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Quadruple Negative Breast Cancers (QNBC) Demonstrate Subtype Consistency among Primary and Recurrent or Metastatic Breast Cancer

Anusha Angajala[*,1], Essynce Mothershed[*,1], Melissa B. Davis[†], Shweta Tripathi[*], Qinghua He[‡], Deepa Bedi[§], Windy Dean-Colomb[¶] and Clayton Yates[*]

[*]Department of Biology and Center for Cancer Research, Tuskegee University, Tuskegee, AL, 36088; [†]Department of Surgery, Weill Cornell Medicine, New York, NY, USA 10065; [‡]Department of Chemical Engineering, Auburn University, AL 36849; [§]Department of Biological Sciences, College of Veterinary Medicine, Tuskegee University, AL; [¶]Department of Hematology/Oncology, Our Lady of Lourdes Regional Medical Center, Lafayette, LA, USA

Abstract

PURPOSE: Despite the availability of current standards of care treatments for triple negative breast cancer (TNBC), many patients still die from this disease. Quadruple negative tumors, which are TNBC tumors that lack androgen receptor (AR), represent a more aggressive subtype of TNBC; however, the molecular features are not well understood. *METHODS:* Immunohistochemistry of estrogen receptor (ER), progesterone receptor (PR), HER2, and AR was determined in 244 primary and 630 recurrent/metastatic site biopsies. Expression was correlated with a panel of 25 cancer-related genes and proteins by IHC and in situ hybridization (ISH). *RESULTS:* We observed that 80.2% (65 of 81) of primary TNBC tumors and 75.7% (159 of 210) of recurrent/metastatic TNBC tumors are QNBC. Bivariate fit analysis demonstrated that QNBC (n = 224) significantly ($P < .03$) correlated with younger aged patients at initial biopsy compared to AR positive TNBC patients (n = 51). In paired primary tissue samples and primary to recurrent/metastatic samples, at least 70% Luminal, HER2 enriched, and QNBC subtype did not change molecular profile. But, TNBC seems to be the "unstable" subtype. Within the total cohort, discordance in molecular profiles was identified in both synchronous (20%) and asynchronous (21%) intra-individual analyses. Irrespective of sample type, (Synchronous or Asynchronous), QNBC demonstrated higher concordant than TNBC. IHC and ISH results of the cancer related genes, demonstrated that gene/protein expression differ by molecular profile: TNBC (HR-/HER2-, AR+) and QNBC (HR-/HER2-, AR-). IHC in metastatic tumors, showed that the percentage of tumors positive of EGFR were higher, while PTEN and TLE3 were lower in QNBC compared to TNBC. *CONCLUSION:* Standard treatment of Breast Cancer (BC) relies on reliable assessment by IHC analysis of ER, PR, and HER2. Our analyses suggest that the heterogeneity of TNBC is at least partially associated with the presence or absence of AR expression, suggesting that QNBC should be considered as a clinically relevant BC subtype. IHC analysis of AR appears to be a practical assay to determine the most aggressive TNBC subtypes and identifies tumors that could benefit from available targeted therapies.

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Introduction

Nearly 30% of women diagnosed with early-stage breast cancer will develop metastatic disease. Discordance in ER, PR and HER2 expression between primary and metastatic breast cancer has been frequently reported [1–5]. Several studies have demonstrated that serial biopsies of tumors across a patient's cancer continuum can result in varying expression of these standard biological markers. In case of hormone receptor positive cancer, ER, PR and Her2 discordance

Address all correspondence to: Clayton Yates, PhD, Department of Biology and Center for Cancer Research, Tuskegee University, Carver Research Foundation, Rm # 22, Tuskegee, Alabama 36088, USA. E-mail: cyates@tuskegee.edu
[1]These authors contributed equally to this work.
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between primary tumor to metastasis site is 12.7%, 38.3% and 15.1% respectively [6]. Furthermore, differences in these standard biomarkers between primary and metastatic tumors has been associated with differences in outcomes; thus, making the selection of the optimal treatment in these patients very complex [7]. As a result, the National Cancer Consortium Network (NCCN) guidelines suggest that additional biopsies should be done at the time of each recurrence. Unfortunately, the acquisition of fresh tissue from suspected breast cancer metastases are not always performed in routine practice. Therefore, therapeutic decisions in the metastatic setting are often based on the features of the primary tumor. This can have a significant impact on patient outcomes, especially when more favorable hormone-receptor positive breast cancers switch and become the more aggressive TNBC phenotype, which occurs in about 20% of cases [8]. Thus, there is a need to have set of reliable biomarkers through the progression of disease that may help optimize patient management [9–12].

Determination of hormone receptors [estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status in the primary tumor is clinically relevant to define breast cancer subtypes, choice of therapy and clinical outcome. TNBC accounts for 15–20% of newly diagnosed invasive breast cancer cases [13,14] and are defined by the absence of ER, PR, and HER2 receptor expression. Due of the lack of defined targets, the current standard of care for treatment of this disease is chemotherapy. Unfortunately, these tumors are often aggressive with many patients diagnosed with incurable, metastatic disease within 2–3 years of initial diagnosis and death within 4–5 years [15,16].

Due to the success of targeted therapies in hormone-receptor and Her2-positive breast cancers, enormous efforts have been extended in identifying therapeutic targets in TNBC. Androgens are known to play a role in normal breast physiology and androgen receptor (AR) signaling is becoming increasingly recognized as an important contributor of breast carcinogenesis [17]. Many studies have been performed to elicit better understanding of AR signaling. In breast cancer, different phenomena such as AR intramolecular interaction through the single AR molecule or the AR V7 isoform, the subcellular localization of AR and its interaction with other proteins (p21, MAPK signaling through phosphorylation of ERK) is important to understand [17]. The four intrinsic subtypes (Luminal A/B, HER2-enriched, Basal-like) of breast cancer, defined by differential expression of 50 genes (PAM50), have been shown to be predictive of risk of recurrence and benefit of hormonal therapy and chemotherapy [18]. Previously, we reported that AR-negative patients have 66% greater odd ratio (95% CI, 32–146) of being the more aggressive basal-like phenotype compared to other PAM50 subtypes Furthermore, these AR-negative breast tumors are associated with a decreased time-to-progression and decreased overall survival [19,20]

Traditionally, despite being a very heterogenous group, all TNBC have been treated the same. However, genomic profiling studies have identified multiple subtypes of TNBC that have led to clinical trials with targeted therapies based on the TNBC subtype [21]. AR expression in TNBC has received a lot of attention recently and it's estimated that 24.8% of TNBC cases express AR [22]. Enzalutamide, an AR antagonist used in the treatment of prostate cancer, has been shown to significantly reduce cell viability of AR-positive TNBC in in vitro and in vivo models [23].

Furthermore, a recent phase II study found enzalutamide demonstrated clinical activity and was well tolerated in patients with advanced AR-positive TNBC [24]. While most of the studies have focused on the clinical significance of AR-positive TNBC [24,25], we recently reported that AR negative TNBC patients expresses a unique, enriched basal and immune signature, that is increased in African American women [19]. Thus, there is a critical need to further characterize the usefulness of AR as a biomarker for TNBC patients.

These findings support the significance of AR expression in TNBC and provides the rationale for further investigation of the predictive and prognostic impact of this biomarker and its potential role in the development of metastatic disease. In this study, we measured the expression of ER, PR, HER2 and AR by immunohistochemistry (IHC) in 291 TNBC primary and metastatic breast cancer tumors. Additionally, a panel of 25 cancer-related genes and proteins were evaluated using IHC and in situ hybridization (ISH). Our goal was to examine the clinicopathologic significance of AR expression in a subset of patients with early stage breast cancer who later developed metastatic disease. Lastly, we sought to examine the examined the prognostic and predictive use of AR as a biomarker in metastatic breast cancer.

Materials and Methods

Tissue Samples

A cohort of 874 breast cancer samples, comprising 418 unique patients, was profiled at Caris Life Sciences from January 1997 through November 2014. ER, PR, and AR were measured by IHC and HER2 was determined by immunohistochemistry (IHC) with confirmation by in situ hybridization (ISH). Out of these samples, only samples with known ER, PR, HER2, and AR status were evaluated for similarities and differences between subtypes, age of patient at collection date, receptor status, collection time, and gene correlations. The Thresholds for the ER, PR, Her2 and AR was used as previously described [26]. Significant variability was observed in accessing AR expression in literature. The heterogeneity is mainly due to the antibody used or the threshold range (1% or 10%). According to Triana et al. nuclear expression 10% was used as threshold. Similarly, in our study we have considered Nuclear localization of AR [24]. The IHC thresholds (= 0+ or <10% or ≥1+ and ≥10%) is used for AR. Anti-androgen receptor antibody (ab49712, Abcam), was used for AR IHC. Clinicopathological parameters included grade, invasion, and metastatic potential. The expression of 25 cancer-related genes was determined using IHC and ISH.

Classification of Molecular Subtype

Cases that were ER-positive and/or PR positive and HER2 negative/positive were considered as Luminal A/B subtype. Cases that were ER-negative, PR-negative and HER2 positive were considered as HER2 type or HER2 enriched. Cases that were ER-negative, PR-negative, HER2 negative and AR positive considered as Triple negative. Cases that were ER -negative, PR-negative, HER2 negative and AR negative were considered as the Quadruple negative subtype [27].

Statistical Methods

Summary statistics were generated to describe the overall dataset, including the distribution of demographic and clinical variables. These variables included: age, hormone receptor status (defined in the diagnostic pathology reports), AR status (defined by IHC scores), grade, invasion, and metastatic potential of the tumor. Significant differences in distributions of variables within the cohort, stratified by specific clinical or demographic characteristics, were determined using a standard T-test with a significance threshold of alpha < 0.05.

IHC was done for cancer-relevant genes in TNBC and QNBC samples. The dichotomized proportion of positive vs negative expression of each gene was calculated based on IHC results. Fisher's exact test was done to determine statistically significant differences, p-values are reported. Specific comparisons between continuous variables (i.e. age and AR expression) or categorical variables (i.e. tumor's marker status and stage) were measured with a bivariate fit analysis which incorporates a least squares regression analysis between the two variables.

Results

Loss of Androgen Receptor Expression Associated with High-Grade Tumor and Metastasis

Four hundred eighteen breast cancer patients were biopsied multiple times throughout the progression of their disease. Thus, our cohort contained 874 biopsy samples taken from local and numerous metastatic breast cancer sites before and after treatment. Each sample was examined for the standard biomarkers (ER, PR, and HER2 expression) and then evaluated for AR protein expression utilizing IHC. The majority of high grade 3, poorly differentiated samples (46%) were negative for AR expression compared to low grade tumors ($P < .5$). Also, most samples with positive for Ki67 IHC score were AR negative (59.1%, 139/235) (Table 1). Within the luminal A/B subtype, which made up over half of the cohort, most samples were positive for AR IHC expression ($P < .0001$) in both the non-metastatic (62.5%, 80/128) and recurrent/metastatic (62.6%, 199/318) samples. However, in the TNBC subtype, AR negative tumors accounted for 80.2% (65/81) and 75.7% (159/210) of TNBC non-metastatic and recurrent/metastatic, respectively (Figure S1). Thus, examining AR expression within the TNBC subtype appears to be critical, and TNBC tumors that lack AR expression should be classified as a unique phenotype, QNBC.

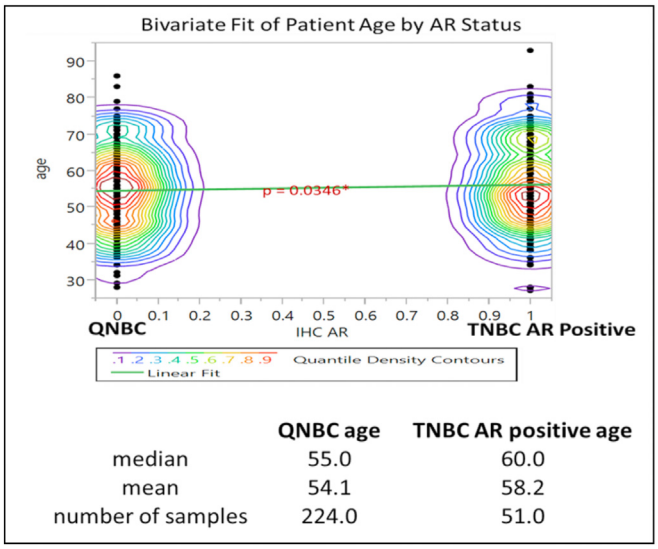

Figure 1. Median and Mean age for QNBC patients is lower than TNBC AR positive patients: Bivariate fit analysis of patients age and AR status determined that AR negative patients were diagnosed at younger ages compares to AR-positive patients ($P < .05$). QNBC is significantly correlated with younger aged patients compared to AR positive TNBC patients.

Lack of AR Expression Associated with Younger Patients in TNBC

Age at diagnosis is a critical clinical feature, which can be associated with a more aggressive breast cancer phenotype. Therefore, we divided samples according to AR positive or

Table 1. Summary of the Clinical Characteristics of the Cohort

	Characteristics		AR- Negative	AR- Positive	Unknown	Total	$P^{\dagger}$
			%; n				
Samples (n = 874)	Age						
	≤ 55.5 (mean)		46.3 (n = 214)	40.5 (n = 187)	13.2 (n = 61)	462	.5009
	> 55.5		43.2 (n = 178)	41.3 (n = 170)	15.5 (n = 64)	412	
	Grade						<.05*
	1		16.7 (n = 1)	83.3 (n = 5)	0.0 (n = 0)	6	
	2		15.0 (n = 3)	80.0 (n = 16)	5.0 (n = 1)	20	
	3		46.0 (n = 57)	37.9 (n = 47)	16.1 (n = 20)	124	
	Ki67 IHC score						<.05*
	0		33.0 (n = 32)	64.9 (n = 63)	2.1 (n = 2)	97	
	1		59.1 (n = 139)	39.6 (n = 93)	1.3 (n = 3)	235	
	Invasion						.5009
	Non-invasive		100.0 (n = 2)	0.00 (n = 0)	0.00 (n = 0)	2	
	Invasive		44.3 (n = 303)	39.6 (n = 271)	16.1 (n = 110)	684	
	Metastasis						.8458
	Non-metastatic		44.7 (n = 109)	39.8 (n = 97)	15.6 (n = 38)	244	
	Metastatic		44.9 (n = 283)	41.3 (n = 260)	13.8 (n = 87)	630	
Non-metastatic samples	HR+ / HER2-	Luminal A/B	28.9 (n = 37)	62.5 (n = 80)	8.6 (n = 11)	128	<.0001*
	HR –/ HER2+	HER2-enriched	50.0 (n = 4)	50.0 (n = 4)	0.0 (n = 0)	8	
	HR- / HER2-	Triple Negative	80.2 (n = 65)	14.8 (n = 12)	4.9 (n = 4)	81	<.0001*
		Total	48.8 (n = 106)	44.2 (n = 96)	6.9 (n = 15)	217	
Recurrent/metastatic samples	HR+ / HER2-	Luminal A/B	30.5 (n = 97)	62.6 (n = 199)	6.9 (n = 22)	318	<.0001*
	HR - / HER2+	HER2-enriched	45.9 (n = 17)	54.1 (n = 20)	0.0 (n = 0)	37	
	HR- / HER2-	Triple Negative	75.7 (n = 159)	18.6 (n = 39)	5.7 (n = 12)	210	<.0001*
		Total	48.3 (n = 273)	45.7 (n = 258)	6.0 (n = 34)	565	

Clinical data was evaluated for 874 samples from 418 breast cancer patients with ER, PR, HER2, and AR IHC expression. The median age at sample collection was 55 years. There is a significant correlation between AR-negativity and high-grade breast tumors, high Ki67 IHC score ($P < .05$). Both non-metastatic Luminal A/B and recurrent/metastatic Luminal A/B significantly correlated with AR expression ($P < .0001$). Unlike TNBC, over 60% of Luminal A/B samples were AR-positive. † *P* values with * were deemed significant. Fishers exact was used and subsequently validated with the C^2 test; odds ratio test was also used to obtain *P* values. Patients or samples missing due to the lack of clinical characteristics were excluded from the analysis.

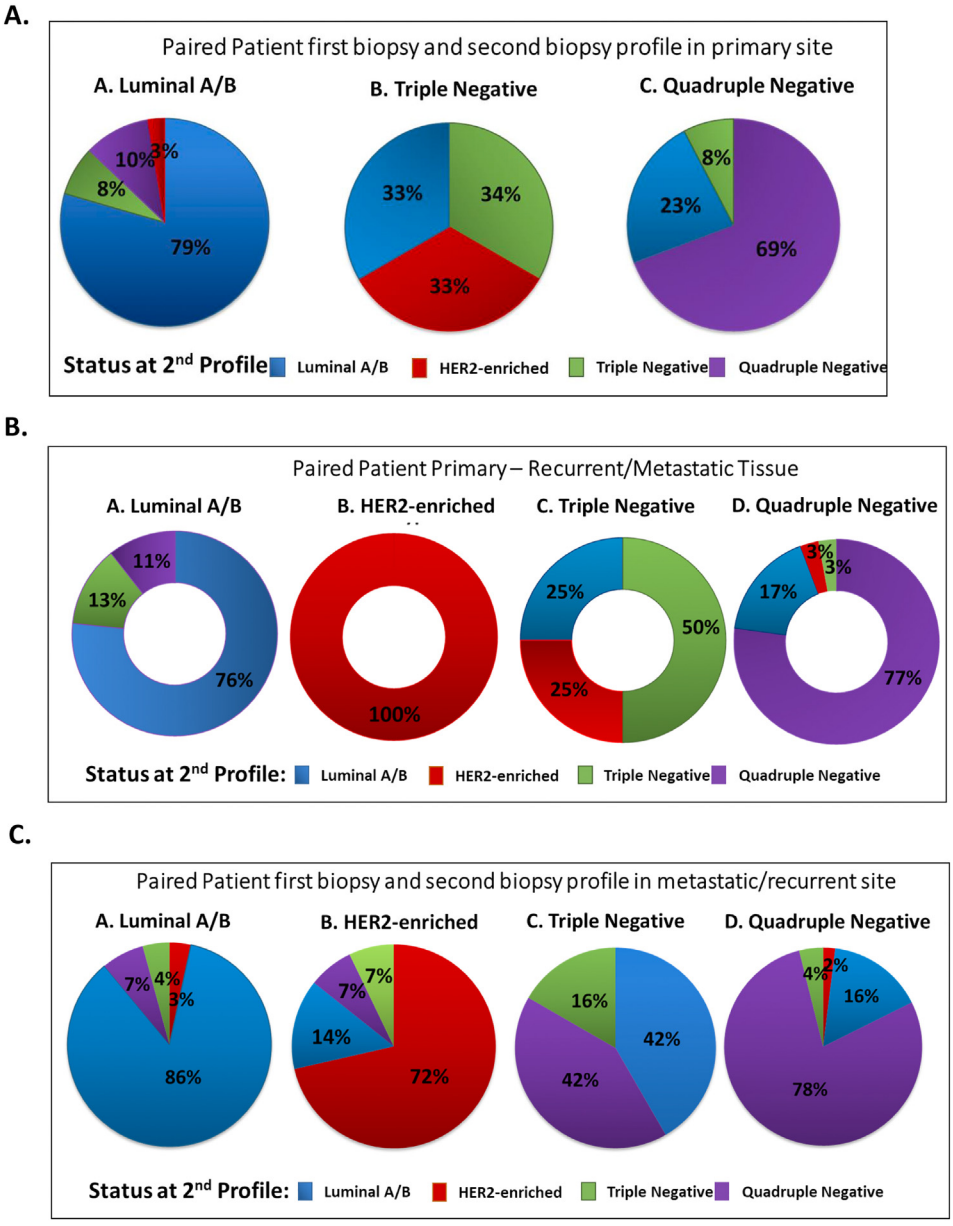

Figure 2. QNBC tumors have decreased discordance. Comparison of the frequency and type of change in ER, PR, HER2, AR status between paired patient profiles. Status at first profile A-D vs. status at 2nd profile (designated by color as shown above). Samples that lack IHC expression of biomarkers were excluded from the analysis. A. This represents comparison between paired patient first biopsy to second biopsy profile at primary site. The number of paired primary biopsies were 55(total), Luminal A/B (n = 39), Her2 type (n = 0), TNBC (n = 3) and QNBC (n = 13). Paired primary tissue samples did not display significant differences in ER, PR, HER2, or AR status for Luminal A/B (79%). The number of AR positive TNBC, those have paired primary biopsies were low. However, within the AR positive TNBC tumors, 66% had an alteration in biomarker status, resulting in a change in molecular profile at second biopsy. Furthermore, in the AR negative QNBC tumors (69%) of the tumors remained QNBC at the second biopsy. B. This illustrates comparison between paired patient primary – recurrent/metastatic samples of all subtypes .Number of paired primary to metastatic/ recurrent biopsies were n = 91 (total), Luminal A/B (n = 47), Her2 type (n = 5), TNBC (n = 4) and QNBC (n = 35).The discordance rate was observed in all subtypes (Luminal A/B (24%, HER2 enriched (0%), and QNBC (23%)), with TNBCs displaying the most discordance (50%). C. This illustrates comparison between paired patient first biopsy and second biopsy profile in metastatic/recurrent site. We further evaluated 195 (total) paired recurrent/metastatic samples based on subtype at first biopsy and second biopsy, Luminal A/B (n = 118), Her2 type (n = 14), TNBC (n = 12) and QNBC (n = 51). We observed that as the cancer progressed to recurrent/metastatic disease, the initial biomarker phenotype often exhibited a more aggressive phenotype, with an increase in QNBC subtype at second profile status. For example, 16% of AR positive TNBC tumors 78% of QNBC tumors not exhibiting any change.

negative expression and compared this to the age of patient at time of sample collection. AR negative TNBC (i.e. QNBC) patients demonstrated the most significant loss of AR expression, we performed bivariate fit analysis on this subtype. QNBC tumors significantly (P = .0346) correlated with younger aged patients at time of biopsy compared to AR-positive TNBC patients (Figure 1). The median age for QNBC patient diagnoses was 55 and TNBC AR positive was 60.

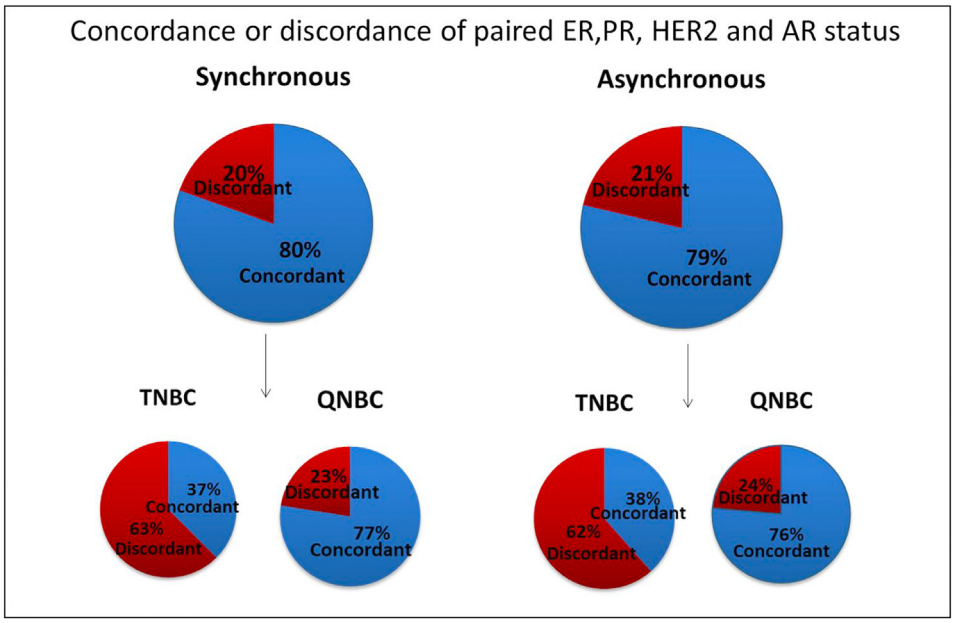

Figure 3. QNBC tumors demonstrate increased concordance in asynchronous and synchronous biopsies. Illustration of the concordance and discordance of paired ER, PR, HER2, and AR status in paired patient tissue samples. Samples that lack IHC expression of biomarkers were excluded from the analysis. The paired samples were then classified based on collection dates as synchronous (taken within 6 months of initial biopsy) or asynchronous (taken more than 6 months of initial biopsy). The number of paired samples were 133 (synchronous) and 192 (asynchronous). Both synchronous and asynchronous paired samples displayed consistent concordant molecular profiles, 80% and 79%, respectively. Despite the concordance of intra-individual samples, discordance of biomarker statuses was illustrated for synchronous (20%) and asynchronous (21%) stratification. The paired synchronous and asynchronous samples were then categorized by TNBC and QNBC (synchronous QNBC (n = 40), synchronous TNBC (n = 8), asynchronous QNBC (n = 55), asynchronous TNBC (n = 13). Within the TNBC subset, in both the asynchronous and synchronous categories, there was a 62% discordance of paired samples. Contrarily, synchronous and asynchronous QNBC tumors only displayed 23% and 24% discordance, respectively.

Concordance of AR Expression in Breast Cancer Subtypes

Although multiple reports have demonstrated that AR expression is important in breast tumors, we are not aware of any reports on the concordance of AR expression throughout breast cancer progression. Therefore, we compared the intra-individual molecular profiles in patients with paired non-metastatic (primary) and recurrent/metastatic, as well as patients with paired recurrent/metastatic samples. Samples missing due to the lack of any IHC biomarkers were excluded from the analysis. Pie charts (Figure 2, *A–C*) were constructed to compare the frequency and type of change in ER, PR, HER2, and AR statuses between first biopsy and second biopsy of paired samples. At least 70% Luminal, HER2 enriched, and QNBC subtype did not change molecular profile. But, TNBC seems to be the "unstable" subtype.

Next, we examined the difference in subtype profile in 1st biopsy to 2nd biopsy in primary site. We analyzed 55 total paired primary biopsies, Luminal A/B (n = 39), TNBC (n = 3) and QNBC (n = 13). Paired primary tissue samples did not display significant differences in ER, PR, HER2, or AR status for Luminal A/B (79%). Although, there were a limited number of AR positive TNBC cases with paired primary biopsies, 66% had an alteration in biomarker status, resulting in a change in molecular profile at second biopsy. Furthermore, in the AR negative QNBC tumors (69%) of the tumors remained QNBC at the second biopsy (Figure 2*A*).

Next, we analyzed only patients with non-metastatic (primary) and recurrent/metastatic paired samples of all subtypes. Number of paired primary to metastatic/ recurrent biopsies were n = 91 (total), Luminal A/B (n = 47), Her2 type (n = 5), TNBC (n = 4) and QNBC (n = 35).The discordance rate was observed in all subtypes (Luminal A/B (24%, HER2-enriched (0%), and QNBC (23%)), with TNBCs displaying the most discordance (50%) (Figure 2*B*).

As previously described, we further evaluated 195 (total) paired recurrent/metastatic samples based on subtype at first biopsy and second biopsy, Luminal A/B (n = 118), Her2 type (n = 14), TNBC (n = 12) and QNBC (n = 51). We observed that as the cancer progressed to recurrent/metastatic disease, the initial biomarker phenotype often progressed into a more aggressive phenotype, with an increase in QNBC subtype at second profile status. For example, 16% of AR positive TNBC tumors 78% of QNBC tumors did not exhibit any change (Figure 2*C*).

QNBC has shown higher concordance than TNBC in both synchronous and asynchronous biopsies

The paired samples were then classified based on collection dates as synchronous (taken within six months of initial biopsy) or asynchronous (taken more than six months of initial biopsy). The number of paired samples were 133 (synchronous) and 192 (asynchronous). Both synchronous and asynchronous paired samples displayed consistent concordant molecular profiles, 80% and 79%, respectively (Figure 3). Despite the concordance of intra-individual samples, discordance of biomarker statuses was illustrated for synchronous (20%) and asynchronous (21%) stratification. We further categorized TNBC and QNBC (synchronous QNBC (n = 40), synchronous TNBC (n = 8), asynchronous QNBC (n = 55), asynchronous TNBC (n = 13). Within the TNBC subset, in both the asynchronous and synchronous categories, there was a 62% discordance

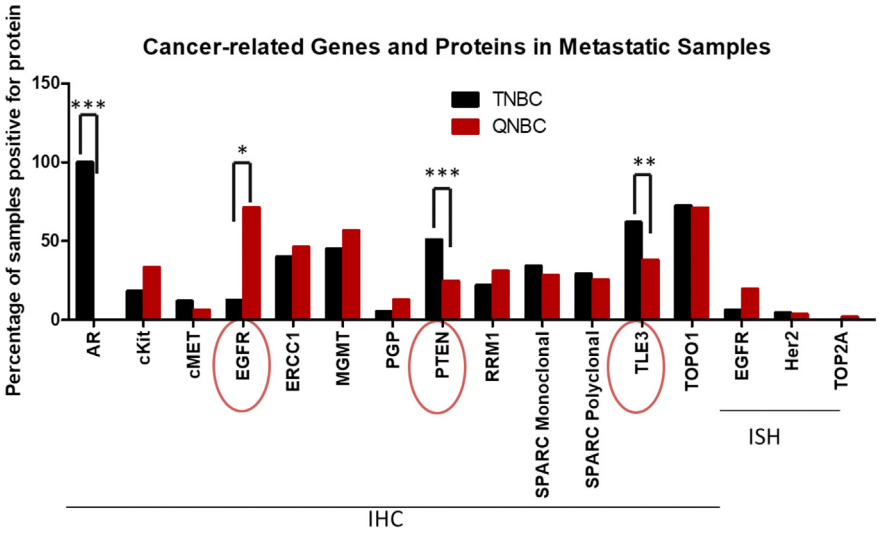

Figure 4. Gene and protein expression in TNBC compared to QNBC tumors in metastatic or recurrent site. The expression multiple cancer-related genes in metastatic TNBC and QNBC tumors was determined by IHC and ISH. EGFR, PTEN and TLE3, demonstrated statistically significance between TNBC (n = 39) and QNBC (n = 159) patients. The number of samples positive or negative for a gene was obtained and the total number of samples that have the IHC for that gene was obtained. The percentage of patients positive for each protein was calculated and statistical significance was obtained using Fisher's exact test (2×2 contingency, two-tailed test). *** ($P < .0001$), **($P < .001$), *($P < .05$).

of paired samples. Contrarily, synchronous and asynchronous QNBC tumors only displayed 23% and 24% discordance, respectively.

QNBC is Associated with Higher EGFR, lower PTEN and TLE3 compared to TNBC in Metastatic Samples

Since, we observed major differences in TNBC and QNBC, all TNBC and QNBC samples were assessed using the CARIS Life sciences panel of 25 cancer related genes and proteins. Both TNBCs and QNBCs were sub-categorized as metastatic or non-metastatic and expression of the cancer-related genes and proteins were evaluated (Figure 4). In both non-metastatic and metastatic QNBCs tumor, we observed a significant increase in loss of AR expression.

Percentage of patients, positive for a gene of interest was calculated and compared. Genes for which sample number was low, excluded in our analysis. In metastatic sample, while QNBC is compared to TNBC, we found that EGFR is increased and TLE3 and PTEN was decreased in QNBC. ($P < .05$). In non-metastatic samples, only AR was statistically significant (Figure S2).

Discussion

It has been well-documented that ER, PR and HER2 expression, which are the essential triad of biomarkers used in the management of breast cancer, can undergo significant gene expression switching across the cancer continuum [28]. This discordance has been estimated to be as high as 40% in patients after neoadjuvant treatment and/or as their disease progresses (recurrence/metastasis). Furthermore, it has shown that discordant cases have poorer survival which has been attributed to inappropriate use of targeted therapies [8]. For example, recent studies have shown that women that switch from an initial ER-positive primary tumor to an ER-negative tumor, have a significant 48% increased risk of death, when compared to women in which the ER/PR/HER2 status at first recurrence is unknown or negative [3,29,30]. Unfortunately, the acquisition of fresh tissue from suspected breast cancer metastases are not always performed in routine practice. This results in treatment decisions being made based upon the biomarker features of the primary tumor, which has often been collected many years, and sometimes, decades earlier. Due to these troubling findings, the NCCN guidelines have highlighted the need that additional biopsies be performed at the time of each recurrence. While the use ER, PR and HER2 status aids towards selection of appropriate therapies, there is still a need to investigate additional markers, such as AR, to potentially benefit those patient tumors that lack expression of classical receptors.

Several studies have reported that AR is closely associated with the occurrence and progression of breast cancers [31–33]. Furthermore, Also, AR positivity is associated with smaller tumor size and a lack of lymph node involvement [31]. In addition, AR positive cells upon AR activation transition from basal to luminal subtype *in vitro* [34]. In humans, over 56% of breast cancers are positive for AR expression regardless of ER status [35]. In previous studies, androgens have been shown to decrease the cell proliferation of AR-positive breast carcinomas. Additionally, the presence of AR is significantly linked to a decrease in the probability of breast cancer recurrence within 5 years and a better overall survival [36]. At the biochemical level this provides evidence that AR could have a functional role in breast tumors.

There are several controversies that exist in the field. The ER to AR ratio has been correlated to breast tumor response to traditional endocrine therapy [37,38]. However, the AR positivity range is broader, depending on criteria used to define positivity and assay employed [39,40]. Most notably, antiandrogen therapy has been shown clinical benefit for LAR-TNBC (19–35%) [24,40–42]. Taken together, we sought to further evaluate AR discordance rates as well as other related tumor markers, important for in the progression of breast cancers.

In our study, we examined the biomarker profile of a cohort of 874 breast cancer samples that consisted of patients with early disease that developed metastases, de novo metastatic disease, and patients with bilateral breast cancer. In both synchronous or asynchronous biopsies there is a discordance in molecular profiles in both synchronous

(20%) and asynchronous (21%) samples by intra-individual analyses. Within an individual patient, ER and HER2 status were not always concordant between lesions within the same breast, between bilateral breast cancers, and between distinct foci in a metastatic organ site. Although, the concordance was much greater between samples, as expected, the fact that a significant degree of discordance occurred in the same patient, emphasizes the need for molecular retesting across the cancer continuum. If not done, patients are at significant risk for not receiving adequate treatment for the most clinically important foci of breast cancer. Thus, this data supports, in accordance with NCCN guidelines, that biopsy with molecular profiling should be performed on tumor sites at both initial diagnosis and at each time of recurrence/progression occurs so that patients receive the most appropriate treatment at any point in time across their cancer continuum.

Our finding suggest that tumors initially identified as TNBCs demonstrated the most change or discordance in receptor status (62%) at second profiling of ER/PR/HER2, compared to all the other molecular subtypes examined, Additionally, we observed that 79% of primary TNBC tumors and 76% of recurrent/metastatic TNBC tumors were negative for AR, which we refer to as quadruple negative breast cancer (QNBC) [19]. Interestingly, however the when AR status was determined to be negative, TNBC discordance decreased. This was also apparent in recurrent or metastatic TNBC patients, where AR-negative status resulted in a 25% decrease in the patients that exhibited a switch in molecular marker status after the subsequent biopsies. A limitation to our study is the low number of paired TNBC AR positive biopsies. Previous results have shown that 13–37% of TNBC are AR positive [43], and exhibit higher discordance rate [44], which is similar to our findings. Thus, despite the low number of cases analyzed in this study, TNBC AR positive seems to exhibit a heterogenous profile.

Bivariate fit analysis demonstrated that AR-negative or QNBC tumors are significantly correlated with younger age at initial diagnosis compared to AR positive TNBC patients. Thus, our analyses suggest that the heterogeneity of TNBC, is at least partially associated with the presence or absence of AR expression, suggesting that QNBC should be considered as a clinically relevant BC subtype. Furthermore, since our previous studies and those of others have shown AR expression is associated with lower tumor grade, along with decreased recurrence and death rates, our findings further support the postulate that AR has prognostic value and can be used as a therapeutic tool in TNBC cases. Moreover, this type of sub-stratification of TNBC also appears to have significant predictive value in these patients and has the potential to identify patients who may benefit from less aggressive therapy. Hence, IHC analysis of AR appears to be a practical biomarker to add to the existing triad of biomarkers for breast cancer and thus provide a more comprehensive model in characterizing the more aggressive subtypes in TNBC patients.

IHC analysis of AR expression appears to be a practical assay to determine the most aggressive TNBC subtypes and identifies tumors that could benefit from available targeted therapies. However, as expected with any new bioassay, determining the relevant cutoffs for AR expression has been challenging. In the literature, AR expression in TNBC has been reported to be as low as 7% and as high as 75%, depending on the study [45–48]. This broad range, at least in part, is probably due to the lack of clearly defined cutoffs for AR expression, with some studies using 1% and 10% used some clinical trials evaluating AR antagonist therapy [41,49,50]. To address this issue, a recent study utilized 135 TNBC cases to determine the appropriate threshold for AR positivity [51]. They utilized AR expression along with clinicopathologic features of TNBCs, such as EGFR expression, and determined that, using several different cutoff points, there was no difference in DFS in patients with AR expression >1%. Thus, they concluded that 1% is the optimal cut point in evaluating AR immunoexpression, which is the same cutoff used to evaluate ER and PR expression per the ASCO/CAP guidelines and the cutoff used in several other studies [52].

This study not only evaluated change in biomarker status between specimens, but also examined some of the genetic changes that occur. The underlying etiology of biomarker switching is still not well understood, thus several plausible explanations for biomarker discordance have been put forth such as PI3K kinase pathway activation, along with TOP2A and ER genes expression dysregulation [26,53–58]. We observed in metastatic samples that EGFR expression was increased in QNBC tumors. Upregulation of EGFR expression is associated with poor outcome, early relapse and death [59]. Additionally, as previously reported EGFR was elevated in AR negative TNBC tumor, which is similar to our findings [60]. PTEN, a tumor suppressor and plays role in cell cycle arrest [61,62].PTEN is downstream of EGFR signaling [63]. Interestingly in our results, PTEN is decreased in QNBC. Lastly, we observed decreased TLE3 protein expression in QNBC tumors. TLE3 negative TNBC patients [64] have significantly poorer outcome, and this loss may be associated with Wnt signaling and adipogenesis. [65,66]. This evidence of molecular changes in QNBC vs TNBC patients, further support that QNBC tumor are an aggressive subtype.

In summary, this study provides confirmation of biomarker switching that occurs across the breast cancer continuum and supports the NCCN recommendations that new biopsies should be obtained at each sign of recurrence or development of metastatic disease. Our study also highlights the need for biopsy of all suspicious sites in synchronous cases so that final treatment will address the most clinically significant phenotype. However, given that the standards of care in treatment of breast cancer, is based upon this triad of biomarkers, there is a critical need to identify additional biomarkers that can be used to complement the existing triad and thus provide a more comprehensive and reliable predictive and/or prognostic model for metastatic disease [56,57,67].

A limitation of this study is that we did not have race information available for this patient cohort, which is important since it's well established that TNBC is very common in young African-American women and our previous studies have shown that QNBC in African American have a more aggressive course hallmarked by a unique basal-like and immune signature. Furthermore, since this data was obtained from a molecular profiling company, we did not have survival or outcome data for this patient cohort either, thus we were not able to fully address the predictive impact of AR as a biomarker. However, when taken together, this study clearly illuminates that QNBC is a distinct tumor phenotype from TNBC, which lends itself for further exploration of new therapeutic targets for this aggressive breast cancer subtype. Our findings warrant further validation in a larger cohort of TNBC and ultimate evaluation of AR utility in a prospective, randomized clinical trial.

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Authors Contributions

Concept and design: CY and WDC. MD EM, QH, ST Generated data and performed the analyses: MD QH. Analyzed the data: CY EM MD ST. Wrote and edited the manuscript.

WDC CY ST MD AA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.11.008.

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