57.08</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>30.3</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>12.3</td>
</tr>
<tr>
<td><strong>LN status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>84</td>
<td>55.26</td>
</tr>
<tr>
<td>Positive</td>
<td>68</td>
<td>44.74</td>
</tr>
<tr>
<td><strong>Ki-67 (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-40</td>
<td>41</td>
<td>28.67</td>
</tr>
<tr>
<td>41-80</td>
<td>43</td>
<td>30.07</td>
</tr>
<tr>
<td>81-100</td>
<td>39</td>
<td>21.26</td>
</tr>
<tr>
<td><strong>Tumor size(cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2</td>
<td>67</td>
<td>40.61</td>
</tr>
<tr>
<td>2.1-4</td>
<td>89</td>
<td>53.33</td>
</tr>
<tr>
<td>4.1-5</td>
<td>10</td>
<td>6.06</td>
</tr>
<tr>
<td><strong>LVI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>100</td>
<td>62.69</td>
</tr>
<tr>
<td>Positive</td>
<td>59</td>
<td>37.31</td>
</tr>
<tr>
<td><strong>Adjuvant Chemotherapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>110</td>
<td>69.16</td>
</tr>
<tr>
<td>Yes</td>
<td>49</td>
<td>30.82</td>
</tr>
</tbody>
</table>

*Abbreviations: LN, lymph node; LVI, lymphovascular invasion.*

Table 11.5 Clinico-pathological and treatment characteristics among EH TNBC patients.
Table 11.6 Antibodies and dilutions used for RARα immunohistochemistry biomarker staining.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cohort</th>
<th>Subcellular location</th>
<th>UK</th>
<th>Source</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARα (Hscore)</td>
<td>UK</td>
<td>Nucleus</td>
<td>C-20</td>
<td>Santa Cruz</td>
<td>polyclonal rabbit</td>
<td>1:20</td>
</tr>
<tr>
<td></td>
<td>Emory</td>
<td></td>
<td>C-20</td>
<td>Santa Cruz</td>
<td>polyclonal rabbit</td>
<td>1:20</td>
</tr>
</tbody>
</table>

Abbreviations: RARα, Retinoic acid receptor α; Nper, nuclear percentage.
Figure 11.1 Normalized gene expression levels of RARα differ between TNBCs and non-TNBCs.

Wilcoxon rank sum tests were performed to determine differences in normalized nuclear RARα gene expression levels between TNBC and non-TNBC patients in the (A) METABRIC and (B) TCGA BC datasets, represented in a box and whisker plot. Small circles represent outliers.

Figure 11.2 Normalized gene expression levels of RARα differ between AA vs. EA BC patients.

Wilcoxon rank sum tests were performed to determine differences in normalized RARα gene expression levels between AA and EA (A) BC and (B) TNBC patient in TCGA BC dataset, represented in a box and whisker plot.
Figure 11.3 Nuclear RARα IHC expression differs between biogeographically-distinct TNBC patients.

Box and whisker plot comparing nuclear RARα IHC expression among between AA, EA, and European patients treated at EH and NH, respectively. One-way Anova test was performed to determine significant differences between the racial groups. \( p<0.05 \) was the level of significance. Small circles represent outliers.
Figure 11.4 Nuclear RARα expression is associated with better prognosis among NH TNBC patients.

Kaplan-Meier survival curves were generated to assess the impact of nuclear RARα Hscore on clinical outcomes among NH TNBC patients (n=166). Log-rank tests were performed to identify the optimal cutpoints to stratify patients into high and low nuclear RARα Hscore subgroups for
each clinical outcome. Impact of nuclear RARα Hscore on (A) overall survival (optimal cutpoint=180), 5-year breast cancer specific survival (BCSS) (optimal cutpoint=180), 10-year BCSS (optimal cutpoint=180), (D) 15-year BCSS (optimal cutpoint=200), (E) disease-free survival (optimal cutpoint=180), and distant metastasis-free survival (optimal cutpoint=180).

Figure 11.5 Nuclear RARα expression is associated with better prognosis among EH TNBC patients.
Kaplan-Meier survival curves were generated to assess the impact of nuclear RARα percentage on clinical outcomes among EH TNBC patients (n=106). Log-rank tests were performed to identify the optimal cutpoints to stratify patients into high and low nuclear RARα percentage subgroups for each clinical outcome. Representative micrographs of nuclear RARα IHC staining (A). Impact of nuclear RARα Hscore on (B) overall survival (optimal cutpoint=60) and (C) breast cancer specific survival (optimal cutpoint=80).

11.6 References


12 DISCUSSION

Women of African descent continue to disproportionately suffer from BC compared to women of other ethnic backgrounds worldwide. This global racially disparate burden in BC can be largely attributed to distinctions in inherent tumor biology between patients of African ancestry and other ethnicities. African and AA women are more likely to acquire aggressive breast tumor phenotypes such as TNBC and QNBC, which lack expression of pharmacologically-targetable biomarkers, leaving this patient population with no approved targeted therapeutic options. My work seeks to address inherent tumor biological differences between breast tumors of African and European descent to ascertain more aggressive tumor biology among African patients. This work may also proffer alternative risk-predictive biomarkers and therapeutic targets for this patient population to improve their clinical disease course. My work proposes a three-pronged strategy to addressing inherent differences in breast tumor biology between ethnically-distinct patient populations.

ITH has been widely reported to be the culprit of the acquisition of aggressive phenotypes and drug resistance and has been shown to be more prevalent among BC and TNBC patients of African descent. However, quantitating and therapeutically targeting ITH remains a challenge in the clinic. My first approach involves investigating racial disparities in key drivers of ITH, mitotic propensity and CA, among BC patients. Mitotic propensity captures the frequency of actively dividing cells among the proliferating cell population. Increased mitotic turnover can lead to increased erroneous mitosis and thus, the generation of diverse cellular phenotypes or ITH. Our laboratory recently proposed a novel metric termed, M:P ratio, that can potentially allow clinicians to quantitate mitotic propensity in a clinical setting. Hence, we
proposed analyzing differences in M:P ratio between breast tumors of African and European
descent and the role of M:P ratio in a more aggressive disease course among AA BC patients.

We discovered this metric to be higher among early-stage AA compared to EA BC patients and
that among several confounding variables (i.e. race, grade, stage, receptor status), race was the
only factor significantly associated with M:P ratio. CA is a hallmark of cancer cells and recently
reported to be associated with more aggressive BC such as high grade, stage, Ki67, and a TNBC
phenotype. CA has been suggested to be a key driver of ITH through inducing aberrant
multipolar mitosis and subsequently increased aneuploidy. Measuring centrosomal aberrations is
feasible in the clinic through non-invasive methods such as fine-needle aspirate cytology and
IHC. Furthermore, amplified centrosomes can be targeted therapeutically through cancer cell-
selective agents such as putative centrosome declustering drugs (i.e. griseofulvin and noscapine),
commercially available HSET inhibitors (i.e. CW069 and AZ82), and poly-ADP ribose
polymerase inhibitors (i.e. GF-15). Hence, we also compared centrosomal profiles between
breast tumors of African and European descent and investigated the role of CA in a more
aggressive disease course among AA patients. Our findings collectively suggest that women of
African descent may exhibit enhanced mitotic propensity and greater centrosomal aberrations
compared to women of European descent, which may underlie the acquisition of more aggressive
phenotypes among AA BC patients. Our work also demonstrated that QNBCs, more prevalent
among women of African descent, may exhibit greater centrosomal aberrations than TNBCs and
underlie a more aggressive disease course in this group of TNBCs. This work suggests that BC
patients of African descent may be selectively susceptible to CA-targeting agents and both CA
and M:P ratio may be able to improve risk-stratification of this patient population in the clinic for
optimal treatment paths.
The tumor immune microenvironment has been implicated in disease progression and drug resistance. Emerging evidence suggest that disparities in the presence of inflammatory mediators exists among breast tumors of African and European descent and may be implicated in the stark racial disparity in survival. Cancer immunotherapy has emerged as one of the leading therapeutic strategies for cancer. TILs (i.e. T, B, and NK cells) are key mediators in the antitumoral inflammatory response and have been harnessed for therapeutic purposes. The presence of TILs have been associated with better clinical outcomes and pCR post neoadjuvant chemotherapy but more aggressive disease, such as TNBC. Hence, we investigated differences in the presence of lymphocytic infiltration among AA and EA TNBC patients. Our findings suggest patients of African descent may exhibit a greater presence of TILs compared to patients of European descent and that high TIL levels were associated with longer OS and DFS among AA patients irrespective of confounding variables. This racial disparity and survival trend persisted among QNBC patients. TIL levels were also discovered to be higher among AR-negative compared AR-positive TNBCs and a greater presence of TILs were associated with more favorable clinical outcomes among QNBCs patients. Thus, my work has uncovered a previously unrecognized racial disparity in the breast tumor immune microenvironment that may be exploited therapeutically to help reduce the racially disparate burden in breast cancer. Adoptive T Cell therapy has emerged as a promising immunotherapeutic strategy to augment TIL levels to improve patient outcomes. Thus, this work suggest patients of African descent with low TIL levels may be selectively susceptible to TIL immunotherapy. Furthermore, TIL levels, which can be easily assessed in the clinic via H&E staining and quantitated according to the International TILs Working Group 2014 recommendations, may help risk-stratify TNBC patients of African ancestry for ideal treatment paths.
As previously mentioned, the greater incidence of triple and quadruple negative receptor statuses among breast cancer patients of African descent has left this patient population with no approved targeted therapies, underlying their more aggressive disease course and poorer prognosis. This lack has increased my interest in investigating alternative growth receptors that can be therapeutically targeted and potentially adapted for enhanced risk-prognostication among this patient population. As TNBCs and QNBCs are characterized by a lack of HER2 expression, I wanted to investigate racial disparities in the expression of the other three members in the HER family including, EGFR, HER3, and HER4 and their potential as alternative risk-prognostic and therapeutic targets among TNBC patients. Interestingly, in addition to HER2, the other three HER family members, also showed lower expression among TNBC patients of African compared to TNBC patients of European descent. Particularly, HER4, which has been demonstrated to elicit antiproliferative activity, and EGFR-HER4 expression directly decreased with increasing presumed African ancestry with Nigerian TNBC patients exhibiting the lowest expression. EGFR and HER4 mRNA expression was also found to be expressed lower among TNBC patients of African compared to European descent. Furthermore, lack of HER4 and EGFR-HER4 expression was associated with a poor prognosis among TNBC patients of both African and European ancestry suggesting that lack of HER4 signaling among Africans may be underlying their more aggressive disease course compared to Europeans. Thus, in addition to ER, PR, and HER2, lack of HER4 among TNBC patients may predict a poor prognosis suggesting it may be useful to also assess HER4 expression among TNBC patients, particularly of African descent, to guide clinical decision-making. Moreover, we discovered through gene expression analysis, that PPAR signaling was more upregulated among TNBC patients low in EGFR-HER4 mRNA expression suggesting the PPAR pathway could be exploited for alternative therapeutic
purposes among TNBC patients lacking HER4 signaling. We also investigated the role of an additional receptor in the nuclear retinoid family, RARα, as another potential alternative therapeutic target or prognostic biomarker among TNBC patients of African descent. As opposed to most of the HER family members, RARα signaling has been reported to suppress tumor growth and proliferation. Hence, we also observed significantly lower IHC expression of RARα among TNBC patients of African compared to European descent suggesting lack of tumor growth suppression via RARα signaling may also be contributing to disproportionately higher mortality rates observed among African TNBCs. Furthermore, lack of nuclear RARα IHC expression was able to predict poor prognosis among TNBC patients in two independent cohorts. Thus, my findings also suggest that it may be beneficial to assess nuclear RARα expression among TNBC patients, particularly of African descent, in the clinic to improve risk-prognostication.

BC in Africans continues to be characterized by what it lacks making it challenging for clinicians to manage this patient population. My work proposes a three-pronged approach in identifying novel actionable biomarkers associated with African ancestry that can improve risk-prognostication and treatment for this population. In addition to the lack of ER, PR, HER2, and AR expression being disproportionately higher among BC patients of West African compared to European descent, we discovered an additional biomarker, HER4, to previously unrecognized to be expressed lower among Nigerian and AA TNBCs compared to European and EA TNBCs and its lack of IHC expression associated with a poorer prognosis. Hence, we discovered another novel biomarker disproportionately lacking in expression among TNBC patients of African ancestry. However, owing to its antiproliferative activity as opposed to the other biomarkers, HER4 may be exploited therapeutically among patients of African descent lacking HER4
expression to improve their clinical outcomes. Future studies delineating mechanisms and pathways upregulated among low HER4-expressing patients may also be exploited therapeutically to improve outcomes in this TNBC patient population. Our multi-pronged approach also uncovered a previously unrecognized racial disparity in levels of lymphocytic infiltration between ethnically-distinct TNBC patients and revealed that TILs may also improve risk-prognostication for TNBC of African descent in the clinic. Moreover, this work has suggested a novel, cancer immunotherapeutic approach to treating TNBC in AA patients, such as adoptive T cell therapy, which is currently being tested in clinical trials for BC. With ITH being a major interference in the clinic in regards to treatment and being reported to be more prevalent among BC patients of African compared to European descent, targeting ITH may be critical to reducing disproportionate mortality rates among this patient population. However, measuring and counteracting ITH in the clinic presents with many obstacles and challenges. Lastly, my work has also revealed potential racial disparities in key drivers of ITH, mitotic propensity and CA, that can more easily assess levels of ITH and be therapeutically targeted in a clinical setting, suggesting a more clinically-facile strategy to reducing ITH and subsequently an aggressive disease course among BC patients of African descent. Collectively, my findings proffer alternative, actionable biomarkers that may circumvent obstacles in bridging the gap in survival rates between BC patients of 4African and European descent and allow patients of West African descent, disproportionately lacking in conventional actionable BC biomarkers, with alternative treatment options. However, validation of our findings in additional cohorts and further investigation into the mechanisms causing disparities in these novel biomarkers will be necessary to achieving these aims.

**Future Directions**
This work primarily seeks to augment clinical management and risk-prognostication of breast cancer patients of African descent, however it has broader clinical implications. Discovery of previously unrecognized racial distinctions in breast tumor biology may lead to the identification of novel therapeutic strategies for aggressive breast cancer patients irrespective of ethnicity, which may attenuate global breast cancer mortality. Furthermore, this work inspires investigation into alternative avenues of addressing the global racially disparate burden in breast cancer through -omic approaches such as metabolomics, proteomics, and epigenomics. These novel technologies may uncover unique distinctions in breast tumor biology between racially-distinct patients and spur the development of novel therapeutic strategies for disadvantaged populations. Additionally, this work sparks interest into the investigation of the role of non-genetic risk factors in the global racial divide in mortality, such as lifestyle and environment, as well as the interplay between these risk factors and alterations in the genetic landscape of racially-distinct populations. Ultimately, our findings as well as other breakthroughs in cancer health disparity research provide an incentive to increase personalization of patient care according to each individual’s unique tumor genetic, epigenetic, and metabolic profiles to more efficaciously reduce the global breast cancer burden.