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ORIGINAL ARTICLE

Tumor mutational burden and immune infiltration as independent predictors of response to neoadjuvant immune checkpoint inhibition in early TNBC in GeparNuevo

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Background: The predictive value of tumor mutational burden (TMB), alone or in combination with an immune gene expression profile (GEP), for response to neoadjuvant therapy in early triple negative breast cancer (TNBC) is currently not known, either for immune checkpoint blockade (ICB) or conventional chemotherapy.

Patients and methods: We obtained both whole exome sequencing and RNA-Seq data from pretreatment samples of 149 TNBC of the recent neoadjuvant ICB trial, GeparNuevo. In a predefined analysis, we assessed the predictive value of TMB and a previously developed immune GEP for pathological complete remission (pCR).

Results: Median TMB was 1.52 mut/Mb (range 0.02—7.65) and was significantly higher in patients with pCR (median 1.87 versus 1.39; $P = 0.005$). In multivariate analysis, odds ratios for pCR per mut/Mb were 2.06 [95% confidence intervals (CI) 1.33—3.20, $P = 0.001$] among all patients, 1.77 (95% CI 1.00—3.13, $P = 0.049$) in the durvalumab treatment arm, and 2.82 (95% CI 1.21—6.54, $P = 0.016$) in the placebo treatment arm, respectively. We also found that both continuous TMB and immune GEP (or tumor infiltrating lymphocytes) independently predicted pCR. When we stratified patients in groups based on the upper tertile of TMB and median GEP, we observed a pCR rate of 82% (95% CI 60% to 95%) in the group with both high TMB and GEP in contrast to only 28% (95% CI 16% to 43%) in the group with both low TMB and GEP.

Conclusions: TMB and immune GEP add independent value for pCR prediction. Our results recommend further analysis of TMB in combination with immune parameters to individually tailor therapies in breast cancer.

Key words: neoadjuvant immune checkpoint inhibition, triple negative breast cancer, tumor mutational burden, exome sequencing

INTRODUCTION

Combining immune checkpoint blockade (ICB) with chemotherapy increased response rates in patients with metastatic triple negative breast cancer (mTNBC).[1,2] For early triple negative breast cancer (TNBC), recent results of the neoadjuvant randomized phase II GeparNuevo study suggest that the addition of durvalumab to anthracycline/taxane-based neoadjuvant chemotherapy increases the rate of pathological complete remission (pCR), mainly in the subgroup of patients treated with durvalumab alone before the start of chemotherapy.[3]

In several cancer types, predictive factors for ICB response include programmed death-ligand 1 (PD-L1) expression,[1] gene expression profiles (GEP) of infiltrating immune cells,[4] as well as microsatellite instability (MSI)[5] and tumor mutational burden (TMB).[4,6–9] Based on results from bucket trials of ICB in metastatic cancer, Cristescu et al. recently proposed a combination of TMB with immune GEP as a predictor of response.[10]

In early TNBC, immune GEP and tumor infiltrating lymphocytes (TIL) have both prognostic and predictive values for response to chemotherapy.[11,12] A high TMB could increase neoantigens inducing immune response. Indeed, in pooled analyses of diverse cancer types, overall a positive

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association of TMB and tumor immune cell infiltration has been observed.[13] Similarly, comparison of breast cancer subtypes reveals that both high TMB and high immune infiltration are more frequent in TNBC compared with luminal breast cancers.[14,15] However, within the TNBC subgroup, no such positive correlation of TMB and immune infiltration was detected, which may be attributed to immunoediting.[16]

The predictive value of TMB, alone or in combination with immune GEP, for response to neoadjuvant therapy in early TNBC is currently not known, either for ICB or conventional chemotherapy. It is especially not clear whether TMB will add to the predictive value of immune GEP. Therefore, our goal was to study these two parameters in pretreatment samples of TNBC from the neoadjuvant ICB trial GeparNuevo. We found that both parameters add independent value for pCR prediction for chemotherapy with and without ICB. Our results recommend further analysis of TMB in combination with immune parameters to individually tailor therapies in breast cancer.

METHODS

All analyses were carried out according to the reporting recommendations for tumor marker prognostic studies (REMARK) criteria.[17] A CONSORT type diagram[18] of the flow of samples through the study is shown in supplementary Figure S1 (available at *Annals of Oncology* online).

Patients

The details of the GeparNuevo study (NCT02685059) have been described in a recent publication.[3] In total, 174 patients were treated with nab-paclitaxel followed by dose-dense epirubicin/cyclophosphamide chemotherapy and were randomly assigned to either simultaneous treatment with durvalumab or placebo. The study was approved by the ethics committee and the competent authority. All patients provided written informed consent for study conduct, biomaterial collection, and analysis. In the window phase, durvalumab/placebo alone was given 2 weeks before the start of nab-paclitaxel; 117 of the 174 participated in this window phase. Randomization was stratified by stromal tumor infiltrating lymphocyte (sTILs). Patients with primary cT1b-cT4a-d disease, centrally assessed sTILs, and confirmed TNBC were included. The primary objective was pCR (ypT0 ypN0). The pCR rate with durvalumab was 53.4% (42.5% to 64.1%) versus placebo 44.2% (33.5% to 55.3%; $P = 0.287$), corresponding to odds ratio (OR) = 1.45 (0.80−2.63, unadjusted Wald $P = 0.224$). The durvalumab effect was significant in the window cohort (pCR 61.0% versus 41.4%, OR = 2.22, 1.06−4.64, $P = 0.035$; interaction $P = 0.048$).[3] The complete set of molecular parameters was successfully obtained only for a subset of the patients: TMB was available for 149 patients (supplementary Figure S1, available at *Annals of Oncology* online).

TIL scoring and PD-L1 immunohistochemistry

TIL scoring and PD-L1 immunohistochemistry were carried out as previously described.[3] In brief, PD-L1 status was determined using the Ventana SP263 antibody. We evaluated the PD-L1 expression as the percentage of tumor cells with membranous staining and percentage of TILs with membranous or cytoplasmic staining (relative to total TILs).[3] Stromal TILs were evaluated based on the standardized guidelines of the international TIL working group.[19] When TILs are included as continuous scores in logistic regression analysis, odds ratios are presented per 10% increase.

Whole exome sequencing for TMB and mutational signatures

Whole exome sequencing (WES) was conducted on fresh-frozen pre-therapeutic core biopsies and patient-matched blood samples with Illumina HiSeq 4000 (Illumina Inc., San Diego, CA). High-quality data were obtained for 149 (85.6%) of the 174 patients from the trial (supplementary Figure S1, available at *Annals of Oncology* online). Full details on the methods are given in the supplementary Methods (available at *Annals of Oncology* online). For TMB calculation, we used the final list of 12 314 non-synonymous single-nucleotide variants (SNVs) and indels, obtained after filtering, together with an effective DNA coverage of 46 Mb to determine mutations per Mb for each sample. Mutational signatures were identified as described by Alexandrov et al.[20] R package SomaticSignatures was employed to estimate the proportion of each sample's mutations that have been assigned to each of the 21 mutational signatures.[21]

RNA sequencing

RNA sequencing was carried out on formalin-fixed paraffin-embedded (FFPE) tissue using an HTG EdgeSeq instrument (HTG Molecular Inc., Tucson, AZ) with the HTG EdgeSeq Oncology Biomarker Panel (2549 genes) based on an RNA-extraction-free chemistry and a nuclease protection assay.[22] The tumor area was marked on a slide stained with hematoxylin and eosin and the area of invasive breast cancer recorded. From a corresponding unstained slide, 15 mm^2 tissue was scraped and used for library preparation according to the manufacturer's instructions. Libraries were quantified, pooled, and sequenced on an Ion Torrent S5 instrument (Thermo Fisher Scientific, Waltham, MA). Count tables were generated using the HTG parsing tool. Full details are given in the supplementary Methods (available at *Annals of Oncology* online). RNA sequencing data from pre-therapeutic cores were available for 159 of the 174 patients (supplementary Figure S1, available at *Annals of Oncology* online).

Molecular subtyping from RNA-Seq and immune GEP

Molecular subtyping from RNA-Seq was carried out using the AIMS method[23] (details in supplementary Methods, available at *Annals of Oncology* online). We also evaluated a predefined immune GEP predictive for neoadjuvant response that was created from a list of genes we previously identified in the GeparSixto study (GeparSixto immune signature: *CXCL9, CCL5, CD8A, CD80, CXCL13, IDO1, PDCD1, CD274, CTLA4, FOXP3*).[4] The genes *CD21* and *IGKC*

were omitted because they were not covered by the mRNA sequencing assay. The immune GEP was calculated as the mean of the expression of the genes from the signature.

Aggregation of WES and HTG-RNA-Seq data

The analyses of the pseudonymized genomic datasets were carried out fully blinded to any clinical or pathological sample information. The final blinded whole exome sequencing (WES) and HTG-RNA-Seq datasets were transferred to German Breast Group (GBG) headquarters. WES and RNA-Seq were available for 149 and 159 patients, respectively, with both data available for 136 patients (supplementary Figure S1, available at *Annals of Oncology* online). A comparison of the complete trial cohort, the WES cohort, and the WES+RNA-Seq cohort is provided in supplementary Table S1 (available at *Annals of Oncology* online).

Statistical analysis

All clinical data, including age, stage, histological grade, treatment arm, window treatment, TILs, PD-L1, and pCR (ypT0 ypN0), were extracted from the clinical study database at GBG headquarters and represent central assessment. Both TMB and the immune GEP (GeparSixto immune signature) from RNA-Seq were used as continuous parameters in univariate and multivariate logistic regression. In addition, we present results of predefined dichotomized TMB and GEP for illustration of pCR frequencies and comparison with previous studies. For GEP, a median split of the cohort was used. TMB was dichotomized at the upper tertile ($\geq$95 mutations) based on the previously suggested pan-cancer cutoff for ICB response prediction (>100 mutations).[10] Pearson's chi-square and Fisher's exact test were applied to assess associations between categorical parameters. To analyze the predictive value of molecular markers for pCR, univariate and multivariate logistic regression models adjusted for prespecified variables were used. The R software environment (version 3.3.2, http://www.r-project.org/) and SPSS[24] (http://www.ibm.com/) were used for all analyses. All confidence intervals (CI) reported are 95%. All *P*-values are two-sided and $P \leq 0.05$ was considered as significant.

RESULTS

TMB and correlation with pCR in the GeparNuevo trial

We successfully obtained high-quality WES data from the tissue of 149 (86%) of the 174 patients from the GeparNuevo study (supplementary Table S1, available at *Annals of Oncology* online, compares clinical data of the WES cohort and the complete study cohort). Median TMB was 1.52 mut/Mb (range 0.02–7.65). As shown in Table 1, we found significantly higher median TMB values in older patients ($P < 0.001$) and a trend for higher values in patients with higher stage of cancer ($P = 0.086$). Numerically, we saw lower values for median TMB in tumors with high numbers of TILs and in those without PD-L1 expression, but this was not significant. Patients with a pCR displayed a significantly higher TMB (median with pCR 1.87 versus 1.39 without pCR; $P = 0.005$). Figure 1 shows the distribution of TMB values of patients with and without a pCR. Interestingly, a high TMB among pCR cases was especially seen in the placebo (chemotherapy alone) treatment arm (Figure 1).

Table 1. Differences in tumor mutational burden (TMB) according to clinical parameters of the GeparNuevo samples

Parameter	Category	Median TMB	*P* value (Wilcoxon test)
Age	<40 years	1.11	**<0.001**
	≥40 years	1.74	
Stage	0–I	1.43	0.086
	IIA or higher	1.62	
Histol. grade	G2	1.56	0.826
	G3	1.52	
Treatment arm	Placebo	1.59	0.672
	Durvalumab	1.47	
Window treatment	No	1.70	0.303
	Yes	1.46	
TILs	<60%	1.61	0.190
	≥60%	1.35	
PD-L1	Negative	1.43	0.989
	Positive	1.59	
Response	RD	1.39	**0.005**
	pCR (ypT0 ypN0)	1.87	

pCR, pathological complete remission; PD-L1, programmed death-ligand 1; RD, residual disease; TILs, tumor infiltrating lymphocytes.
Significant P values are given in bold.

As shown in Table 2, we detected a predictive value of continuous TMB for pCR among all patients both in univariate (OR 1.62, 1.20–2.20; $P = 0.002$) and multivariate logistic regression (including age, stage, grading, stromal TILs, PD-L1 status, and window treatment, OR 2.06, 1.33–3.20; $P = 0.001$). When we separately analyzed the two treatment arms, we detected a significant positive correlation between TMB and pCR in both arms in multivariate analyses; in the univariate analysis we found a strong trend in the durvalumab arm and a significant correlation in the placebo arm (Table 2). We did not observe an interaction with the treatment arm ($P = 0.439$ and $P = 0.436$ for univariate and multivariate analysis, respectively, Table 2).

We also present results for TMB dichotomized using the upper tertile of our cohort (2.05 mut/Mb), which was similar to a previously published cutoff from a pan-cancer analysis of ICB response prediction.[10] As shown in Table 2, dichotomized TMB significantly predicted pCR among all patients (univariate OR 2.22, 1.11–4.43, $P = 0.024$; multivariate OR 3.45, 1.41–8.48, $P = 0.007$). Also in this analysis we found no significant interaction with the treatment arm (Table 2). After dichotomization of TMB at the top tertile, 50 patients had high TMB and 29 of these (58%) achieved a pCR, while 99 had low TMB and only 38 of these (38%) had a pCR ($P = 0.007$) (supplementary Figure S2, available at *Annals of Oncology* online). In the durvalumab treatment arm, pCR rates were 17/27 (63%) and 19/47 (40%) for high and low TMB, respectively ($P = 0.028$), and in the placebo arm 12/23 (52%) for high, and 19/52 (37%) for low TMB ($P = 0.232$, supplementary Figure S2, available at *Annals of Oncology* online).

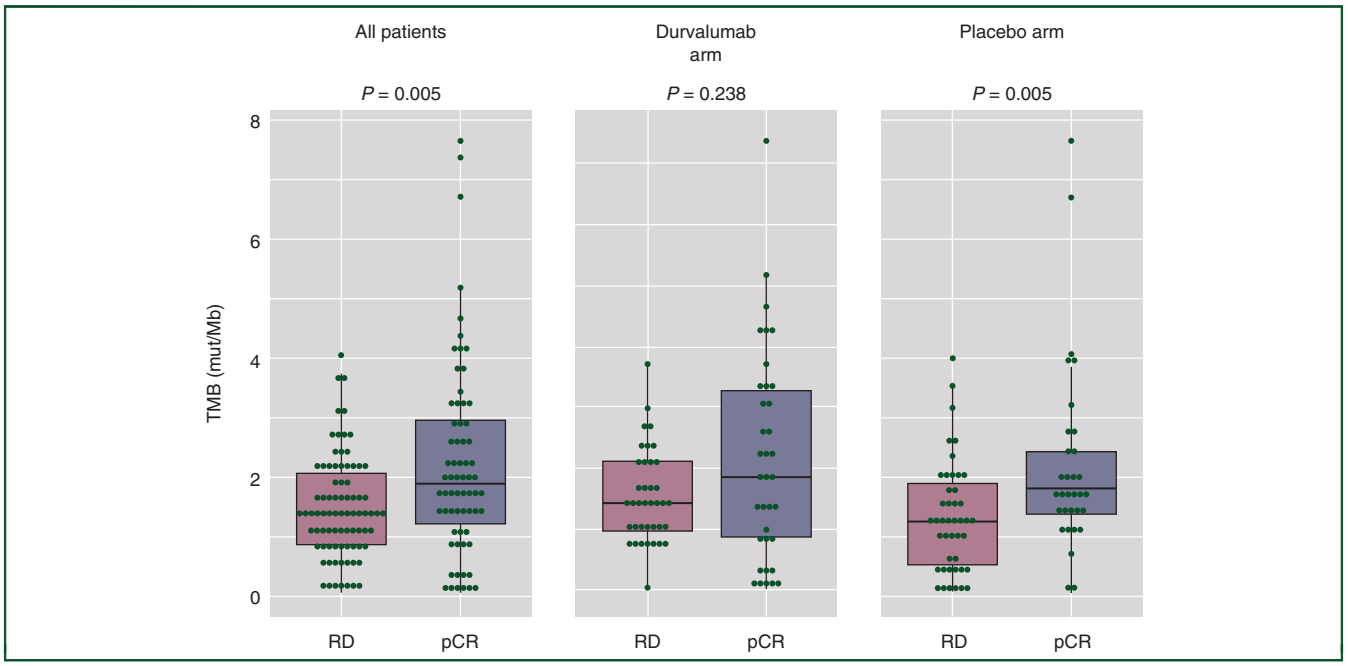

Figure 1. Association of pathological complete response and tumor mutational burden (TMB) in GeparNuevo.
Distribution of TMB values in pretreatment samples of GeparNuevo patients stratified as either residual disease or pathological complete remission (pCR) among all patients and separately in the durvalumab and placebo arm, respectively.

Joint relationship of TMB and immune gene expression profile with pCR in GeparNuevo

We previously reported that TILs and immune GEP predicted pCR in the GeparNuevo study.[3,25] For 136 patients, both WES and RNA-Seq data were available (supplementary Figure S1, available at *Annals of Oncology* online). Both a predefined immune GEP (GeparSixto immune signature) and TILs predicted pCR in this cohort in multivariate analysis (OR 1.73, 2.16-2.59, $P = 0.008$ and OR 1.32, 1.08−1.60, $P = 0.006$ for GEP and stromal TILs, respectively). Therefore, we also analyzed whether GEP or TILs correlate with continuous TMB. We found no significant correlation of either the immune GEP (Spearman's rho +0.03, −0.14 to +0.19, $P = 0.771$) or TILs (Spearman's rho −0.09, CI −0.25 to +0.07, $P = 0.269$) with TMB. In contrast, we could clearly detect that age, for example, was strongly correlated with continuous TMB (Spearman's rho +0.36, 0.21−0.49, $P < 0.001$). The observed independence of TMB and immune GEP or TILs from each other suggests that both factors may add predictive value for response, as has been suggested for other cancer types.[10] Figure 2 demonstrates this contribution of both factors for TMB and GEP (see supplementary Figure S3, available at *Annals of Oncology* online, for individual treatment arms). In a multivariate model, both continuous parameters were independent significant predictors of pCR (supplementary Table S2, available at *Annals of Oncology* online). As shown in Figure 2B, the pCR rate among patients with both high TMB and high GEP in pretreatment samples was 82% (60% to 95%) compared with only 28% (16% to 43%) in the groups with both low TMB and GEP. When we used TILs instead of immune GEP, we obtained similar results with a pCR rate of 83% (36% to

Table 2. Predictive value of tumor mutational burden (TMB) for pathological complete remission (pCR) in GeparNuevo

			All patients	Durvalumab	Placebo	Test for interaction
Continuous TMB (mut/Mb)	Univariate	*n*	149	74	75	
		OR (95% CI)	1.62 (1.20−2.20)	1.45 (0.99−2.14)	1.87 (1.13−3.08)	
		P value	**0.002**	0.060	**0.014**	0.439
	Multivariate[a]	*n*	133	64	69	
		OR (95% CI)	2.06 (1.33−3.20)	1.77 (1.00−3.13)	2.82 (1.21−6.54)	
		P value	**0.001**	**0.049**	**0.016**	0.436
Dichotomized TMB (upper tertile)	Univariate	*n*	149	74	75	
		OR (95% CI)	2.22 (1.11−4.43)	2.51 (0.95−6.64)	1.89 (0.70−5.12)	
		P value	**0.024**	0.065	0.208	0.694
	Multivariate[a]	*n*	133	64	69	
		OR (95% CI)	3.45 (1.41−8.45)	4.66 (1.18−18.48)	2.21 (0.60−8.12)	
		P value	**0.007**	**0.028**	0.232	0.438

CI, confidence interval; OR, odds ratio.
Significant P values are given in bold.
[a] Including age, stage, grading, stromal tumor infiltrating lymphocytes, PD-L1 status, and window treatment.

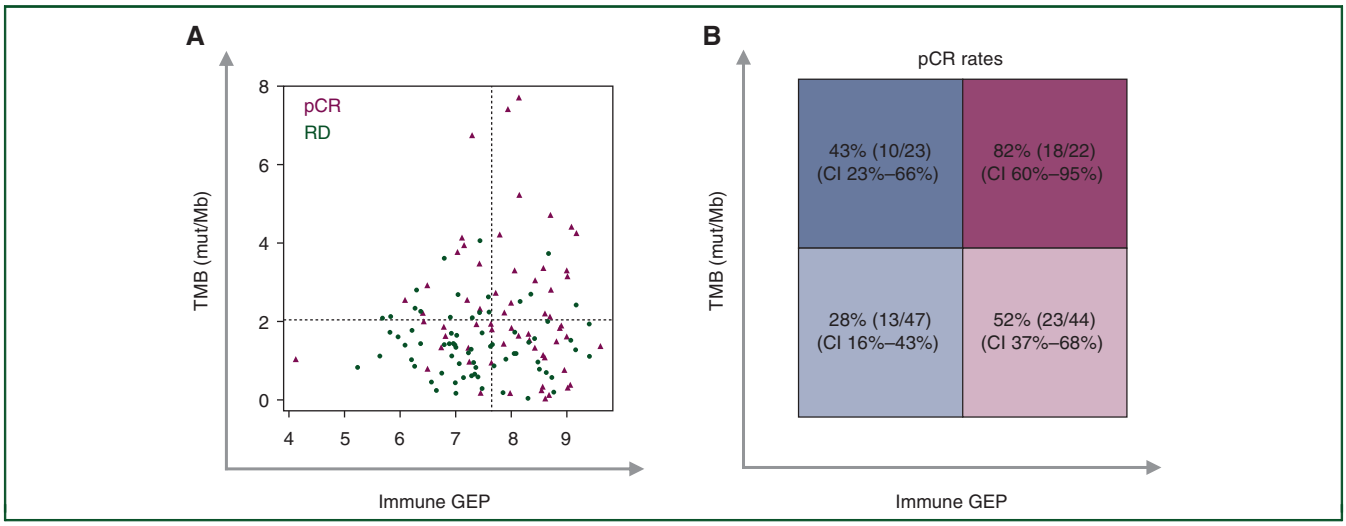

Figure 2. Joint relationship of tumor mutational burden (TMB) and immune gene expression profile (GEP) with pCR in GeparNuevo.
(A) Scatter plot of TMB and immune GEP in pretreatment biopsies of GeparNuevo patients colored by response [burgundy triangles, pathological complete remission (pCR); green circles, residual disease (RD)]. Cutoffs of median GEP and upper tertile of TMB are given by dashed vertical and horizontal lines, respectively. (B) pCR rates in percentages and 95% confidence intervals (CI) in subgroups defined by the cutoffs given as dashed lines in A.

100%) in the group with both high TMB and high TILs compared with 33% (23% to 44%) in the low/low group (supplementary Figure S4, available at *Annals of Oncology* online), and again both parameters were independent significant pCR predictors (supplementary Table S3, available at *Annals of Oncology* online).

TMB as a potential surrogate marker for biologically distinct groups of tumors

We next asked whether a high TMB may be a surrogate marker for a specific type of genomic alteration or for molecular subtypes, which could add mechanistic explanations for its association with response. We studied continuous scores of the mutational signatures 2, 3, 6, and 13 of Alexandrov et al.[20] and binary classifications of tumors characterized by specific driver mutations as well as RNA-based molecular subtypes (AIMS method).[23] We observed a strong positive correlation of TMB with continuous scores for mutational signature 2 (APOBEC-related, rho = 0.44, 0.30—0.56, $P < 0.001$) and signature 3 [homologous recombination deficiency (HRD)-related, rho = 0.37, 0.22—0.50, $P < 0.001$] and a weak negative correlation with signature 6 (MMR-related, rho = −0.18, −0.33 to −0.02, $P = 0.030$). As shown in supplementary Table S4 (available at *Annals of Oncology* online), we also compared the median TMB in groups of tumors characterized by specific driver mutations or molecular subtype. We found no difference in median TMB between TNBC of the 'basal-like' or 'HER2-enriched' subtype. In contrast, we detected significant higher TMB values in tumors with mutations in *BRCA2, TP53,* and *ARID1A* ($P = 0.004$, $P = 0.011$, and $P = 0.040$, respectively) and a trend for *BRCA1, NOTCH1,* and *PTEN* ($P = 0.091$, $P = 0.063$, and $P = 0.099$, respectively) but no differences for *ATM, CCNE1, MYC,* and *PIK3CA* (supplementary Table S4, available at *Annals of Oncology* online). Moreover, a predefined panel of 16 genes involved in HRD identified 45 mutated tumors with significantly higher TMB (1.89 versus 1.37 mut/Mb, $P < 0.001$; supplementary Table S4, available at *Annals of Oncology* online).

A recent paper identified specific copy number gains in immune-related genes in those breast cancers with high TMB but poor immune cell infiltration.[15] When we analyzed GeparNuevo samples characterized by these copy number gains, we observed a non-significantly higher TMB (supplementary Figure S5A, available at *Annals of Oncology* online). However, we were not able to detect an association with a reduced immune infiltration in high TMB patients in our sample cohort (supplementary Figure S5B, available at *Annals of Oncology* online).

DISCUSSION

The predictive value of TMB for response to ICB has been reported for several cancers, mostly in the metastatic setting.[6–9] However, its value for response to neoadjuvant therapy in early TNBC is not known, especially in combination with other parameters as immune GEP or TILs either for ICB or for neoadjuvant chemotherapy alone. We analyzed the predictive value of TMB for pCR (ypT0 ypN0) both alone and in combination with an immune GEP or TILs in a randomized neoadjuvant ICB trial in TNBC. We found that both TMB and immune GEP or TILs add independent value for pCR prediction. Interestingly, this result was obtained for both arms of the trial, that is for ICB in combination with chemotherapy and for chemotherapy alone.

These are novel results for breast cancer. But knowledge about TMB and response to chemotherapy is also limited in other cancers. In ovarian cancer TCGA data, a positive prognostic effect of high TMB was confined to *BRCA1/2* mutated cases.[26] Among more than 5000 different metastatic cancers without ICB treatment, no prognostic effect could be detected.[9] In contrast, in colon cancer treated with

chemotherapy, high TMB was associated with improved survival, independent of its association with hypermutated tumors.[27] In the latter case, high TMB could be a surrogate for increased immune cell infiltration. However, our study indicates that both parameters seem to contribute independent prognostic value.

TMB values among more than 100 000 tumors differed largely (0—1241 mut/Mb) with medians between different cancer types from 0.8 up to 45.2 mut/Mb.[20,28] Moreover, different platforms and analysis pipelines influence numerical TMB values.[9,28] Therefore development of TMB cutoffs is challenging.[9,10,28] While targeted sequencing approaches used different fixed cutoff values, most WES studies applied percentiles of cohorts.[6—10,28] We have applied both continuous TMB and a dichotomized score. The upper tertile we used includes samples with $\geq$95 total mutations, not far from the optimized cutoff of $>$100 mutations in a pan-cancer analysis of ICB.[10] In fact, 41 TNBC from our study (28%) surpass this published cutoff.[10] In a recent study of breast cancers from TCGA, the prognostic value of an immune GEP was detected only in samples with TMB $>$1.63 mut/Mb.[15] Again, this cutoff value is similar to the upper tertile of TNBC (1.75 mut/Mb) in the respective TCGA dataset. Despite clinical demand for dichotomized TMB cutoffs, optimization resembles that of other continuous parameters such as TILs and Ki67 in breast cancer[19,29] and the quest for the 'right cutoff' may be difficult.

Importantly, our results suggest that a combination of TMB and immune GEP can increase the precision of response prediction. We also obtained similar results when we replaced GEP with TIL scoring, which could be easier to incorporate into routine settings. TMB did not correlate significantly with TILs or with immune GEP and contributes independent prognostic information.

We were also interested in whether TMB represents a surrogate for distinct biological groups of tumors. We observed no difference between TNBC of 'basal-like' or 'HER2-enriched' subtype but found three driver genes significantly associated with TMB: *BRCA2, TP53,* and *ARID1A* (supplementary Table S4, available at *Annals of Oncology* online). Interestingly, these genes were also among the top 7% of TMB-associated genes in a previous comprehensive study of more than 100 000 tumors.[28] However, we detected no predictive value for pCR of these individual genes: *BRCA2* $P = 0.977$, *TP53* $P = 0.408$, *ARID1A* $P =$ not available, HRD panel $P = 0.407$. This suggests that TMB captures predictive information beyond individual driver genes.

The strengths of our study include the use of samples from a prospectively randomized controlled trial with central TNBC confirmation and TIL assessment. In addition, we focused on a biologically motivated, limited set of prespecified variables and our WES dataset represents the largest so far for early breast cancers with neoadjuvant ICB treatment. However, our study clearly has limitations. First, the sample size is still small and the complete set of molecular data was only available for a subset of the trial patients, which could introduce bias even if we detected no differences in baseline parameters. Second, the clinical impact of the study is limited by the fact that neither TMB or immune GEP or TILs had a specific predictive effect for the addition of immunotherapy but identified patients with a high chance of pCR in both arms of the trial. Finally, a proper validation dataset for our findings is missing.

In conclusion, our study shows for the first time that TMB and immune infiltration add independent value for pCR prediction both for ICB and for chemotherapy. Our results merit validation and recommend further study of both parameters to individually tailor therapies in breast cancer.

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DISCLOSURE

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