Clinical Cancer Research

Mutational Diversity and Therapy Response in Breast Cancer: A Sequencing Analysis in the Neoadjuvant GeparSepto Trial

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Abstract

Purpose: Next-generation sequencing (NGS) can be used for comprehensive investigation of molecular events in breast cancer. We evaluated the relevance of genomic alterations for response to neoadjuvant chemotherapy (NACT) in the GeparSepto trial.

Experimental Design: Eight hundred fifty-one pretherapeutic formalin-fixed paraffin-embedded (FFPE) core biopsies from GeparSepto study were sequenced. The panel included 16 genes for mutational (*AKT1*, *BRAF*, *CDH1*, *EGFR*, *ERBB2*, *ESR1*, *FBXW7*, *FGFR2*, *HRAS*, *KRAS*, *NRAS*, *SF3B1*, *TP53*, *HNF1A*, *PIK3CA*, and *PTEN*) and 8 genes for copy-number alteration analysis (*CCND1*, *ERBB2*, *FGFR1*, *PAK1*, *PIK3CA*, *TOP2A*, *TP53*, and *ZNF703*).

Results: The most common genomic alterations were mutations of *TP53* (38.4%) and *PIK3CA* (21.5%), and 8 different amplifications (*TOP2A* 34.9%; *ERBB2* 30.6%; *ZNF703* 30.1%; *TP53* 21.9%; *PIK3CA* 24.1%; *CCND1* 17.7%; *PAK1* 14.9%; *FGFR* 12.6%). All other alterations had a prevalence of less than 5%. The genetic heterogeneity in different breast cancer subtypes [lum/HER2neg vs. HER2pos vs. triple-negative breast cancer (TNBC)] was significantly linked to differences in NACT response. A significantly reduced pathologic complete response rate was observed in *PIK3CA*-mutated breast cancer [*PIK3CA*mut: 23.0% vs. wild-type (wt) 38.8%, $P < 0.0001$] in particular in the HER2pos subcohort [multivariate OR = 0.43 (95% CI, 0.24–0.79), $P = 0.006$]. An increased response to nab-paclitaxel was observed only in *PIK3CA*wt breast cancer, with univariate significance for the complete cohort ($P = 0.009$) and the TNBC ($P = 0.013$) and multivariate significance in the HER2pos subcohort (test for interaction $P = 0.0074$).

Conclusions: High genetic heterogeneity was observed in different breast cancer subtypes. Our study shows that FFPE-based NGS can be used to identify markers of therapy resistance in clinical study cohorts. *PIK3CA* mutations could be a major mediator of therapy resistance in breast cancer.

Introduction

The mutational landscape of breast cancer has been described in several large-scale international sequencing approaches (1–3). Overexpression and amplification of HER2/ERBB2 has been successfully implemented to define a subgroup of breast cancer with specific targeted therapy. Additional copy-number alterations (CNAs) or point mutations have not yet been integrated into clinical practice in breast cancer.

Although the identification of genomic alterations in breast cancer has been largely completed, the most relevant research questions are now related to the clinical utility of these alterations as markers of prognosis and as indicators of therapy response and

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Translational Relevance

Translational studies investigating the mutational landscape of breast cancer in large clinical trial cohorts with well-defined therapy response are rare. We reported the results of a next-generation sequencing (NGS) analysis conducted in the neoadjuvant GeparSepto trial. A total of 851 pretherapeutic formalin-fixed paraffin-embedded (FFPE) samples were sequenced using a breast cancer–specific hotspot panel of 24 genes, performing a stratified analysis for luminal, HER2pos, and triple-negative tumors. Point mutations and amplifications with high heterogeneity between molecular subtypes were identified. *PIK3CA*-mutated tumors showed a significantly reduced pathologic complete response rate compared with wild-type tumors (23.0% vs. 38.8%, $P < 0.0001$), in particular in the HER2pos subcohort [multivariate OR = 0.43 (95% CI, 0.24–0.79), $P = 0.006$]. This study is, to our knowledge, the first comprehensive FFPE-based NGS analysis of different breast cancer subtypes and provided evidence for using NGS to dissect molecular heterogeneity in clinical trial samples. Our results pointed to *PIK3CA* mutations as a therapy resistance parameter in the HER2pos breast cancer.

resistance in different breast cancer subtypes. In particular, studies investigating the mutational landscape in large clinical trial cohorts with well-defined therapy response are rare. Due to considerable advances in implementation of new technologies, it is now feasible to perform targeted next-generation sequencing (NGS) for analysis of mutations using formalin-fixed paraffin-embedded (FFPE) core biopsy samples from clinical studies (4). In addition, targeted NGS allows new exploratory approaches to analyze CNAs, allowing a more comprehensive view on genomic alterations (5–7). We and others have recently demonstrated that *PIK3CA* mutations predict lower pathologic complete response (pCR) to dual blockade with trastuzumab and lapatinib as well as trastuzumab plus afatinib in HER2pos primary breast cancer (8–13).

Here, we studied a large cohort of 851 pretherapeutic FFPE core biopsies from the neoadjuvant GeparSepto trial (14) by targeted NGS. We investigated a panel of 21 genes in total including 16 genes selected for mutational analysis (*AKT1*, *BRAF*, *CDH1*, *EGFR*, *ERBB2*, *ESR1*, *FBXW7*, *FGFR2*, *HRAS*, *KRAS*, *NRAS*, *SF3B1*, *TP53*, *HNF1A*, *PIK3CA*, and *PTEN*) and 8 genes selected for CNA analysis (*CCND1*, *ERBB2*, *FGFR1*, *PAK1*, *PIK3CA*, *TOP2A*, *TP53*, and *ZNF703*). *PIK3CA*, *TP53*, and *ERBB2* were analyzed for both mutations and CNAs. We investigated the prevalence of the alterations in different breast cancer subgroups as well as the role for response to neoadjuvant chemotherapy (NACT).

Materials and Methods

Patients and treatment

The GeparSepto study (NCT01583426; ref. 13) enrolled women with previously untreated, primary invasive breast cancer after written-informed consent for study participation and biomaterial collection. The relevant authorities and ethics committees approved the studies. The REMARK criteria were followed (15).

Patients were randomized to either weekly nab-paclitaxel (nab-P; Celgene) or solvent-based paclitaxel (P) for 12 weeks followed by 4 cycles of conventionally dosed epirubicin/cyclophosphamide (EC). Patients with HER2pos breast cancer received trastuzumab and pertuzumab every 3 weeks simultaneously to all chemotherapy cycles. Postsurgery trastuzumab continued for a total duration of 1 year. The patient inclusion criteria and treatment information are presented in the Supplementary Data. Pretherapeutic FFPE core biopsies were prospectively collected in the GBG tumor biobank. HER2 status [positive if immunohistochemistry (IHC) 3+ or *in situ* hybridization (ISH) ratio >2.0] and hormone receptor status (ER/PR positive if >1% stained cells) were centrally assessed for all patients prior to randomization.

The biomarker investigations in the GeparSepto trial were performed after approval by the ethics committee of the Charité Hospital (EA1-139-05 Am. 2013; 2015) and conducted in accordance with the International Ethical Guidelines for Biomedical Research involving Human Subjects (CIOMS). Written-informed consent for study participation and biomaterial collection was obtained from all patients.

Strategy for NGS panel development, DNA isolation, and semiconductor sequencing

A hotspot panel was designed to cover the most frequent mutated hotspots in breast cancer with a special focus on the PI3K pathway (see Supplementary Data for details). The breast cancer hotspot (IAD68218_166) panel included the following 17 genes for mutational analysis: *PIK3CA* (7 amplicons), *TP53* (6 amplicons), *ERBB2* (3 amplicons), *CDH1* (2 amplicons), *FBXW7* (2 amplicons), *PTEN* (2 amplicons) as well as *AKT1*, *BRAF*, *EGFR*, *ESR1*, *FGFR2*, *HNF1A*, *HRAS*, *KRAS*, *NRAS*, *SF3B1*, and *ATM* (1 amplicon each). During bioinformatical analysis, we found that the variations in *ATM* were SNPs; therefore, *ATM* was excluded from *post hoc* analysis. For CNAs, we included putative driver genes *CCND1* (3 amplicons), *PAK1* (3 amplicons), *ZNF703* (3 amplicons), *PIK3CA* (7 amplicons), *TP53* (6 amplicons), *ERBB2* (3 amplicons), *FGFR1* (3 amplicons; refs. 2, 16), and *TOP2A* (3 amplicons). We did not include deletions, which are more difficult to detect due to the lower absolute change of copy numbers, especially in the background of normal DNA. DNA was extracted from three 5-μm FFPE sections (at least 20% tumor) using VERSANT kPCR Sample Prep (Siemens) following the manufacturer's DNAext protocol. Following library preparation, samples were either 16-fold multiplexed and sequenced using the Ion 318 chip v2 on an Ion Torrent Personal Genome Machine or 96-fold multiplexed and sequenced using an Ion 540 chip on an Ion S5XL with an adapted standard protocol using 330 flows (ref. 17; see Supplementary Data for details).

NGS data processing

Base calling, alignment to the human genome (hg19), raw data processing, and variant calling were executed with Torrent Suite Software 4.0.3. using the recent Ion Torrent standard protocol (somatic variant calling: low stringency). VCF and BAM files were obtained from the Ion Torrent server. VCF files were imported into Ion Reporter and annotated using the workflow "Annotate variants single sample, version 4.4."

The mean coverage of each amplicon in each sample was obtained from the BAM files. Samples having a minimum coverage of 500 at the 2 most important mutation hotspots in *PIK3CA* (p.542/p.545 and p.1047) were included in the study. Only nonsynonymous COSMIC mutations with allele frequencies $\geq$10% were taken into consideration. CNAs were called using

the Ioncopy method based on amplicon coverage data, which has been published and validated before (6, 18).

Statistical analysis

The project is based on the predefined statistical analysis plan with 2 parts: in part 1, the primary hypothesis is that *PIK3CA* mutations predict non-pCR, whereas part 2 is an exploratory analysis of the different mutation in the whole cohort and the different molecular subsets (for details see Supplementary Methods). The prevalence of all mutations/CNAs was determined in the complete GeparSepto cohort as well as in 3 subcohorts of TNBC, HER2pos, and lum/HER2neg breast cancer. Only those genetic alterations with a prevalence of at least 10% in at least 1 of the 4 cohorts were included in the complete analysis. Associations between the gene status, clinicopathologic characteristics, and pCR rate were investigated with the Fisher exact and χ^2 tests for categorical variables. Univariate analyses using binary logistic regression models were performed: ORs with 95% confidence intervals (CIs) and 2-sided Wald *P* values are presented. In addition, multivariate logistic regression models were conducted to adjust for known predictive factors: age (continuous), tumor stage (T1–2 vs. T3–4), nodal stage (N0 vs. N+), histologic type (ductal vs. lobular vs. other), grading (G1–2 vs. G3), Ki67 (continuous), hormone receptor status (negative vs. positive), HER2 status (negative vs. positive), tumor-infiltrating lymphocytes (TILs, continuous), and treatment (nab-P vs. P). Interaction tests were reported as Wald *P* values from logistic regression models: standard interaction tests were based on regression models with independent variables *PIK3CA* status and treatment and their interaction; adjusted models for multivariate interactions tests contained the predictive factors for adjustment enlisted above. All *P* values are 2-sided, with a P value $\leq$ 5% considered to be statistically significant. No correction for multiple testing was applied. Statistical analysis was performed using SPSS version 24 (IBM Corp.).

Results

Baseline factors

From the 1,085 samples available in the biobank, successful NGS analysis was possible for 851 (78.4%) patients. Among these, 295 had HER2pos breast cancer, 159 TNBC, and 397 luminal/HER2neg (lum/HER2neg) breast cancer (Fig. 1; Supplementary Table S1). The set of successfully sequenced patients did not significantly differ from the GeparSepto patients not sequenced with respect to parameters age, tumor size, nodal status, grading, histologic type, and treatment arm; however, the rate of TNBC was lower in the sequenced patients.

Frequency of mutations and heterogeneity in breast cancer subtypes

The most commonly mutated genes in the GeparSepto cohort of 851 patients were *TP53* (327, 38.4%) and *PIK3CA* (183, 21.5%). Each of the other mutations (*AKT1*, *BRAF*, *CDH1*, *EGFR*, *ERBB2*, *ESR1*, *FBXW7*, *FGFR2*, *HRAS*, *KRAS*, *NRAS*, *SF3B1*, *HNF1A*, and *PTEN*) had a prevalence of less than 2% in this cohort. The most common CNAs were amplifications of *TOP2A* (34.9%), *ERBB2* (30.6%), *ZNF703* (30.1%), *PIK3CA* (24.1%), *TP53* (21.9%), *CCND1* (17.7%), *PAK1* (14.9%), and *FGFR1* (12.6%; Figs. 2 and 3; Supplementary Table S2).

A considerable heterogeneity of mutations and CNAs was observed in the different molecular subtypes (Fig. 2B; Supplementary Table S2). *TP53* mutations were more common in TNBC (57.9%), whereas *PIK3CA* mutations were more frequent in lum/HER2neg (27.5%) and HER2pos tumors (21.4%). Amplification of *ERBB2* was observed in 84.7% of HER2pos tumors, but only in 1.5% of lum/HER2neg breast cancer and 2.5% of TNBCs ($P < 0.0001$). An amplification of *TOP2A* was observed in 69.2% of TNBC, in 46.8% of HER2pos tumors, but only in 12.3% of lum/HER2neg breast cancers ($P < 0.0001$). In summary, a high genetic diversity of mutations and CNAs was observed in the 3 different breast cancer subtypes.

Comparison of prevalence of mutations with METABRIC cohort

Prevalence of most mutations and amplifications in the overall GeparSepto cohort as well as in the molecular subtypes was similar to those published for the METABRIC cohort (2) (Fig. 3B; Supplementary Fig. S1; Supplementary Methods), with a few exceptions: we observed a higher prevalence of *TP53* amplifications in all GeparSepto subcohorts (GeparSepto: lum/HER2neg: 28.2%, TNBC: 35.2%, HER2pos: 6.1% vs. METABRIC: lum/HER2neg: 0.9%, TNBC: 0.6%, HER2pos: 0.8%) suggesting a higher sensitivity of the ion copy method compared with the METABRIC approach. Moreover, *ERBB2* amplification rate was similarly high for HER2pos tumors (GeparSepto: 84.7%, METABRIC: 98.8%), but considerably lower for TNBC (GeparSepto: 2.5%, METABRIC: 10.6%) and lum/HER2neg (GeparSepto: 1.5%, METABRIC: 11.4%). Also, we determined a much higher *TOP2A* amplification rate in the TNBC subtype compared with the METABRIC subcohort (GeparSepto: 69.2%, METABRIC: 1.6%). For lum/HER2neg and HER2pos tumors, the *TOP2A* amplification rates were similar in GeparSepto and METABRIC, suggesting a TNBC-specific difference rather than a general difference in the sensitivity of the method.

Correlation of mutations and CNAs with NACT response (pCR)

In the complete cohort, several genomic alterations were significantly linked to differences in chemotherapy response in univariate analysis (Fig. 4A). The 2 most important mutations (*TP53* and *PIK3CA*) as well as 7 of 8 CNAs (*ERBB2*, *TP53*, *PAK1*, *CCND1*, *TOP2A*, *FGFR1*, and *ZNF703*) were significantly associated with variations in pCR rate. The response to NACT remained statistically significant only for 3 of the genomic alterations (*PIK3CA* mutation, *ERBB2* amplification, and *PAK1* amplification) in multivariate analysis (Fig. 4B). Patients with tumors with an *ERBB2* amplification had a significantly increased pCR rate of 63.8%, compared with 22.8% for patients without an *ERBB2* amplification ($P < 0.0001$). For *PIK3CA* mutations, a significant lower pCR rate was observed (23.0% compared with 38.8% for tumors with wild-type *PIK3CA*, $P < 0.0001$, Fig. 4C).

In the HER2pos subgroup, those patients whose tumors also showed an *ERBB2* amplification had a higher pCR rate than patients without an *ERBB2* amplification (univariate: $P = 0.008$, multivariate: $P = 0.02$, Supplementary Fig. S1). In addition, HER2pos tumors with *PIK3CA* mutations (univariate: $P = 0.007$, multivariate: $P = 0.006$) had significantly lower pCR rates (Supplementary Fig. S2). In TNBC, tumors with a *TOP2A* amplification had a significantly decreased pCR rate compared with tumors without a *TOP2A* amplification (univariate: $P = 0.012$, multivariate: $P = 0.036$; Supplementary Fig. S3). In lum/HER2neg tumors, *PIK3CA* mutations were significantly

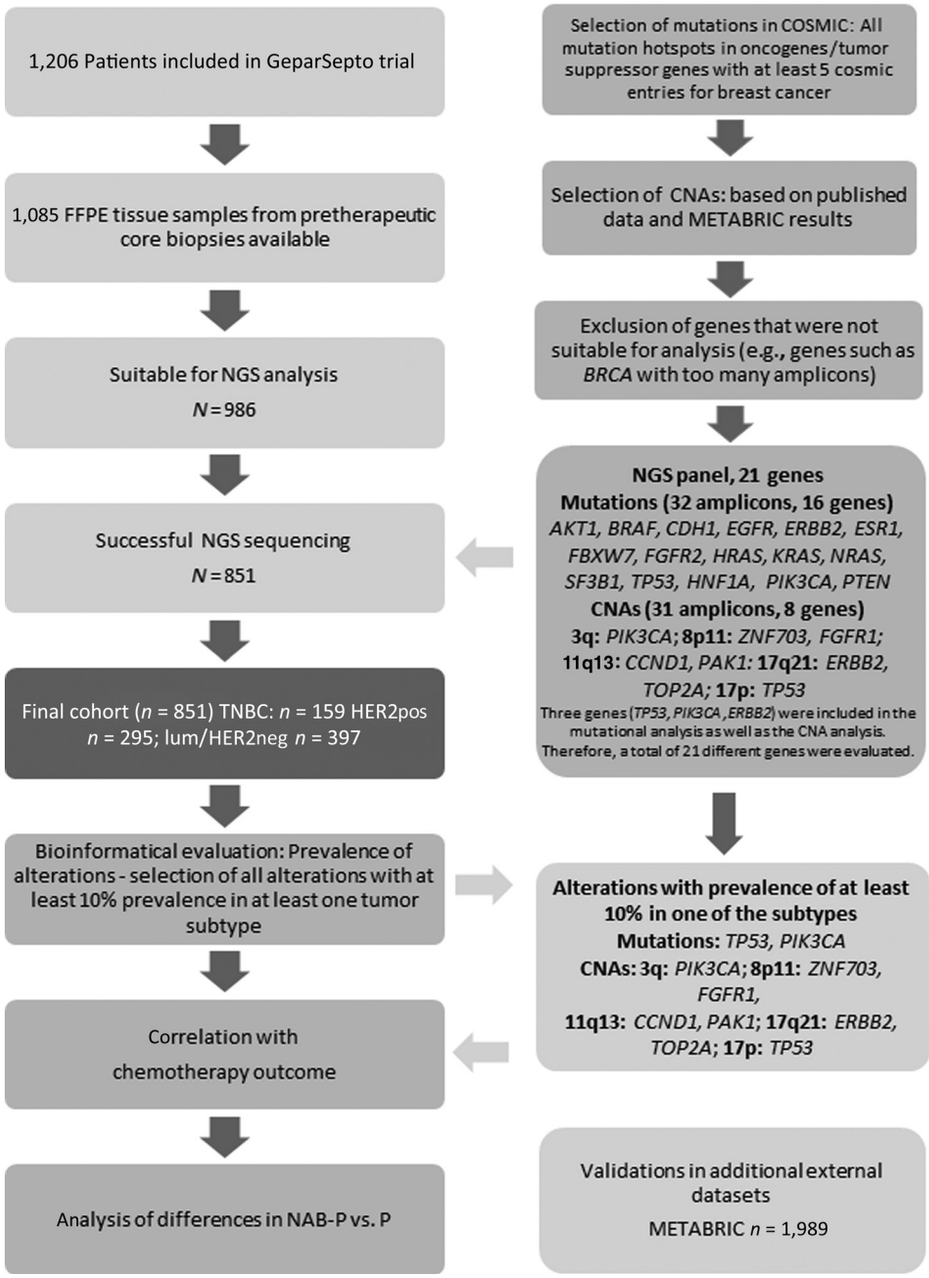

Figure 1.
Overview on consort statement, available samples, and selection of genes for the NGS as well as bioinformatical evaluation strategy. METABRIC cohort was used for additional validations.

associated with reduced pCR in univariate ($P = 0.006$), but not in multivariate analysis (Supplementary Fig. S4).

To validate the analysis of HER2 amplification by NGS, we have compared the CNA data derived from NGS panel with the clinical gold standard, a combination of central IHC and ISH. For each of the 3 amplicons in the HER2 gene, ROC curves showed a very good agreement with clinical HER2 status (Supplementary Fig. S5A–S5C). We observed a very strong correlation between the copy number estimated from different amplicons in a gene *ERBB2* and *CCND1* (Supplementary Fig. S5D–S5I). In Supplementary Table S3, we have analyzed the correlation between the different amplicons of each gene for all genes included in the CNA analysis. For most amplicons, we observed very strong correlations of the different amplicons, but there were some genes, in particular *TOP2A* and *ZNF703*, where 1 of the amplicons showed a poor correlation with the other amplicons. Because this comparison was a *post hoc* analysis, we did not remove the poor performing amplicons from the further analyses.

Correlation of genomic alterations with response to nab-paclitaxel

The primary aim of the GeparSepto trial was the comparison of pCR rates in patients treated either with nab-P or P containing NACT. In the complete NGS cohort of 851 patients, nab-P increased the pCR rate from 31.6% to 38.9% ($P = 0.031$, Fig. 5B), and this effect was particularly strong in the TNBC subgroup (pCR rate

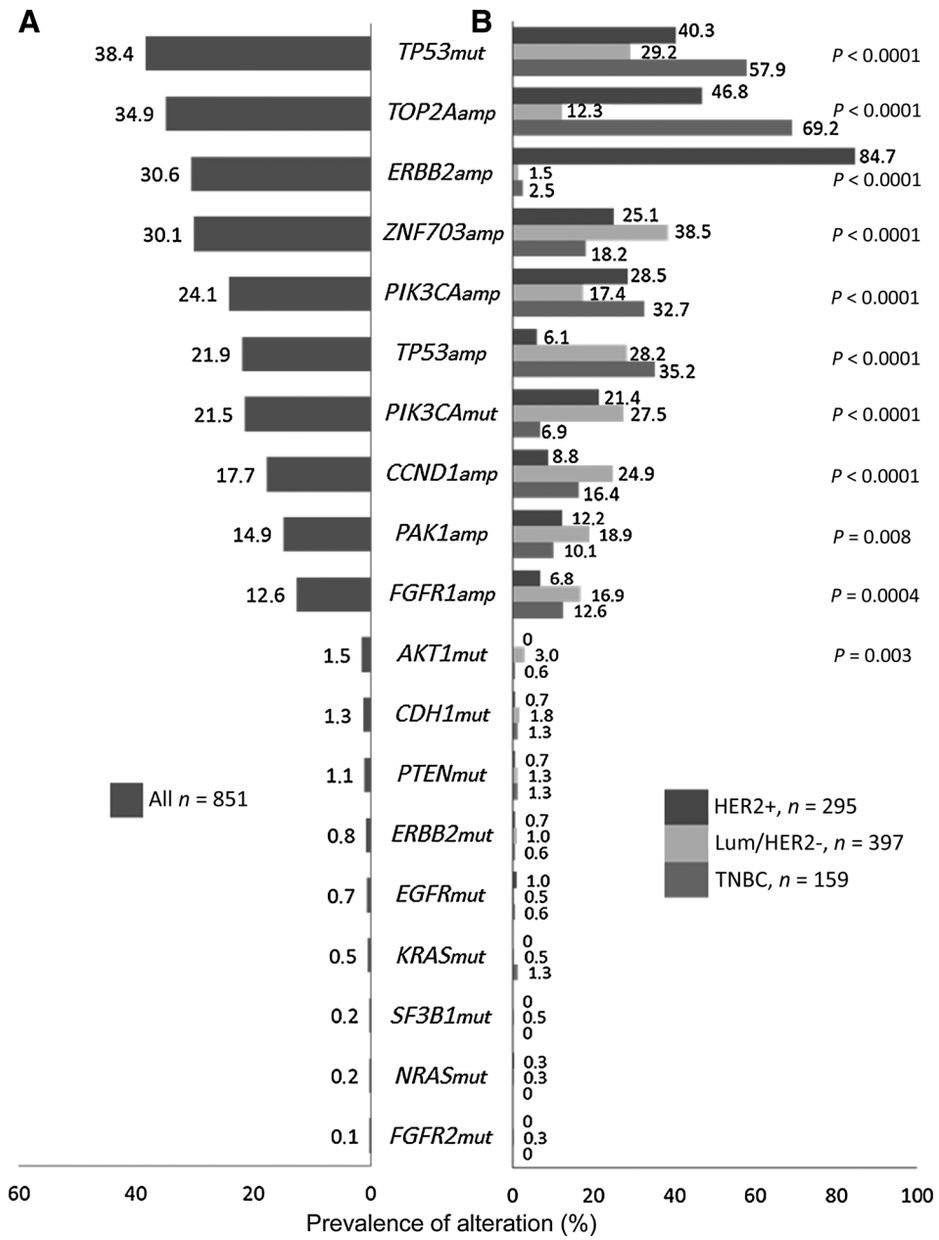

Figure 2.
Molecular diversity of genomic alterations in different subtypes of breast cancer. **A,** Prevalence of mutations and CNAs in the complete cohort. **B,** Comparison of the 3 different molecular subtypes [HER2-positive (HER2pos), triple-negative (TNBC), and luminal/HER2-negative (lum/HER2neg) breast cancer]. *P* values: χ^2 test; the genes *HRAS*, *FBXW7*, *ESR1*, and *HNF1A* are not shown, as they had no detectable mutations.

with nab-P 49.4% vs. 27.5% with P, $P = 0.006$). These results correspond to the main results of GeparSepto; therefore, the subcohort investigated by NGS is representative for the complete GeparSepto cohort (13).

We evaluated the role of different mutations and CNAs for prediction of response to neoadjuvant nab-P. As shown in Fig. 5A and B, the better response rate to nab-P was restricted to the *PIK3CA*wt tumors, and it was not observed in most subgroups of *PIK3CA*-mutant tumors, with the exception of a nonsignificant trend in the few ($n = 11$) TNBCs with *PIK3CA* mutations. The interaction effect between *PIK3CA* status and nab-P response was significant only in the HER2pos *PIK3CA*wt subgroup [*PIK3CA* mut: univariate OR = 0.49 (95% CI, 0.18–1.34); *PIK3CA*wt: univariate OR = 1.64 (95% CI, 0.95–2.83), test for interaction $P = 0.0394$, adjusted multivariate test for interaction $P = 0.0074$]. The comparison of nab-P versus P for the other mutational alterations is shown in Supplementary Figs. S6 and S7.

To further analyze the different tumor biology linked to mutations and CNAs, we compared Ki67 expression (as an indicator of tumor proliferation) and TILs (as an indicator of tumor immunogenicity) in subsets of tumors with detected alterations. TIL levels and Ki67 proliferation rates differed significantly within breast cancer subgroups defined by mutations or CNAs (Supplementary Fig. S8 and Supplementary Tables S4 and S5). Reduced lymphocyte infiltration was significantly correlated with *PIK3CA* mutations (all, lum/HER2neg) and amplification of *PAK1* (all, TNBC), *CCND1* (all, lum/HER2neg, HER2pos), and *ZNF703* (all), whereas *TP53* mutations were linked with an increase of TILs. Increased Ki67 expression was significantly correlated with *TP53* mutations in the total cohort and in lum/HER2neg tumors,

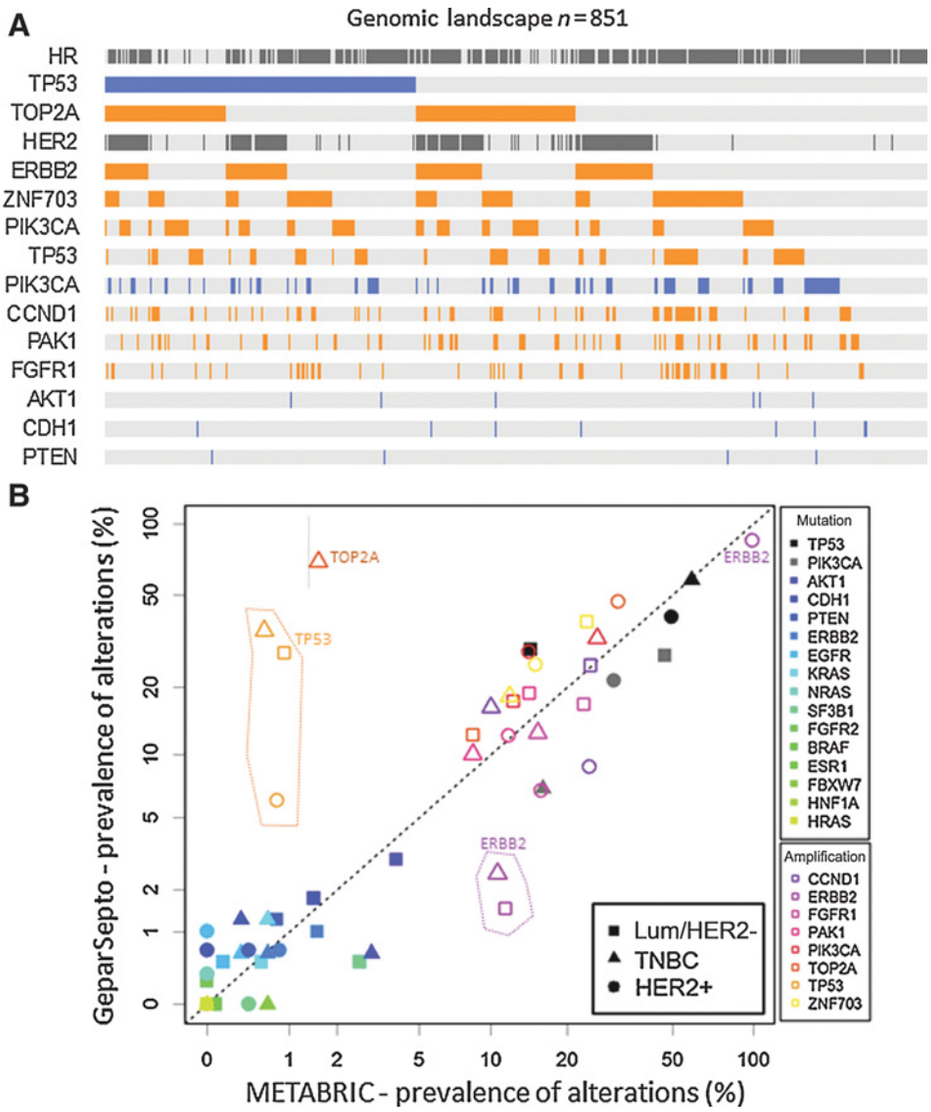

Figure 3.
Overview on distribution of genetic aberrations in the GeparSepto cohort (**A**), all alterations with at least 1% prevalence in the GeparSepto cohort are shown [mutations (blue), amplifications (orange), HER2 and HR status by IHC (gray)]. **B,** Comparison of prevalence of mutations between the GeparSepto study cohort ($n = 851$) and the METABRIC cohort ($n = 1980$). The different tumor subtypes are shown by the form of the symbol, and the different genomic alterations are shown in different colors. Those alterations with differences in the prevalence are marked with dotted encirclements.

whereas *PIK3CA* mutations (all, lum/HER2neg, TNBC) were linked with a reduced number of proliferative cells.

Discussion

In this study, a total of 851 FFPE core biopsies from patients treated within the GeparSepto trial were evaluated by targeted NGS, and the analysis could be conducted in 78.4% of samples from the study population. We interrogated hotspot regions of 16 genes and found as expected *TP53* and *PIK3CA* to be the most commonly mutated genes overall, with differences between the subtypes. CNAs had a much higher prevalence than mutations, which is consistent with the model of breast cancer as a C-type tumor (19) mainly driven by CNAs (2).

To our knowledge, this study is the first comprehensive NGS-based DNA sequencing analysis of a breast cancer neoadjuvant clinical trial cohort including different molecular subtypes using FFPE. In previous studies focusing on HER2pos breast cancer, whole-exome sequencing has been used in 203 frozen tissue samples from the NeoALTTO trial (20), and RNA-Seq has been used in NeoALTTO ($n = 254$; ref. 21) and CALGB 40601 ($n = 265$; ref. 22). The available large sequencing projects such as TCGA (3) and METABRIC (2) have provided extensive data for identification of alterations and classification of mutations, but typically the clinical information is restricted and the therapies have not been standardized.

In the NGS analysis of the GeparSepto study, we were able to investigate the role of mutations and CNAs for response prediction to NACT. A considerable heterogeneity of mutations and CNAs in the different molecular subtypes was observed in our study. Many alterations were linked to differences in chemotherapy response in univariate analysis of the complete cohort, whereas the multivariate analysis for the molecular subtypes revealed only a few significant alterations: *PIK3CA* mutations in HER2pos and univariate in HRpos/HER2neg breast cancer, *ERBB2* amplification in HER2pos breast cancer, and *TOP2A* amplifications in TNBC. This suggests that the high number of genetic alterations which were significantly linked to NACT

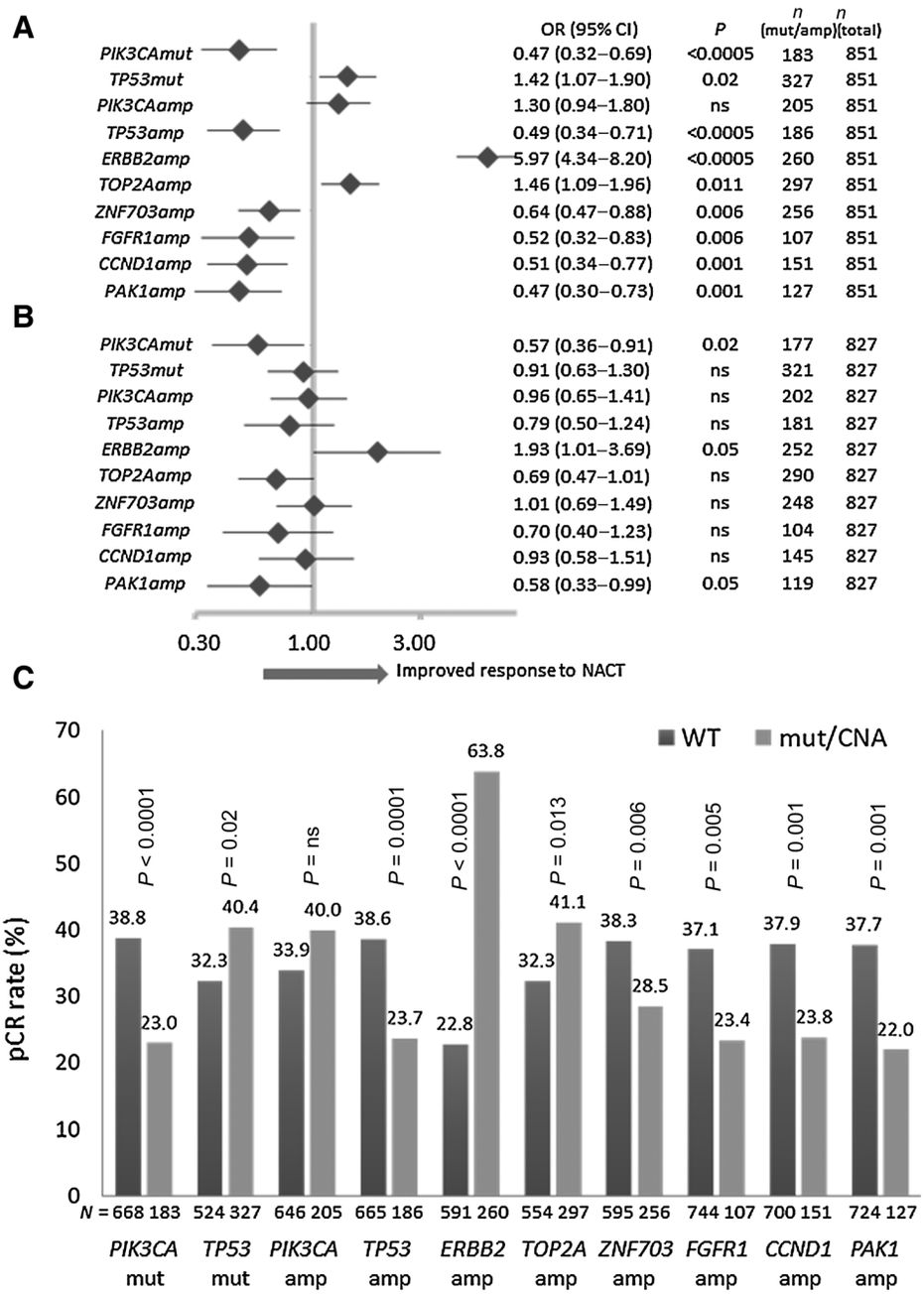

Figure 4.
Genomic alterations and response to neoadjuvant chemotherapy. **A,** Forest plot showing the OR for pCR for different mutations and CNAs based on univariate logistic regression analysis. **B,** Forest plot for multivariate logistic regression analysis. **C,** pCR rates of tumor subsets with or without genomic alterations (*P* values: 2-sided Fisher test). All analyses refer to the complete GeparSepto NGS cohort of 851 tumors (univariate analysis) or 827 tumors with complete data for multivariate analysis, respectively.

response in the complete cohort mainly reflects the molecular differences between tumor subtypes.

We found that in the subgroup of clinically HER2pos tumors, an amplification of *ERBB2* measured by sequencing-based copy-number analysis was still a significant predictor of pCR in the subset of HER2pos tumors, which suggests that NGS-based analysis might provide information on responsive tumors beyond the current state-of-the-art combination of IHC and ISH.

The patients with HER2pos tumors in GeparSepto have received trastuzumab and pertuzumab, and our results showed that *PIK3CA* mutations were linked to significantly reduced therapy response for this type of dual HER2 blockade. In the NeoSphere study based on 417 patients, there was a trend of lower pCR rates in tumors with a *PIK3CA* mutation, but a significance was not reached ($P = 0.1$; ref. 23). The TRYPHAENA study, investigating several chemotherapy regimen with trastuzumab and pertuzumab, also demonstrated a numerically lower pCR rate in the group with *PIK3CA* mutations compared with the wild-type *PIK3CA* status, but the power was too low to show a significant effect (48.7% vs. 64.3%; $P = 0.172$; refs. 24, 25). In our analysis, a significant interaction of mutant *PIK3CA* with the more potent chemotherapy regimen nab-P followed by EC could be demonstrated for HER2pos patients. Although we do not have mechanistic data that explain our statistical results on *PIK3CA*

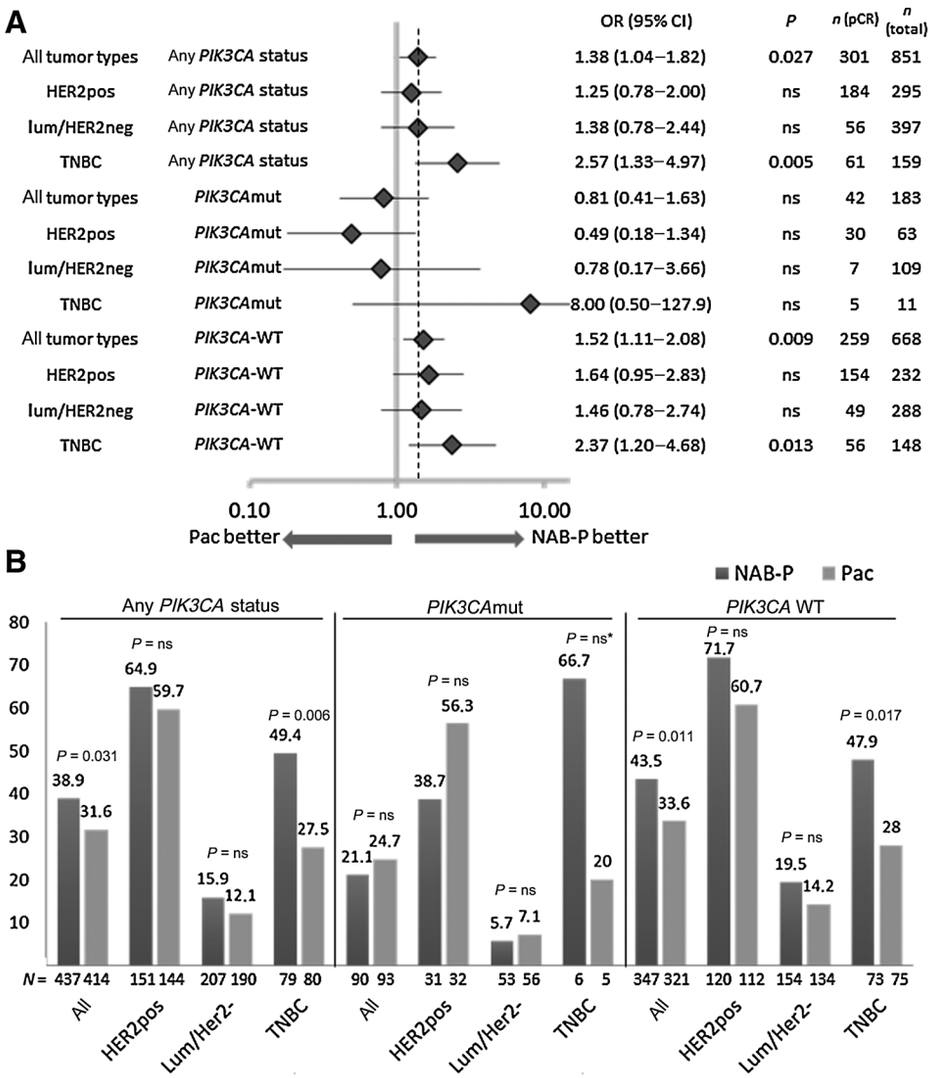

Figure 5.
PIK3CA mutations and response to nab-paclitaxel (NAB-P) vs. paclitaxel (Pac) in different subtypes of tumors. **A,** Forest plot showing the OR for NAB-P vs. Pac in all tumors, *PIK3CA*-mutated tumor, *and PIK3CA* wild-type tumors, with subanalysis for the 3 molecular tumor subtypes. pCR for different mutations and CNAs based on a univariate logistic regression analysis. **B,** pCR rates for NAB-P vs. Pac in different tumor subgroups (*P* value: 2-sided Fisher test). *numbers of cases for TNBC with *PIK3CA* mutations are very low.

mutations and response toward nab-P, several recent studies reported on mutant *PIK3CA* conferring resistance toward chemotherapy regimens via modulating and binding to tubulin isoforms that play a major role in mitosis (26–28). Taken together, these results confirm previous reports of *PIK3CA* mutations as a resistance factor with different types of anti-HER2 treatment, suggesting a dysregulation of *PIK3CA* signaling as a therapy resistance mechanism (8, 9).

In addition, in multivariate analysis *PIK3CA* mutations were linked to a significant reduced response in the complete GeparSepto-NGS cohort, which points to *PIK3CA* as a major mechanism of neoadjuvant therapy resistance in breast cancer. The question if this resistance can be modulated by *PIK3CA* inhibitors is investigated in several clinical trials in HER2neg, hormone receptor–positive breast cancer, such as SOLAR-1 (NCT02437318) and SANDPIPER (NCT02340221), using novel PI3-Kinase inhibitors.

For therapeutic strategies involving AKT inhibitors, currently tested in TNBC, it might be promising to combine *PIK3CA* mutations with other alterations, such as *AKT* and *PTEN*, the combination of these 3 genes has been linked to response to AKT inhibitors in the Lotus and Manta studies (29, 30), and a similar approach was investigated in the neoadjuvant Fairlane study (31). The rate of *PIK3/AKT* pathway alterations ranged from 23% to 43%, which was higher than the 8% in our study, which did not include *PTEN* loss.

It is the first study investigating clinical trial FFPE breast cancer core needle biopsies by targeted NGS. The results underpin that this approach is feasible not only for the detection of mutations but also for CNAs. The samples are from a well-defined prospectively conducted clinical trial. NGS allows interrogation of not only 1 but multiple genes of interest, and provides higher sensitivity than conventional molecular approaches.

Nevertheless, there are some limitations: we have not been able to evaluate all genes that are relevant in breast cancer, and genes described in newer and more comprehensive sequencing studies have not been included (i.e., t*BRCA*) (4, 24, 32). It should be noted that some amplifications, in particular the high prevalence of *TP53* amplifications in all cancer types and the high prevalence of *TOP2A* amplifications in TNBC, have not been described in previous studies and might be related to the specific method used. For these alterations, additional validations are needed.

Important tumor-suppressor genes such as *GATA3* and *BRCA* could not be included due to the size restrictions of the NGS panel. It is important to emphasize that the CNA approach is still more exploratory compared with the standardized mutational analysis. We have been able to validate this approach for HER2 amplification, but additional validations would be needed for additional CAN markers before this would be introduced into clinical practice. Therefore, although the CNA approach is innovative compared with other methods and opens a flexible CNA analysis from different NGS panels, additional validation is needed to achieve a diagnostic standard that is similar to the HER2 assessment. This would be in particular important for markers such as *TOP2A* and *ZNF703*, where our analysis shows that 1 of the amplicons has a poor correlation with the other amplicons.

In summary, targeted NGS on FFPE core biopsies reliably identified point mutations and amplifications with a high heterogeneity between the molecular subtypes. We confirm previous reports of *PIK3CA* mutations as a therapy resistance parameter in HER2pos breast cancer, and our results point to a role for *PIK3CA* mutations in response to nab-P versus P, which should be validated in additional trials.

Disclosure of Potential Conflicts of Interest

A. Stenzinger reports receiving speakers bureau honoraria from Roche, Bristol-Myers Squibb, MSD, AstraZeneca, Thermo Fisher, Illumina, and Novartis, is a consultant/advisory board member for AstraZeneca, Bayer, Bristol-Myers Squibb, Illumina, Thermo Fisher, and Novartis, and reports receiving commercial research grants from Bayer. W. Weichert reports receiving speakers bureau honoraria from Roche, Bristol-Myers Squibb, MSD, Novartis, AstraZeneca, Boehringer Ingelheim, Takeda, Lilly and Amgen, is a consultant/advisory board member for Roche, Bristol-Myers Squibb, AstraZeneca, Novartis, Pfizer and Merck, and reports receiving commercial research grants from Roche, Bristol-Myers Squibb, MSD, and Bruker Daltonics. G. von Minckwitz has ownership interests (including patents) at CARA GmbH, is a consultant/advisory board member for Amgen and Roche, reports receiving commercial research grants from Pfizer, Amgen, Roche, AstraZeneca, Myriad Genetics, Abbvie, and Vifor. C. Jackisch is a consultant/advisory board member for Roche and Novartis. A. Schneeweiss reports receiving other remuneration from Roche and Merck Sharp & Dohme. P.A. Fasching reports receiving speakers bureau honoraria from Roche, Amgen, Novartis, Pfizer, Celgene, Daiichi-Sankyo, Eisai, and Hexal, is a consultant/advisory board member for Roche, Novartis, Pfizer, Puma, Eisai, Merck Sharp & Dohme, AstraZeneca, Teva, Hexal, and reports receiving commercial research grants from BioNTech and Novartis. C. Schem reports receiving speakers bureau honoraria from Celgene. M. van Mackelenbergh reports receiving speakers bureau honoraria from AstraZeneca, and is a consultant/advisory board member for Amgen and Genomic Health. M. Untch is a consultant/advisory board member for Celgene. C. Denkert has ownership interests (including patents) at Sividon Diagnostics, reports receiving speakers bureau honoraria from Teva, Novartis, Pfizer, Roche, and Amgen, and is a consultant/advisory board member for MSD Oncology, Daiichi-Sankyo, and Amgen. No potential conflicts of interest were disclosed by the other authors.

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