Relapsed and De Novo Metastatic HER2-positive Breast Cancer Treated With Trastuzumab: Tumor Genotypes and Clinical Measures Associated With Patient Outcome

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Abstract

Patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer (MBC) present with relapse after adjuvant chemotherapy for early stage disease (R-MBC) or directly with stage IV disease (de novo, dn-MBC). We analyzed tumor mutational profiles from 113 trastuzumab-treated patients with HER2-positive MBC. We identified distinct prognostic impact of tumor genotypes in R-MBC and dn-MBC that may be used in the context of personalized medicine.

Background: We examined tumor genotype characteristics of human epidermal growth factor receptor 2 (HER2)-positive relapsed (R-) and de novo (dn-) metastatic breast cancer (MBC) in trastuzumab-treated patients who were previously not exposed to this agent. **Materials and Methods:** We analyzed genotypes obtained upon deep sequencing from 113 HER2-positive primary tumors from 69 patients with R-MBC and 44 patients with dn-MBC. **Results:** Mutations were observed in 90 (79.6%) tumors, 56 R-MBC and 34 dn-MBC (median number per tumor: 2; mean: 11.2; range: 0-150). The top mutated gene was *TP53* (63.7%) followed by *PIK3CA* (24.8%) and others that were mostly co-mutated with *TP53* (eg, 22 of 28 *PIK3CA* mutated tumors were co-mutated in *TP53*, 17 of these were R-MBC [$P = .041$]). dn-MBC had higher CEN17 average copies ($P = .048$). Tumor mutational burden inversely correlated with average HER2 copies (rho −0.32; $P < .001$). In all patients, *PIK3CA* mutations and higher proliferation rate were independent unfavorable prognosticators. In R-MBC, longer disease-free interval between initial diagnosis and relapse conferred lower risk for time-to-progression ($P < .001$) and death ($P = .009$); *PIK3CA* mutations conferred higher risk

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for death ($P = .035$). In dn-MBC, surgical removal of the primary tumor before any other therapy was favorable for time-to-progression ($P = .002$); higher tumor mutational burden was unfavorable for survival ($P = .026$). **Conclusions:** Except for the overall unfavorable prognostic effect of *PIK3CA* mutations in trastuzumab-treated MBC, our exploratory findings indicate that the outcome of patients with R-MBC is related to patient benefit from the preceding adjuvant chemotherapy and provide initial evidence that tumor mutational burden may be related to prognosis in dn-MBC, which is of potential clinical relevance and merits further investigation.

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Introduction

Breast tumors that overexpress human epidermal growth factor receptor 2 (HER2) represent about 15% to 20% of all cases, affecting younger women with a more aggressive disease course.[1] The yearly rate of recurrence in HER2-positive ($HER2^+$) breast cancer is 5% for HER2 luminal ($HER2^+$ estrogen receptor-positive [ER^+]) to 9% for HER2 enriched ($HER2^+ER^-$) tumors for the first 5 years after diagnosis when not treated with adjuvant anti-HER2 therapy.[2] These relapsed cases account for the majority of patients with metastatic breast cancer (MBC), whereas about 25% of all patients with MBC present with metastases de novo (dn-MBC) being treatment-naive[3]; dn-MBC represent 3% to 4% of new breast cancer diagnoses per year.[4]

The introduction of trastuzumab — the first HER2-targeted therapy — altered the grave prognosis of these tumors in the adjuvant,[5] neoadjuvant,[6] and metastatic[7] setting. However, $HER2^+$ MBC progresses, needs multiple lines of treatment, and remains lethal, with less than 50% of the patients surviving after 5 years.[8,9] Resistance to trastuzumab, primary or acquired, remains a major challenge for physicians. Adding trastuzumab to first-line cytotoxic chemotherapy for $HER2^+$ MBC yields response rates from 50% to 80%,[7,10] but the clinical benefit in the second line is less than 50%.[11] Various processes are involved in trastuzumab resistance, including molecular alterations in HER family members; constitutive activation of the PI3K/AKT pathway or, suppression of the immune response to cancer cells (reviewed in[12]). At present, data on breast cancer genomics[13] and on the evolution of genotypes from primary to metastatic disease[14,15] are used to illuminate the molecular mechanisms related to trastuzumab resistance. However, in everyday practice, primary tumors are usually the only material available in the majority of MBC. Thus, an important question for the treating physician is whether pathologic and molecular features of primary tumors have predicting value for trastuzumab benefit in the metastatic setting.

To address that, we interrogated the impact of tumor characteristics on trastuzumab response in patients with $HER2^+$ MBC. A population of 113 women with $HER2^+$ disease on their primary tumors was retrospectively divided into R-MBC and dn-MBC. None had received trastuzumab or another anti-HER2 regimen in the adjuvant setting. We examined archived formalin-fixed paraffin-embedded (FFPE) primary tumors from these patients for tumor infiltrating lymphocyte (TILs) density,[16] performed targeted deep genotyping with massively parallel sequencing (NGS) for genes usually affected in breast cancer[13] on the same material, and compared these findings with patient clinical data.

Patients, Tissues, and Methods

The study was performed on a previously published patient cohort[17,18] enriched with new cases. Eligibility criteria for this study were: (1) $HER2^+$ tumors upon central laboratory assessment; (2) trastuzumab treatment for metastatic disease only (no prior adjuvant trastuzumab for R-MBC); (3) tissue material obtained before trastuzumab treatment start; and (4) informative NGS data. Patients had been treated with trastuzumab-based combinations between November 1999 and September 2012 in clinical centers affiliated with the Hellenic Cooperative Oncology Group. The translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (#4283; January 14, 2008) under the general title "Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer." All patients included in the study after 2005 provided written informed consent for the provision of biological material for future research studies, before receiving any treatment. Waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

Tissue Processing

Hematoxylin and eosin sections from 229 FFPE tissue blocks that were diagnosed as $HER2^+$ breast carcinomas were histologically reviewed for tissue availability. Two 1.5-mm cores per tumor were transferred into low density tissue microarrays (TMAs), which were used for in situ methods and for DNA extraction. ER, PgR, Ki67, and HER2 protein expression were assessed by immunohistochemistry and HER2 amplification by fluorescence in situ hybridization as described.[18] Stromal TILs were evaluated on core sections according to widely used recommendations,[16] provided that the stromal component was maintained and could be evaluated on the tissue core; the average percentage of 2 tumor cores was used. TMA cores were assessed for tumor cell content, which ranged from 10% to 95% and was > 50% in 81% of the cases. For this study, DNA was extracted from 10-micron thick TMA sections with silica-coated magnetic beads (Versant Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY), according to the manufacturer's instructions.

NGS Genotyping

Samples with $\geq$ 2 ng/μL DNA as measured with the Qubit fluorometer (Thermo-Fisher/Life Technologies, Paisley, UK) and with < 33 Ct values for 2 wild-type control DNA targets upon

quantitative polymerase chain reaction were processed for targeted semiconductor sequencing (n = 147). Block age higher than 10 years in the majority of the present cases may have contributed to the large failure rate at this step. The applied Ampliseq panel (panel ID: IAD47063_31) targeted 373 regions in 61 genes covering a total of 41,477 nts, out of which 34,845 nts in coding regions from 59 genes, as previously described and validated.[19] Samples were sequenced in an Ion Proton sequencer. Variants obtained from Ion Reporter v.4 were extensively filtered out for low quality metrics ($P > .0001$) and GC-stretches to avoid artifacts; amplicon eligibility was set at a minimum of 100 reads; worse case variants were considered for 40 reads at positions covered 100 times. Samples were considered for analysis if > 90% of amplicons were eligible, mean depth was > 400, and uniformity > 60%. For this study, we accepted samples with at least 5 variants; if no mutations were identified, we doubled the mean depth cutoff to 800 for sample eligibility.

Based on the above criteria, informative results were obtained for 146 of 147 tumors. However, cases were further excluded based on centrally confirmed HER2 positivity and on patient clinical data availability; thus, finally, 113 NGS informative tumors were examined. All were primary tumors. Values for mean depth were mean = 2802.99; median = 2373; range = 553 to 23,422, and for uniformity, mean = 79.25%; median = 80.75%; range = 62.31% to 87.52%. The mean number of variants per tumor was 50.75 (median, 25), whereby 10% of the tumors had < 18 and another 10% > 128 variants.

Variants were called mutations if amino acid or splice site changing with Minor Allelic Frequency < 0.1% (< 0.001), according to dbSNP and 5000Exomes. In the tumors examined, tumor cell content ranged from 10% to 95% and was > 50% in 81% of the cases. "Clonal" mutations were considered for variant allelic frequencies ≥ 20% in the present series; this distinction was made in order to examine mutations less likely to represent FFPE artifacts owing to deamination during fixation,[20] although, as more recently described, these seem to be unpredictable and unavoidable.[21] Since no germline data were available, the somatic nature of the examined mutations was assumed but could not be addressed.

Statistical Analysis

Categorical data are presented as frequencies with corresponding percentages, whereas the mean, standard deviation, median, and range values are presented for the continuous variables. Comparisons between categorical variables were performed using the χ^2 or Fisher exact (where appropriate) tests, whereas the Wilcoxon rank-sum or Kruskal-Wallis tests were used to detect differences between categorical and continuous variables. Spearman correlations were performed to examine the association between continuous variables.

Disease-free interval (DFI) was defined as the time (in months) from first breast cancer diagnosis to the time of diagnosis of metastatic disease and was examined among patients with R-MBC only. Surgical removal of the primary tumor before any treatment start was considered against biopsy without tumor removal in patients with dn-MBC only. Time to progression (TTP) was defined as the time (in months) from the initiation of trastuzumab treatment for metastatic disease (with or without simultaneous chemotherapy/hormonal therapy) to the date of documented disease progression. Patients without progression were censored at the date of their last contact. Survival was measured from the initiation of trastuzumab treatment to the date of death, with alive patients censored at the time of last contact. TTP and survival curves were produced using the Kaplan-Meier method and compared across groups with the log-rank test.

The TTP and survival analyses were performed in the entire study population as well as in the subgroups of patients with dn-MBC and R-MBC. Univariate and multivariate Cox proportional hazard regression models were applied to assess the association of clinical and genotype variables with progression/mortality rates. Multivariate analyses were performed in each of the 3 study groups (ie, entire cohort, R-MBC, and dn-MBC). The independent prognostic significance of genotype variables that had been associated with outcome in univariate analyses was adjusted against clinicopathologic variables relevant in each group. The final models included variables remaining from a backwards selection procedure with a removal criterion of $P > .10$.

Results of this study are presented according to reporting recommendations for tumor marker prognostic studies.[22] All tests are 2-sided at an alpha 5% level of significance. Analyses were performed using the SAS (version 9.3, SAS Institute Inc, Cary, NC) software.

Results

Of the 113 patients that were finally examined, 44 (38.9%) were diagnosed at stage IV (dn-MBC), and 69 (61.1%) were diagnosed at earlier stages of the disease (R-MBC). Selected patient characteristics are presented in Table 1. In the entire cohort, the median age of the patients was 57 years; 106 (93.8%) patients received trastuzumab as first-line treatment either with concurrent chemotherapy (n = 100) or hormonal therapy (n = 6). Seven patients received trastuzumab as second- or third-line regimen, one of them as monotherapy. In comparison with patients with R-MBC, those with dn-MBC had more frequently $HER2^+/ER^-$ tumors (HER2-enriched); more unstable tumors as revealed by the higher number of CEN17 copies[23]; and more metastatic sites. TIL density was generally low with only 5 tumors, among them only 1 dn-MBC in the lymphocyte predominant category (≥ 50% TILs).

A total of 1010 mutations (797 in R-MBC; 213 in dn-MBC) were distributed in 90 (79.6%) of the 113 tumors, corresponding to 56 R-MBC and 34 dn-MBC. Of all mutations, only 228 were clonal (173 in R-MBC; 55 in dn-MBC), distributed in 74 (65.5%) tumors, 46 R-MBC and 28 dn-MBC. Mutation patterns are shown in Figure 1A. The most frequently mutated genes were *TP53*, *PIK3CA*, *CDH1*, and *GATA3*. As shown though, most of the mutations in genes other than *TP53* occurred in parallel with mutations in *TP53*. *PIK3CA* mutations were present in 28 tumors, 22 of which were co-mutated in *TP53*; similarly, of 14 tumors with clonal *PIK3CA* mutations, 10 had clonal mutations in *TP53* as well.

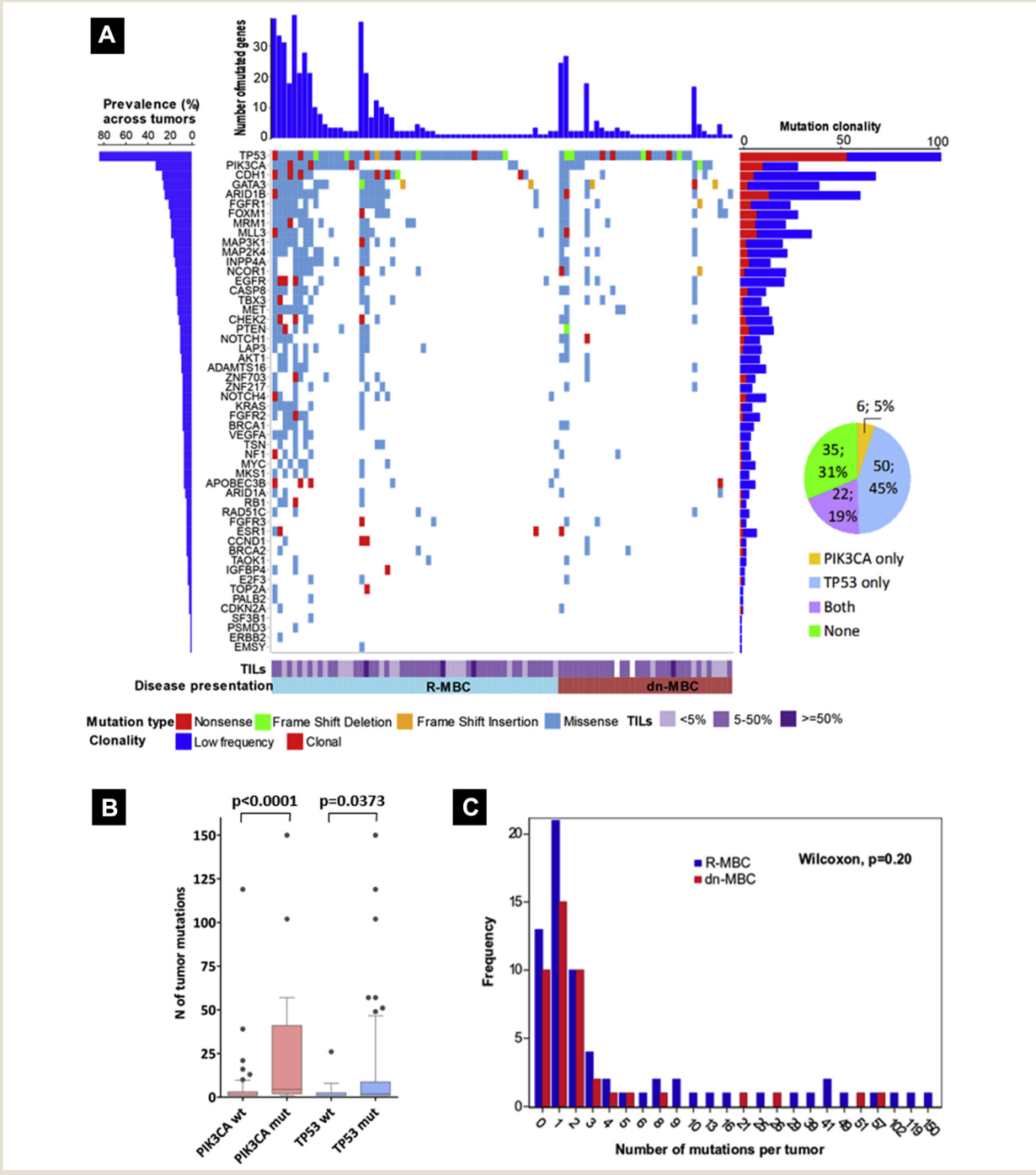
Tumors with a high number of mutations were noticed in both the R-MBC and dn-MBC groups, but the majority of tumors carried 1 to 2 mutations in an equal number of genes, whereas 10% of all tumors exhibited > 40 mutations in > 19 genes. In the 90 mutated tumors, *TP53* and, particularly, *PIK3CA*, mutations were associated with a higher numbers of mutations (Figure 1B). The overall distribution of mutational burden did not differ between the

Table 1 Patient Characteristics in the Entire Cohort and Separately in HER2$^+$ R-MBC and dn-MBC

	Entire Cohort (N = 113), n (%)	R-MBC (N = 69), n (%)	dn-MBC (N = 44), n (%)	*P* Value
Age,[a] y				
Median (range)	57.0 (28.9-95.00)	55.2 (36.0-95.0)	59.1 (28.9-83.7)	.14[e]
Ki67, %				
Median (range)	35.0 (1.0-90.0)	35.0 (1.0-90.0)	40.0 (10.0-85.0)	.55[e]
TILs				
Median (range)	10.0 (1.0-80.0)	10.0 (1.0-80.0)	11.0 (1.0-78.0)	.21[e]
HER2 average copies				
Median (range)	12.4 (3.6-38.6)	12.8 (3.6-35.3)	11.6 (5.2-38.6)	.66[e]
CEN17 average copies				
Median (range)	2.2 (1.1-4.9)	2.1 (1.1-4.4)	2.5 (1.1-4.9)	**.048**[e]
HER2 CEN17 ratio				
Median (range)	5.3 (2.2-26.6)	5.5 (2.2-18.3)	5.2 (2.3-26.6)	.24[e]
Menopausal status[a]				
Premenopausal	26 (23.0)	16 (23.2)	10 (22.7)	.92[f]
Postmenopausal	86 (76.1)	52 (75.4)	34 (77.3)	
Unknown	1 (0.9)	1 (1.4)	0 (0.0)	
Number of metastatic sites[a]				
≤3	108 (95.6)	69 (100.0)	39 (88.6)	**.008**[f]
>3	5 (4.4)	0 (0.0)	5 (11.4)	
Visceral metastases[a]	82 (72.6)	48 (69.6)	34 (77.3)	.37[f]
Bone metastases[a]	36 (31.9)	22 (31.9)	14 (31.8)	.99[f]
Nodal metastases[a,b]	17 (15.0)	7 (10.1)	10 (22.7)	.068[f]
Surgery[c]				
Mastectomy			30 (68.2)	
Biopsy only			14 (31.8)	
Subtypes central				
HER2-enriched	41 (36.3)	20 (29.0)	21 (47.7)	**.043**[f]
Luminal HER2	72 (63.7)	49 (71.0)	23 (52.3)	
Histologic grade				
I-II	40 (35.4)	29 (42.0)	11 (25.0)	.072[f]
III	69 (61.1)	38 (55.1)	31 (70.5)	
Unknown	4 (3.5)	2 (2.9)	2 (4.5)	
TILs				
<5%	27 (23.9)	19 (27.5)	8 (18.2)	.28[f]
5%-50%	75 (66.4)	42 (60.9)	33 (75.0)	
≥50%	5 (4.4)	4 (5.8)	1 (2.3)	
Unknown	6 (5.3)	4 (5.8)	2 (4.5)	
Treatment line				
First	106 (93.8)	64 (92.8)	42 (95.5)	.69[f]
Second	6 (5.3)	4 (5.8)	2 (4.6)	
Third	1 (0.9)	1 (1.5)	0 (0.0)	
Adjuvant CT[d]		53 (76.8)		
Adjuvant HT[d]		49 (71.0)		
Adjuvant RT[d]		34 (49.3)		

Bold *P* values iindicates statistically significant.
Abbreviations: CT = chemotherapy; dn-MBC = de novo metastatic breast cancer; HER2 = human epidermal growth factor receptor; HT = hormonal therapy; MBC = metastatic breast cancer; R-MBC = relapsed metastatic breast cancer; RT = radiotherapy; TILs = tumor-infiltrating lymphocites.
[a]At initiation of trastuzumab treatment.
[b]Nodal metastases other than in loco-regional lymph nodes.
[c]Only for patients with de novo metastatic breast cancer.
[d]Only for patients with relapsed metastatic breast cancer.
[e]Wilcoxon rank-sum test.
[f]χ^2/Fisher exact test.

Figure 1 Mutations and Mutation Patterns in R-MBC and dn-MBC. A, Map Showing the Distribution of Mutations in Single Genes and of Mutation Patterns Per Tumor. The Majority of Mutations Were Missense (91%) Followed by Nonsense (6.5%) and Frameshifts. *TP53* Was the Most Frequently Affected Gene and Was Mutated in 72 (63.7%) Tumors; Followed by *PIK3CA* in 28 (24.8%); *CDH1* in 23 (20.4%); *GATA3* in 22 (19.5%); *ARID1B* in 21 (18.6%), and *FOXM1* in 17 (15.0%) Tumors. The Majority of *TP53* Mutated Tumors Had at Least 1 Clonal Mutation in This Gene (70 Clonal Mutations in 60 Tumors), Whereas 20 *PIK3CA* Clonal Mutations Were Identified in 14 (12.4%) Tumors and 19 Clonal *ARID1B* Mutations in 8 (7.1%) Tumors. Most Genes Were Co-Mutated With *TP53*. This Also Applied for the 28 *PIK3CA* Mutant Tumors in the Entire Cohort, Out of Which Only 6 Did Not Carry Mutations in *TP53* (Pie Graph on the Right). B, Tumor Mutational Burden According to *PIK3CA* and *TP53* Mutations in the 90 Tumors With Detected Mutations. C, No Difference With Respect to Tumor Mutational Burden Between R-MBC and dn-MBC



Abbreviations: dn-MBC = de novo metastatic breast cancer; R-MBC = relapsed metastatic breast cancer.

Table 2 Associations Between Genotypes and Clinicopathologic Variables

	Case With Mutation		
	No	Yes	*P* Value
HER2 average copies			
Mean	16.45	13.66	.087[a]
SD	8.20	7.21	
Median	15.20	11.60	
Min-max	5.75-38.55	3.58-35.25	
Histologic grade			
I-II	13 (56.5)	27 (31.4)	.026[b]
III	10 (43.5)	59 (68.6)	

	PIK3CA		
	Wild-type	Mut	*P* Value
TILs (categorical)			
<10%	29 (36.7)	16 (57.1)	.024[b]
10%-19%	30 (38.0)	5 (17.9)	
20%-29%	9 (11.4)	1 (3.6)	
30%-49%	6 (7.6)	6 (21.4)	
≥50%	5 (6.3)	0 (0.0)	

	PIK3CA, Clonal		
	Clonal	Other	*P* Value
HER2 average copies			
Mean	9.43	14.90	.007[a]
SD	3.42	7.64	
Median	9.95	12.55	
Min-max	4.28-15.65	3.58-38.55	

	TP53		
	Wild-type	Mut	*P* Value
Histologic grade			
I-II	22 (53.7)	18 (26.5)	.004[b]
III	19 (46.3)	50 (73.5)	

	TP53, Clonal		
	Clonal	Other	P-value
Histologic grade			
I-II	16 (27.6)	24 (47.1)	.035[b]
III	42 (72.4)	27 (52.9)	

	FOXM1		
	Wild-type	Mut	*P* Value
TILs			
Mean	16.96	8.97	.042[a]

	ARID1B		
	Wild-type	Mut	*P* Value
HER2 average copies			
Mean	14.73	11.68	.044[a]
SD	7.69	5.75	
Median	12.65	9.90	
Min-max	3.58-38.55	6.63-27.00	
Ki67			
Mean	42.64	33.00	.048[a]
SD	22.01	16.01	
Median	40.00	30.00	
Min-max	1.00-90.00	15.00-75.00	

	CDH1		
	Wild-type	Mut	*P* Value
HER2 average copies			
Mean	14.86	11.58	.035[a]
SD	7.75	5.55	
Median	12.75	10.05	
Min-max	3.58-38.55	4.00-27.00	
TILs			
Mean	16.88	11.35	.029[a]
SD	17.33	15.04	
Median	11.00	5.00	
Min-max	1.00-80.00	1.00-65.00	

	MLL3 (KMT2C)		
	Wild-type	Mut	*P* Value
HER2 average copies			
Mean	14.75	10.96	.025[a]
SD	7.72	4.66	
Median	12.75	9.80	
Min-max	3.58-38.55	6.63-25.95	
Age, y			
≤50	25 (25.8)	8 (50.0)	.048[b]
>50	72 (74.2)	8 (50.0)	
Ki67			
Mean	42.56	30.33	.021[a]
SD	21.66	15.75	
Median	40.00	25.00	
Min-max	1.00-90.00	15.00-75.00	

	GATA3		
	Wild-type	Mut	*P* Value
TILs			
Mean	16.89	11.04	.046[a]

Table 2 Continued

	FOXM1				GATA3		
	Wild-type	Mut	*P* Value		Wild-type	Mut	*P* Value
SD	17.77	9.48		SD	17.36	14.69	
Median	10.00	5.00		Median	10.00	5.00	
Min-max	1.00-80.00	1.00-40.00		Min-max	1.00-80.00	1.00-65.00	

	TP53/PIK3CA Clonal Status					
	PIK3CA Only	TP53 Only	Both Mut	No Mut	Both Wild-type	*P* Value
HER2 average copies						
Mean	8.81	15.17	9.68	16.45	13.07	.028[a]
SD	3.64	7.48	3.49	8.20	7.40	
Median	8.41	12.55	10.05	15.20	10.90	
Min-max	5.15-13.25	4.00-30.55	4.28-15.65	5.75-38.55	3.58-35.25	
Ki67						
Mean	65.00	44.40	36.00	43.09	30.77	.013[a]
SD	15.81	22.56	20.66	22.41	13.47	
Median	67.50	35.00	40.00	40.00	30.00	
Min-max	45.00-80.00	2.00-90.00	1.00-85.00	1.00-85.00	10.00-60.00	

Abbreviations: Mut = mutant; SD = standard deviation.
[a]Wilcoxon rank-sum/Kruskal-Wallis test.
[b]χ^2/Fisher exact test.

2 groups (Figure 1C). The majority of mutations and 70% of clonal mutations in *TP53* were located in exons 5, 7, and 8, within the DNA-binding domain of the protein; mutations in hot spots were observed but these were maximally recurrent only up to 3 times. The majority of mutations and 75% of clonal mutations in *PIK3CA* were located in the known hot spots p.E542 and p.E545 in coding exon 9 corresponding to the helical domain and p.H1047 in exon 20 corresponding to the kinase domain of the protein.

Associations of Tumor Genotypes With Clinicopathologic Characteristics

The number of mutations was proportional to the number of mutated genes; both parameters inversely correlated with the number of HER2 average copies (Spearman rho, −0.32; $P < .001$). Tumors with mutations tended to have lower average HER2 copies; this was pronounced in tumors carrying clonal mutations in *PIK3CA* and also in *CDH1*, *MLL3*, and *ARID1B* (Table 2). Tumors with mutations in *PIK3CA*, *CDH1*, *FOXM1*, and *GATA3* had mostly lower TIL density, whereas those with mutations in *ARID1B* and in *MLL3* had lower proliferation rate. Further, the presence of mutations was associated with higher histologic grade, a feature mostly contributed by *TP53* mutations, because 73.5% of such tumors were grade III. The latter tumors had an overall higher number of mutated genes compared with lower grade tumors (mean value, 5.25 vs. 3.58; Wilcoxon rank-sum $P = .039$).

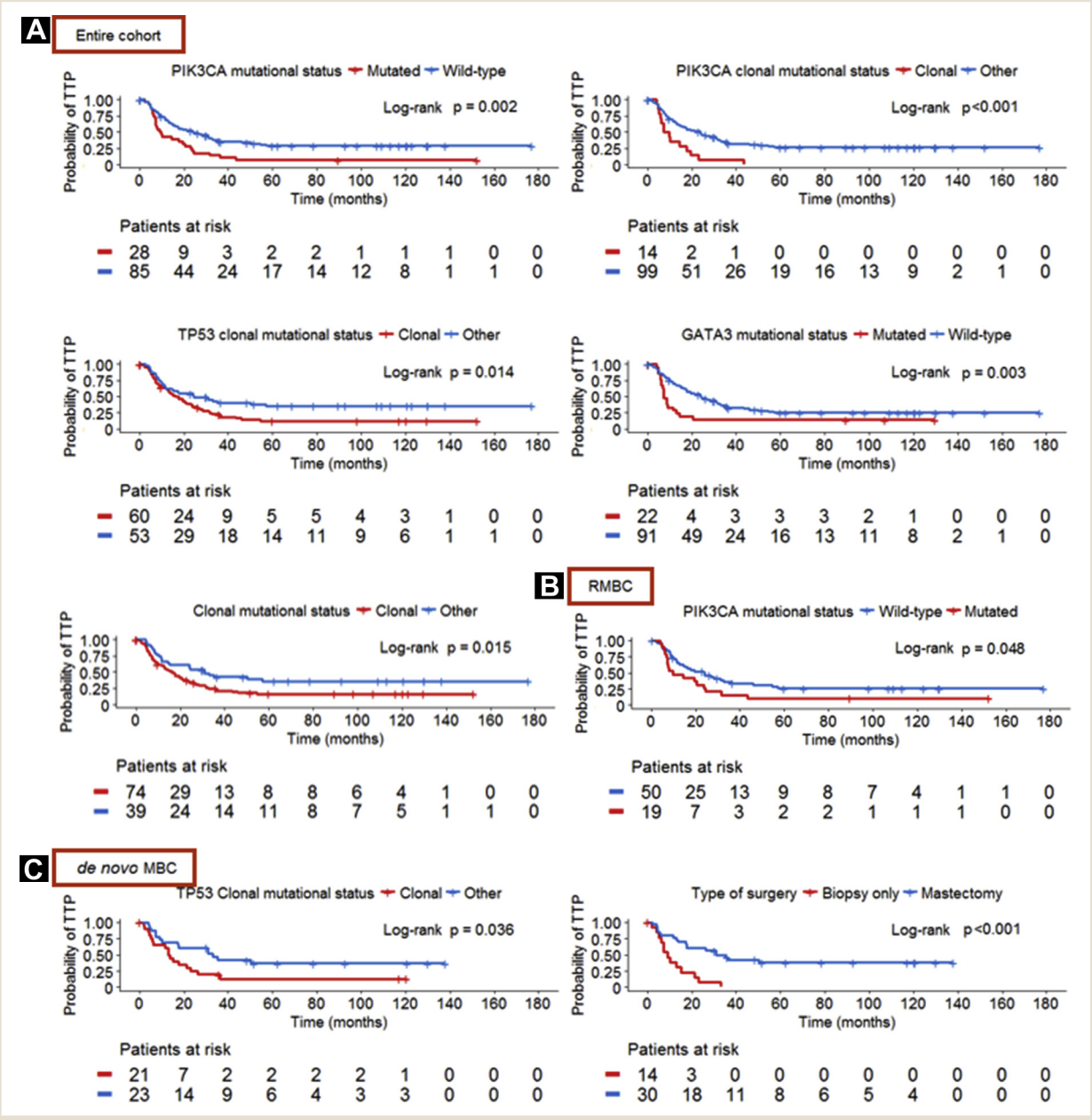
PIK3CA and *TP53* co-mutated tumors did not exhibit particular clinicopathologic characteristics. Of note though, 17 of 22 co-mutated tumors were R-MBC, whereas 4 of 6 tumors with *PIK3CA* mutations only were dn-MBC (Fisher $P = .041$). In addition, in the presence of clonal mutations in both genes, the average HER2 copies were similar to those contributed by *PIK3CA* mutations. The 4 tumors with clonal mutations in *PIK3CA* without *TP53* co-mutation exhibited particularly high Ki67 labeling index (Table 2).

With respect to clinicopathologic parameters, TIL density was vaguely associated with higher average HER2 copies (Spearman rho, 0.22; $P = .025$) and higher Ki67 index (rho, 0.26; $P = .007$), whereas HER2 copies and Ki67 also exhibited a weak positive correlation with each other (rho, 0.24; $P = .012$). No further significant associations were noticed.

Clinicopathologic Characteristics and Tumor Genotypes on Outcome of Trastuzumab-treated Patients With MBC

Patients were followed-up for a median of 120.3 months (95% confidence interval [CI], 105.1-130.2 months). Follow-up time was significantly longer for patients with R-MBC (median, 129.9 months; 95% CI, 107.8-158.6 months) as compared with patients with dn-MBC (median, 105.1 months; 95% CI, 71.5-130.1 months; log-rank $P = .029$). Overall, 78 (69.0%) patients died, and 82 (72.6%) patients experienced disease progression (51 patients with R-MBC and 31 patients with dn-MBC). Median TTP and survival were 19.5 months (95% CI, 13.0-24.3 months) and 48.5 months (95% CI, 37.2-59.8 months), respectively, whereas no difference was observed between patients with R-MBC and dn-MBC in either TTP (log-rank $P = .76$) or survival ($P = .48$). With respect to DFI, which was examined in patients with R-MBC only, the median time from diagnosis to initiation of trastuzumab therapy for metastatic disease was 30.2 months (95% CI, 30.1-43.7 months). Five patients with R-MBC relapsed within 1 year from initial breast cancer diagnosis, 36 within 3 years, and 45 within 5 years since the initial diagnosis. In R-MBC, DFI did not differ

Figure 2 Genotype Characteristics Associated With TTP in HER2$^+$ MBC. A, Clonal Mutations Without Gene Specificity, PIK3CA Mutations, Clonal or Not, TP53 Clonal Mutations, and GATA3 Mutations Were Significantly Associated With Worse TTP in the Entire Cohort. B, PIK3CA Mutations Were the Only Significant Genotype Parameter in R-MBC. C, Clonal TP53 Mutations and Primary Tumor Removal Were Respectively Associated With Poor and Favorable Prognosis in dn-MBC



Abbreviations: dn-MBC = de novo metastatic breast cancer; HER2$^+$ = human epidermal growth factor receptor 2-positive; R-MBC = relapsed metastatic breast cancer; TTP = time-to-progression.

between luminal-HER2 and HER2-enriched patients (Mann-Whitney $P = .4430$).

Among all patients with MBC, clonal mutations, *PIK3CA* mutations (particularly clonal), clonal *TP53*, and *GATA3* mutations were all associated with shorter TTP (Figure 2A). Patients with tumors exhibiting any clonal mutation (N = 74; Wald $P = .016$), *PIK3CA* mutations (N = 28; $P = .002$), clonal *PIK3CA* mutations (N = 14; $P < .001$), clonal *TP53* mutations (N = 60; $P = .015$), *GATA3* mutations (N = 22; $P = .004$), and with higher proliferation rate ($P = .031$) had higher risks for disease progression compared with those without such features (see Supplemental Table 1 in the online version). Except for *GATA3*, all above parameters were also associated with a higher risk for death.

In R-MBC, *PIK3CA* mutations were the only mutational parameter associated with shorter TTP (Figure 2B). This feature, and particularly clonal mutations in the same gene, conferred a

Table 3 Multivariate Analyses (Backwards Selection Models) for the Entire Cohort (A), Patients With R-MBC (B), and Patients With dn-MBC (C) With Respect to TTP and Survival

Parameter/*Categories*	No. Patients	No. Events	HR	95% CI	*P* Value
A. Entire cohort					
TTP					
First model (N = 106 patients)					
Ki67[a]			1.01	1.00-1.02	.035
PIK3CA mutational status					
Mutant vs. wild-type	26 vs. 80	24 vs. 52	2.21	1.36-3.61	.002
Second model (N=106 patients)					
Ki67[a]			1.01	1.00-1.02	.089
Central subtypes					
Luminal HER2 vs. HER2-enriched	68 vs. 38	45 vs. 31	0.66	0.41-1.06	.089
PIK3CA clonal mutational status					
Clonal vs. other	14 vs. 92	14 vs. 62	3.17	1.70-5.92	<.001
Survival					
First model (N = 106 patients)					
Ki67[a]			1.01	1.00-1.02	.053
PIK3CA mutational status					
Mutant vs. wild-type	26 vs. 80	21 vs. 52	1.74	1.04-2.91	.034
Second model (N = 106 patients)					
PIK3CA clonal mutational status					
Clonal vs. other	14 vs. 92	13 vs. 60	1.83	0.99-3.40	.055
TP53 clonal mutational status					
Clonal vs. other	55 vs. 51	44 vs. 29	1.62	1.00-2.62	.050
B. R-MBC					
TTP					
First model (N = 49 patients)					
Disease-free interval[a]			0.97	0.95-0.98	<.001
Second model (N = 49 patients)					
Disease-free interval[a]			0.97	0.95-0.99	<.001
Clonal mutational status					
Clonal vs. other	33 vs. 16	33 vs. 16	3.00	0.84-10.73	.092
TP53 clonal mutational status					
Clonal vs. other	30 vs. 19	30 vs. 19	0.31	0.09-1.09	.067
Survival					
First model (N = 49 patients)					
Disease-free interval[a]			0.98	0.96-1.00	.011
PIK3CA mutational status					
Mutant. vs. wild-type	16 vs. 33	15 vs. 26	2.07	1.05-4.06	.035
Second model (N = 49 patients)					
Disease-free interval[a]			0.98	0.97-0.99	.009
PIK3CA clonal mutational status					
Clonal vs. other	9 vs. 40	9 vs. 32	2.47	1.12-5.45	.025
C. dn-MBC					
TTP					
First model (N = 43 patients)					
Number of mutations per tumor[a]			1.03	1.00-1.06	.067
Type of surgery					
Mastectomy vs. biopsy	29 vs. 14	17 vs. 13	0.27	0.12-0.61	.002
Second model (N = 43 patients)					

Table 3 Continued

Parameter/*Categories*	No. Patients	No. Events	HR	95% CI	*P* Value
Number of mutated genes per tumor[a]			1.07	1.00-1.14	.061
Type of surgery					
Mastectomy vs. biopsy	29 vs. 14	17 vs. 13	0.28	0.12-0.63	.002
Survival					
First model (N = 43 patients)					
Number of mutations per tumor[a]			1.04	1.00-1.07	.026
TP53 clonal mutational status					
Clonal vs. other	20 vs. 23	15 vs. 11	2.11	0.95-4.68	.065
Second model (N = 43 patients)					
Number of mutated genes per tumor[a]			1.09	1.01-1.18	.025
TP53 clonal mutational status					
Clonal vs. other	20 vs. 23	15 vs. 11	2.03	0.91-4.50	.082

Abbreviations: CI = confidence interval; dn-MBC = de novo metastatic breast cancer; HR = hazard ratio; No. = number; R-MBC = relapsed metatstatic breast cancer; TTP = time to progression.
[a]Continuous variable.

higher risk for disease progression and death (see Supplemental Table 2 in the online version); however, only 9 R-MBC tumors had clonal *PIK3CA* mutations. In this group of patients, monthly increases in DFI reduced the risk for disease progression by 2% (Wald $P < .001$) and the risk for death by 1% ($P = .016$).

In dn-MBC, the presence of clonal mutations and the presence of *TP53* mutations (Figure 2C) were associated with shorter TTP. These parameters, along with the number of mutations and mutated genes per tumor, conferred higher risk for disease progression; clonal mutations and *TP53* mutations also conferred a higher risk for death (see Supplemental Table 3 in the online version). By contrast, surgical removal of the primary breast tumor instead of biopsy only was associated with longer TTP (Figure 2C). Surgery reduced the risk for progression by 74%; however, it had no effect on survival in patients with dn-MBC (see Supplemental Table 3 in the online version).

The models applied for multivariate analyses are shown in Supplemental Table 4 (in the online version), and their rationale is described in Methods. As shown in Table 3, *PIK3CA* mutations of any clonality status retained an independent unfavorable effect on TTP in the entire cohort, along with proliferation rate. The same parameters tended to confer increased risk for death, along with clonal *TP53* mutations. In R-MBC, clonal *PIK3CA* mutations were associated with higher risk for death. In these patients, DFI was a significant independent favorable predictor of outcome. In dn-MBC, the number of mutations retained their unfavorable prognostic effect, whereas clonal *TP53* mutations tended to confer a higher risk for death. In these patients, the application of surgery for the primary tumor was an independent favorable predictor for longer TTP, although it was unrelated to patient survival.

Discussion

Genotypes of primary tumors from patients with HER2$^+$ MBC yielded the expected mutation profiles for HER2$^+$ breast cancer[13]: a high prevalence of *TP53* followed by *PIK3CA*, and far less *CDH1* and *GATA3* mutations. As described, the examined tumors had been collected before any treatment. In dn-MBC, the observed mutation prevalence and patterns may represent the actual tumor status to treat; in R-MBC, however, the primary tumor was obtained before the application of adjuvant treatment, in most cases years before the manifestation of metastatic disease, which as a rule substantially differs from the primary tumor.[14] On this basis, it may appear irrelevant to examine the primary tumor in patients with R-MBC but the latter, unfortunately, still constitutes the real-life practice; biopsies from metastatic sites are not routinely processed, and the full genomic profile of metastatic disease cannot be obtained with existing methods. Thus, any information that would potentially help predicting for response to the treatment applied in the metastatic setting is useful. Nevertheless, looking into the primary tumor may not be completely irrelevant because the basic drivers identified therein are usually retained in metastatic sites,[15] whereas clonal expansion seems based on positive mutation selection in breast cancer.[15,24]

Here we report that a high mutational burden and the presence of clonal mutations, particularly in *PIK3CA,* are unfavorable prognosticators for benefitting from trastuzumab treatment in dn-MBC and R-MBC, respectively. The unfavorable impact of high mutational burden is a novel observation in MBC in general and in HER2$^+$ MBC in particular. Tumors with a high mutational burden have a higher potential of clonal expansion, resulting in a diverse and hence more difficult to treat disease. A high mutational burden has been reported in a fraction of most types of solid tumors, mostly but not exclusively linked to DNA repair deficiency (eg, mismatch repair)[25]; when accompanied by stalled immune response, it may provide the basis for immunotherapeutic interventions (eg, in non–small-cell lung cancer[26] and in diverse cancer types).[27] These studies suggest that the assessment of tumor mutational burden should be addressed only with large panels, such as the 1.1MB comprehensive genomic profiling panel utilized therein, unlike the small one we used in the present study. We did not apply any cutoff for assessing this marker and provide initial evidence that with increasing numbers of tumor mutations, treatments may more likely fail. Tumors in the present study were characterized by low TIL density, which may have accounted for the adverse effect of

increased mutation number, as previously shown.[27,28] Evidently, owing to the small number of examined patients, our study is exploratory and hypothesis-generating, and the results need to be prospectively validated in large patient series. Studies on even smaller patient cohorts, using different NGS panels and different approaches for classifying mutational burden have indeed yielded contradictory prognostic associations for high tumor mutational burden (eg, in breast cancer); unfavorable in 53 patients[29] but favorable in 46 patients.[30] Nevertheless, as discussed in the literature,[31,32] it appears important to pursue tumor mutational burden in HER2$^+$ dn-MBC and to investigate its role in R-MBC as well, especially in light of possible immunotherapeutic interventions in prospective clinical trials.

The unfavorable impact of *PIK3CA* mutations and of PI3K activation on trastuzumab resistance is well-established.[17,33-37] Of note, however, *PIK3CA* almost always coexisted with *TP53* mutations in the present series, a feature that was associated with unfavorable outcome in the adjuvant setting in a previous study of our group.[38] Further, *PIK3CA* mutations were strongly associated with high mutational burden and with mutations in genes within the ER-pathway. Co-mutation in the same tumor does not necessary mean co-mutation in the same cell or clone, particularly before treatment; it rather reflects the coexistence and possible cooperation of these alterations in the same tumor. This has been shown on a single-cell basis for *PIK3CA* mutations versus HER2 amplification[39] and has been predicted with recent algorithms for mutations in *PIK3CA* and *TP53*.[40] Based on our findings, *PIK3CA* mutations rather appear as a surrogate of complex mutational profiles in HER2$^+$ MBC. The unfavorable effect attributed to *PIK3CA* may in fact be contributed by *TP53* mutations or by mutations in genes within the ER pathway, which should be considered when testing PI3K inhibitors, for example, in luminal-HER2 tumors.

Further notable features of MBC genotypes in association with clinicopathologic characteristics concerned HER2 copy numbers and TIL density. We noticed an inverse correlation between HER2 average copies and the number of mutations per tumor. This is compatible with the proposed classification of tumors into "mutation" and "copy number" types,[41] whereby tumors with a high mutational load usually have few copy number changes, and vice versa. A higher mutational load in tumors with low HER2 copies may perhaps justify the occasionally reported low efficiency of trastuzumab in low HER2 copy tumors in the adjuvant setting.[42] The present series was too limited to reliably examine the role of TILs for trastuzumab benefit in the metastatic setting. Nevertheless, the rate of tumors with very high TIL density (lymphocytic predominance) in the entire cohort was lower than the expected range for HER2$^+$ tumors,[43] particularly in dn-MBC tumors. This may explain the early dissemination of tumor cells in patients diagnosed with de novo stage IV disease. TIL density was inversely associated with *PIK3CA* mutations, as expected from our previous study in a different cohort,[38] and also with mutations in other ER-phenotype related genes such as *GATA3* and *CDH1*. However, we did not observe an association between TIL density and trastuzumab benefit in the entire cohort or in the particular disease presentation groups. This is in contrast to relevant findings for survival of patients with HER2$^+$ MBC from the CLEOPATRA (Clinical Trial to Evaluate the Efficacy and Safety of Pertuzumab and Trastuzumab + Docetaxel) trial,[44] where the ratio between R-MBC and dn-MBC was close to 1:1, and most examined tumors were primary tumors. The current small sample size and the possibility that TIL density may be found decreased in breast cancer metastases may account for our controversial finding.

We did not observe any difference in the outcome of patients with R-MBC and dn-MBC, although the latter are generally expected to fare better, as shown irrespectively of HER2 status and treatment[8,45,46] or in the context of HER2$^+$ disease only.[47] Our results are rather in line with those by Rossi et al[48] who found no difference in the outcome of trastuzumab-treated patients with dn-MBC and R-MBC. Of note, none of the traditional clinicopathologic parameters (eg, grade and HER2 subtypes) were independent predictors of outcome in our series. This is important for excluding sample selection bias, particularly in the R-MBC subgroup, which was enriched in patients with luminal-HER2 tumors that are considered of a more favorable outcome compared with HER2-enriched.[49] Instead, in these previously trastuzumab-naive patients with R-MBC, the DFI before the appearance of metastases was the most important factor affecting progression and death in the metastatic setting, in line with a previous work.[45] DFI collectively depends on the biological characteristics of the tumor, the applied treatment, and the host response to treatment. In the present era of (neo)adjuvant trastuzumab, DFI may be considered as analogous to the trastuzumab-free period before the diagnosis of metastasis.[50] In both cases, it seems that patients are more likely to benefit from trastuzumab in the metastatic setting if they have benefitted from the preceding adjuvant treatment, whether it includes trastuzumab or not.

An independently significant parameter affecting the TTP of our trastuzumab-treated patients with HER2$^+$ dn-MBC appeared to be the application of locoregional surgery before treatment start. In comparison, this practice was considered of no benefit for HER2$^+$ dn-MBC in the prospective Turkish study.[51] Our result comes from a retrospective analysis and is in line with the relevant literature for HER2$^+$ cancer[47] or for patients with MBC independently of breast cancer subtypes.[52,53] We have not assessed response to first-line chemotherapy, thus our finding cannot be directly compared with the results from the prospective TBCRC 013 (Translational Breast Cancer Research Consortium)[54] and from an open-label randomized trial,[55] again stating that the factor determining dn-MBC outcome is the initial response to treatment and not locoregional surgery itself. Designing prospective clinical trials to assess the net effect of surgery in HER2$^+$ dn-MBC could shed light on this clinical dilemma.

In conclusion, according to our findings, patients with HER2$^+$ MBC may not benefit from trastuzumab combinations if their primary tumors are highly mutated and have clonal mutations, particularly in *PIK3CA* and less so in *TP53*, 2 frequently co-mutated genes. We cannot conclude that any of these parameters is related to trastuzumab failure, because this agent is administered together with cytotoxic chemotherapy or hormonal therapy. However, we provide initial evidence that the impact of *PIK3CA* mutations on patient outcome may differ in patients with R-MBC and dn-MBC. Further, although we only used a targeted panel, we also indicate that the outcome of dn-MBC may be related to tumor mutational burden. These exploratory findings, if validated in larger

studies, will be important when designing trials for testing anti-PI3K combined with anti-HER2 interventions. We emphasize on the necessity to decipher the genomic differences between R-MBC and dn-MBC in order to decide for the most suitable treatment approaches in the context of these clinically challenging conditions.

Clinical Practice Points

- $HER2^+$ MBC remains a lethal disease with median survival less than 5 years. Systemic therapy for $HER2^+$ R-MBC and dn-MBC is the same. For R-MBC, treatment is still based on characteristics of the early tumor. The genomic and phenotypic characteristics of dn-MBC, compared with tumors that have initially presented at early stage and then relapsed (ie, R-MBC) remain unknown.
- For approaching the latter issue, we applied deep sequencing on a series of tumors from patients with R-MBC and dn-MBC who received trastuzumab in the metastatic setting only. The obtained genotype patterns correspond: in R-MBC, to the early disease that has been treated with adjuvant chemotherapy in the past; in dn-MBC, to the metastatic disease to be treated in the present.
- Main novel findings in patients with $HER2^+$ MBC include:
  - Compared with R-MBC, dn-MBC appear more genetically unstable, which is compatible with the observed larger number of metastatic sites;
  - Tumors with higher numbers of mutations and mutated genes (mutational burden) have lower numbers of HER2 copies. Tumor mutational burden may affect prognosis in dn-MBC;
  - *PIK3CA* mutations are a valid marker for unfavorable outcome, but these are rather surrogates in $HER2^+$ disease, because they occur in co-mutation with *TP53* and genes within the estrogen receptor pathway and are not independent prognostic factors in dn-MBC.
- These exploratory findings, if validated in larger studies, will be helpful in the design of trial testing for new agents in combination with trastuzumab, potentially including immunotherapeutics and synthetic lethality approaches.

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Disclosure

G. Lazaridis reports honoraria from BMS, Roche, Merck, MSD; an advisory role with Merck; and travel from BMS. C. Christodoulou reports an advisory role with Merck, Zeincro, Genesis Pharmaceuticals, Pfizer, Novartis, Roche, AstraZeneca, and Bristol Myers Squibbs; honoraria from Roche and Bristol Myers Squibbs; and research funding from Abbvie, PPD Inc, Novartis, Parexel, and AstraZeneca. G. Pentheroudakis reports an advisory role with Roche; honoraria from Roche; speaker bureau with Roche; and grants from Amgen. A. Koutras reports an advisory role with Roche, Genesis, and Astra-Zeneca; and travel from Novartis, Merck, Sanofi-Aventis, Astellas, Genesis, BMS, and Pfizer. E. Razis reports an advisory role with Roche, Amgen, AstraZeneca, Janssen-Cilag, Astellas Pharma, Novartis, Bristol-Myers Squibb, Merck, Pfizer, and Zeincro; travel from Genesis Pharmaceuticals, LEO Pharma, Pfizer, Roche, GlaxoSmithKline, Sanofi, Amgen, Bristol-Myers Squibb, Genekor, Eisai, Merck, Pierre Fabre, and Novartis; honoraria from Roche, AstraZeneca, GlaxoSmithKline, Amgen, Novartis, Zeincro, Merck, Pfizer, and Bristol-Myers Squibb; and research funding from Sanofi, Roche/Genentech, Novartis, AstraZeneca, Demo Pharmaceutical, Celldex; and Parexel. G. Fountzilas reports a role on the advisory board of Pfizer, Sanofi, and Roche; and honoraria from Astra-Zeneca.

Supplemental Data

Supplemental tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2018.10.014.

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Supplemental Data

Supplemental Table 1 Cox Univariate Regression Analysis With Respect to TTP and Survival for the Entire Study Population

Parameter	Categories	TTP			Survival				
		HR	95% CI	*P* Value	No. Patients	No. Events	HR	95% CI	*P* Value
Presense of mutations	Yes vs. no	1.19	0.69-2.06	.53	90 vs. 23	65 vs. 13	1.43	0.79-2.60	.24
Clonal mutational status	Clonal vs. other	1.80	1.12-2.91	**.016**	74 vs. 39	57 vs. 21	1.81	1.10-2.99	**.020**
Disease presentation status	dn-MBC vs. R-MBC	0.93	0.60-1.46	.76	44 vs. 69	27 vs. 51	0.85	0.53-1.35	.48
Histologic grade	III vs. I II	0.97	0.62-1.53	.90	69 vs. 40	47 vs. 29	1.09	0.68-1.73	.72
PIK3CA mutational status	Mutant vs. wild-type	2.07	1.29-3.32	**.002**	28 vs. 85	22 vs. 56	1.60	0.97-2.63	.064
PIK3CA clonal mutational status	Clonal vs. other	2.73	1.51-4.93	**<.001**	14 vs. 99	13 vs. 65	2.09	1.15-3.81	**.016**
Central subtypes	Luminal HER2 vs. HER2-enriched	0.77	0.50-1.20	.25	72 vs. 41	48 vs. 30	0.81	0.52-1.29	.38
TP53 mutational status	Mutant vs. wild-type	1.18	0.75-1.85	.49	72 vs. 41	54 vs. 24	1.52	0.94-2.46	.089
TP53 clonal mutational status	Clonal vs. other	1.74	1.11-2.71	**.015**	60 vs. 53	49 vs. 29	1.86	1.17-2.95	**.008**
ARID1B mutational status	Mutant vs. wild-type	1.40	0.81-2.42	.23	21 vs. 92	14 vs. 64	1.06	0.60-1.90	.84
CDH1 mutational status	Mutant vs. wild-type	0.94	0.54-1.65	.83	23 vs. 90	15 vs. 63	0.96	0.55-1.69	.90
FOXM1 mutational status	Mutant vs. wild-type	0.97	0.51-1.83	.92	17 vs. 96	9 vs. 69	0.79	0.39-1.58	.50
GATA3 mutational status	Mutant vs. wild-type	2.18	1.28-3.70	**.004**	22 vs. 91	16 vs. 62	1.48	0.85-2.57	.16
MLL3 KMT2C mutational status	Mutant vs. wild-type	1.12	0.61-2.07	.72	16 vs. 97	10 vs. 68	0.89	0.46-1.73	.73
MRM1 mutational status	Mutant vs. wild-type	1.21	0.66-2.24	.54	16 vs. 97	10 vs. 68	1.01	0.52-1.96	.98
HER2 average copies[a]		1.01	0.98-1.04	.41			1.01	0.97-1.04	.75
CEN17 average copies[a]		0.94	p.72-1.25	.68			0.95	0.72-1.25	.70
HER2CEN17 ratio[a]		1.01	0.96-1.06	.63			1.00	0.95-1.05	.94
Age[a]		0.99	0.97-1.00	.16			1.00	0.99-1.02	.64
Ki67[a]		1.01	1.00-1.02	**.031**			1.01	1.00-1.02	**.043**
No. mutations per tumor[a]		1.00	1.00-1.01	.34			1.00	0.99-1.01	.87
No. mutated genes per tumor[a]		1.02	0.99-1.04	.17			1.00	0.98-1.03	.81
TILs[a]		1.00	0.99-1.01	.70			0.99	0.98-1.01	.34

Bold *P* values iindicates statistically significant.
Abbreviations: CI = confidence interval; dn-MBC = de novo metastatic breast cancer; HER2 = human epidermal growth factor receptor 2; HR = hazard ratio; No = number; R-MBC = relapsed metastatic breast cancer; TILs = tumor-infiltrating lymphocytes; TTP = time to progression.
[a]Continuous variables.

Supplemental Table 2 Cox Univariate Regression Analysis With Respect to TTP and Survival for Patients With R-MBC

		TTP					Survival				
Parameter	**Categories**	**No. Patients**	**No. Events**	**HR**	**95% CI**	***P* Value**	**No. Patients**	**No. Events**	**HR**	**95% CI**	***P* Value**
Presence of mutations	Yes vs. no	56 vs. 13	41 vs. 10	0.81	0.40-1.62	.55	56 vs. 13	42 vs. 9	0.98	0.47-2.02	.95
Clonal mutational status	Clonal vs. other	46 vs. 23	35 vs. 16	1.22	0.68-2.21	.51	46 vs. 23	36 vs. 15	1.31	0.72-2.40	.38
Histologic grade	III vs. I-II	38 vs. 29	28 vs. 22	1.14	0.65-1.99	.66	38 vs. 29	29 vs. 21	1.24	0.71-2.17	.46
PIK3CA mutational status	Mutant vs. wild-type	19 vs. 50	17 vs. 34	1.79	1.00-3.22	.051	19 vs. 50	16 vs. 35	1.51	0.83-2.74	.18
PIK3CA clonal mutational status	Clonal vs. other	9 vs. 60	9 vs. 42	2.85	1.37-5.95	**.005**	9 vs. 60	9 vs. 42	2.43	1.17-5.04	**.017**
Central subtypes	Luminal HER2 vs. HER2-enriched	49 vs. 20	32 vs. 19	0.58	0.33-1.03	.064	49 vs. 20	33 vs. 18	0.62	0.35-1.11	.11
TP53 mutational status	Mutant vs. wild-type	46 vs. 23	35 vs.16	1.13	0.63-2.05	.68	46 vs. 23	37 vs. 14	1.47	0.79-2.73	.22
TP53 clonal mutational status	Clonal vs. other	39 vs. 30	32 vs. 19	1.51	0.85-2.67	.16	39 vs. 30	33 vs. 18	1.66	0.93-2.95	.086
Adjuvant CT	No vs. yes	16 vs. 53	8 vs. 43	0.56	0.26-1.20	.14	16 vs. 53	10 vs. 41	0.72	0.36-1.44	.36
Adjuvant RT	No vs. yes	34 vs. 34	22 vs. 28	0.78	0.45-1.37	.39	34 vs. 34	27 vs. 23	1.35	0.77-2.38	.30
Adjuvant HT	No vs. yes	20 vs. 49	14 vs. 37	1.10	0.59-2.04	.76	20 vs. 49	16 vs. 35	1.41	0.78-2.56	.25
HER2 average copies[a]				1.01	0.98-1.04	.58			1.02	0.98-1.06	.30
CEN17 average copies[a]				1.32	0.88-1.96	.18			1.20	0.81-1.77	.37
HER2 CEN17 ratio[a]				0.99	0.93-1.06	.87			1.02	0.95-1.09	.64
DFI[a]				0.98	0.96-0.99	**<.001**			0.99	0.98-1.00	**.016**
Age[a]				0.98	0.96-1.01	.14			1.01	0.98-1.03	.68
Ki67[a]				1.02	1.00-1.03	**.027**			1.02	1.00-1.03	**.011**
Number of mutations per tumor[a]				1.00	0.99-1.01	.78			0.99	0.98-1.01	.43
Number of mutated genes per tumor[a]				1.01	0.98-1.04	.63			0.99	0.96-1.02	.57
TILs[a]				1.00	0.99-1.02	.69			1.00	0.99-1.01	.99

Bold *P* values iindicates statistically significant.
Abbreviations: CI = confidence interval; CT = chemotherapy; DFI = disease-free interval; HER2 = human epidermal growth factor receptor 2; HR = hazard ratio; HT = hormonal therapy; No = number; R-MBC = relapsed metastatic breast cancer; RT = radiotherapy; TILs = tumor-infiltrating lymphocytes; TTP = time to progression.
[a]Continuous variables.

Supplemental Table 3 Cox Univariate Regression Analysis With Respect to TTP and Survival for Patients With dn-MBC

Parameter	Categories	TTP					Survival				
		No. Patients	No. Events	HR	95% CI	*P* Value	No. Patients	No. Events	HR	95% CI	*P* Value
Preserve of mutations	Yes vs. no	34 vs. 10	25 vs. 6	1.88	0.76-4.60	.17	34 vs. 10	23 vs.4	2.44	0.84-7.09	.10
Clonal mutational status	Clonal vs. other	28 vs. 16	23 vs. 8	3.22	1.42-7.33	**.005**	28 vs. 16	21 vs. 6	3.20	1.28-8.00	**.012**
Histologic grade	III vs. I-II	31 vs. 11	21 vs. 8	0.77	0.34-1.75	.54	31 vs. 11	18 vs.8	0.91	0.39-2.09	.82
PIK3CA mutational status	Mutant vs. wild-type	9 vs. 35	9 vs. 22	2.69	1.21-5.98	**.015**	9 vs. 35	6 vs.21	1.76	0.70-4.41	.23
PIK3CA clonal mutational status	Clonal vs. other	5 vs. 39	5 vs. 26	2.48	0.92-6.68	.073	5 vs. 39	4 vs. 23	1.61	0.56-4.68	.38
Central subtypes	Luminal HER2 vs. HER2-enriched	23 vs. 21	16 vs. 15	1.03	0.51-2.08	.94	23 vs. 21	15 vs.12	1.15	0.54-2.45	.73
TP53 mutational status	Mutant vs. wild-type	26 vs. 18	18 vs. 13	1.22	0.60-2.49	.59	26 vs. 18	17 vs.10	1.62	0.74-3.56	.2
TP53 clonal mutational status	Clonal vs. other	21 vs. 23	17 vs. 14	2.12	I.03-4.35	**.040**	21 vs. 23	16 vs. 11	2.33	1.07-5.05	**.033**
Type of surgery	Mastectomy vs. biopsy	30 vs. 14	18 vs. 13	0.26	0.12-0.57	**<.001**	30 vs. 14	18 vs. 9	2.04	0.91-4.57	.084
HER2 Average copies[a]				1.01	0.96-1.07	.58			0.98	0.92-1.04	.43
CEN17 Average copies[a]				0.71	0.46-1.10	.12			0.79	0.52-1.21	.28
HER2 CEN17 Ratio[a]				1.03	0.96-1.10	.42			0.97	0.87-1.07	.49
Age[a]				1.00	0.97-1.02	.75			1.01	0.98-1.03	.72
Ki67[a]				1.01	0.99-1.03	.43			1.00	0.98-1.02	.79
Number of mutations per tumor[a]				1.03	1.01-1.06	**.013**			1.04	1.01-1.08	**.011**
Number of mutated genes per tumor[a]				1.09	1.02-1.15	**.008**			1.10	1.03-1.19	**.009**
TILs[a]				0.98	0.96-1.01	.25			0.97	0.93-1.00	.072

Bold *P* values iindicates statistically significant.
Abbreviations: CI = confidence interval; DFI = disease-free interval; dn-MBC = de novo metastatic breast cancer; HER2 = human epidermal growth factor receptor 2; HR = hazard ratio; No = number; TILs = tumor-infiltrating lymphocytes; TTP = time to progression.
[a]Continuous variables.

Supplemental Table 4 Multivariate Models Applied in the Present Study

A. Entire Cohort	
Disease presentation (dn-MBC vs. R-MBC)	
Histologic grade (III vs. I-II)	
Subtype classification (luminal HER2 vs. HER2-enriched)	
Age	
Ki67	
Model 1	**Model 2**
Clonal mutations (clonal vs. other)	Clonal (clonal vs. other)
PIK3CA mutations (mutant vs. wild-type)	*PIK3CA* clonal mutations (clonal vs. other)
TP53 mutations (mutant vs. wild-type)	*TP53* clonal mutations (clonal vs. other)
GATA3 mutations (mutant vs. wild-type)	*GATA3* mutations (mutant vs. wild-type)
B. R-MBC	
Age	
Ki67	
DFI	
Clonal mutations (clonal vs. other)	
Model 1	**Model 2**
PIK3CA mutations (mutant vs. wild-type)	*PIK3CA* clonal mutations (clonal vs. other)
TP53 mutations (mutant vs. wild-type)	TP53 clonal mutations (clonal vs. other)
C. dn-MBC	
Age	
Ki67	
Surgery (mastectomy vs. biopsy)	
Model 1	**Model 2**
PIK3CA clonal mutations (clonal vs. other)	*PIK3CA* clonal mutations (clonal vs. other)
TP53 clonal mutations (clonal vs. other)	TP53 clonal mutations (clonal vs. other)
Number of mutations per tumor	Number of mutated genes per tumor

Abbreviations: DFI = disease-free interval; dn-MBC = de novo metastatic breast cancer; HER2 = human epidermal growth factor receptor 2; R-MBC = relapsed metastatic breast cancer.