Genomic Complexity Predicts Resistance to Endocrine Therapy and CDK4/6 Inhibition in Hormone Receptor–Positive (HR+)/HER2-Negative Metastatic Breast Cancer

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ABSTRACT

Purpose: Clinical biomarkers to identify patients unlikely to benefit from CDK4/6 inhibition (CDK4/6*i*) in combination with endocrine therapy (ET) are lacking. We implemented a comprehensive circulating tumor DNA (ctDNA) analysis to identify genomic features for predicting and monitoring treatment resistance.

Experimental Design: ctDNA was isolated from 216 plasma samples collected from 51 patients with hormone receptor–positive (HR+)/HER2-negative (HER2−) metastatic breast cancer (MBC) on a phase II trial of palbociclib combined with letrozole or fulvestrant (NCT03007979). Boosted whole-exome sequencing (WES) was performed at baseline and clinical progression to evaluate genomic alterations, mutational signatures, and blood tumor mutational burden (bTMB). Low-pass whole-genome sequencing was performed at baseline and serial timepoints to assess blood copy-number burden (bCNB).

Results: High bTMB and bCNB were associated with lack of clinical benefit and significantly shorter progression-free survival (PFS) compared with patients with low bTMB or low bCNB (all $P < 0.05$). Dominant APOBEC signatures were detected at baseline exclusively in cases with high bTMB (5/13, 38.5%) versus low bTMB (0/37, 0%; $P = 0.0006$). Alterations in *ESR1* were enriched in samples with high bTMB ($P = 0.0005$). There was a high correlation between bTMB determined by WES and bTMB determined using a 600-gene panel ($R = 0.98$). During serial monitoring, an increase in bCNB score preceded radiographic progression in 12 of 18 (66.7%) patients.

Conclusions: Genomic complexity detected by noninvasive profiling of bTMB and bCNB predicted poor outcomes in patients treated with ET and CDK4/6*i* and identified early disease progression before imaging. Novel treatment strategies including immunotherapy-based combinations should be investigated in this population.

Introduction

The combination of endocrine therapy (ET) and cyclin-dependent kinase 4/6 inhibition (CDK4/6*i*) has emerged as the standard-of-care, first-line treatment for patients with hormone receptor–positive (HR+)/HER2-negative (HER2−) metastatic breast cancer (MBC). This treatment indication is based on the significant improvement in survival outcomes and extended chemotherapy-free interval across all clinical and pathologic subgroups (1–5). Therefore, outside of clinical trials or impending organ failure, patients in the United States and Europe are offered CDK4/6*i* and ET as first-line treatment. Despite this advancement in care for patients with HR+/HER2− MBC, a subset of patients rapidly progress, and biomarkers to predict efficacy and resistance are lacking.

Analysis of circulating tumor DNA (ctDNA) using next-generation sequencing (NGS) enables the noninvasive assessment of genomic alterations during tumor progression and has led to the identification of biomarkers for predicting and monitoring response to treatment (6–10). In 2019, the FDA approved a ctDNA-based companion diagnostic test for the detection of *PIK3CA* mutations to select patients for treatment with alpelisib, leading to increased utilization of ctDNA tests in clinical practice (11). Both tissue and blood-based NGS profiling have identified individual alterations associated with resistance in patients treated with ET and CDK4/6*i*, including alterations in *CCNE1*, *FGFR1*, *FAT1*, *PTEN*, and *RB1* (12–17). However, to date, no clinical, pathologic, or genomic signatures have been identified as predictive at baseline to define a subset of patients who benefit from alternative treatment strategies. We hypothesized that a comprehensive NGS-based liquid biopsy approach encompassing assessment of ctDNA mutation and copy-number burden (CNB) could identify prognostic and predictive biomarkers in patients with HR+/HER2− MBC and track response to ET and CDK4/6*i* treatment. To accomplish this, we utilized a combination assay that provides targeted coverage of 600 cancer genes in addition to whole-exome sequencing (WES) to enable comprehensive genomic profiling, evaluation of mutational

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Translational Relevance

Clinical biomarkers are needed to identify patients with hormone receptor–positive (HR+)/HER2-negative (HER2−) metastatic breast cancer (MBC) who are unlikely to respond to endocrine therapy combined with CDK4/6 inhibition. Circulating tumor DNA (ctDNA) samples from a phase II clinical trial (NCT03007979) were profiled to define subsets of patients with high tumor mutational burden (TMB) and genome-wide copy-number burden (CNB) levels associated with poor clinical outcomes. This work extends whole-exome sequencing (WES) to blood to define resistance at baseline and clinical progression. On the basis of the prior tumor-agnostic FDA drug approval for immune checkpoint inhibition based on tissue TMB, our work identifies HR+/HER2− patients with high blood TMB who may benefit from immunotherapy. Demonstration of concordant TMB profiles using targeted sequencing panels also offers a cost-effective method for future adoption in the clinic. Measurement of CNB levels with low-pass whole-genome sequencing may constitute a low-cost approach for treatment monitoring to detect early progression.

signatures, and derivation of bTMB at baseline and progression timepoints. In addition, we implemented low-pass whole-genome sequencing (LP-WGS) to derive a novel measure of genome-wide copy-number variation (CNV).

Tumor mutational burden (TMB) is a measure of the number of mutations per megabase of sequenced DNA with WES considered the gold standard for TMB measurement (18). The rationale for developing TMB as a clinical biomarker, initially derived from tissue, was based on the observation that tumor types with high tissue TMB [tTMB; e.g., non–small cell lung cancer (NSCLC) in smokers, melanoma associated with ultraviolet radiation, and mismatch repair deficient tumors] respond well to immune checkpoint inhibitor (ICI) therapy (19–22). tTMB has shown promise as a potential surrogate biomarker for neoantigen load to predict response to ICI monotherapy and as a nonoverlapping biomarker in conjunction with PD-L1 expression on tumor or immune cells (23). Blood tumor mutational burden (bTMB) was initially explored as a noninvasive method of TMB determination in NSCLC given the difficulty of obtaining adequate tissue for sequencing in some cases. Several studies performed with NGS targeted cancer gene panels have demonstrated that patients with NSCLC with high bTMB preferentially responded to ICI over chemotherapy (24–26). However, due to technical challenges and cost considerations, the application of WES to measure TMB in blood samples has been performed less widely (27, 28). Relative to other malignancies, evaluation of TMB in breast cancer has been less extensive, with most studies assessing tTMB. The evaluation of tTMB has demonstrated that while patients with breast cancer have a relatively low median tTMB, tTMB is higher in metastatic versus primary tissue. Importantly, early data indicate that a subset of patients with breast cancer with high TMB benefit from PD-1 inhibitors with or without anti-CTLA-4 (29–31). In addition, parallel assessment of mutational signatures in hypermutated malignancies has revealed the presence of APOBEC (alipoprotein B mRNA-editing enzyme catalytic polypeptide-like) mutational signatures in patients with high tTMB, which have been associated with response to ICI (29, 32–34).

Blood CNB (bCNB), derived from the PredicineCNB assay, is a comprehensive measure of CNV via LP-WGS, including amplifications and deletions across the entire genome. While current strategies for blood-based treatment response monitoring have primarily tracked individual ctDNA mutations or changes in allele frequency to evaluate tumor response to systemic therapy, early evidence has demonstrated that the integration of copy number changes and whole-genome methylation can provide an early signal of response for patients treated with a variety of systemic therapies prior to standard-of-care imaging (35–39). Given that LP-WGS is less expensive compared with other NGS methods and therefore more feasible for serial testing from a cost perspective, the technique may offer clinical applications for monitoring global CNV levels during the course of treatment. Studies evaluating this technique in patients with MBC treated with CDK4/6*i* are limited.

Here, we describe the use of two novel, genome-wide ctDNA assays that combine sequencing breadth and depth to profile patients with HR+/HER2− MBC undergoing combined ET and CDK4/6*i* treatment in a prospective phase II interventional clinical trial. The goal of this work was to identify resistance biomarkers that define patients who may be candidates for novel treatment strategies and to explore the potential for serial ctDNA monitoring to predict early disease progression. Our comprehensive approach identified bTMB and bCNB levels that predicted poor patient outcomes, identified APOBEC signatures exclusively in hypermutated patients, defined an expanded list of candidate alterations that may mediate resistance at baseline and clinical progression, and demonstrated the potential for bCNB to predict and monitor early disease progression.

Materials and Methods

Patient cohort

Patient ctDNA samples were retrospectively analyzed from a prospective, single-arm, phase II study (NCT03007979) that was conducted at the Washington University School of Medicine and the University of Nebraska Medical Center. Patients with HR+/HER2− MBC treated with 0 to 1 lines of prior systemic therapy without prior use of CDK4/6*i* were enrolled. Patients received palbociclib 125 mg daily, on a continuous 5-days-on/2-days-off weekly schedule in combination with letrozole or fulvestrant per physician's choice with goserelin administration for premenopausal patients. Each treatment cycle was 28 days. Research blood samples were collected in Streck tubes at baseline, cycle 1 day 15 (C1D15), C2D1, and C4D1, then on D1 of every 3 cycles (with tumor imaging) until disease progression. Fifty-four patients were enrolled to the study, of which 51 patients were evaluable for response and included in this analysis. At data cutoff, 29 patients went off study due to disease progression, and therefore samples were available for 29 patients at disease progression. For these patients, plasma samples collected at the timepoints immediately prior to clinical progression were also included in this analysis. The results of the primary endpoint (rate of grade 3 or 4 neutropenia), and clinical response were reported previously (40). The study was approved by the institutional review board at each site and conducted according to the ethical guidelines set forth in the Declaration of Helsinki. Informed written consent was obtained from all patients to allow correlative research on their blood samples.

ctDNA analysis

Patient samples were analyzed using two comprehensive NGS platforms, PredicineWES+ and PredicineCNB (Predicine, Inc.), to generate genomic profiles, perform mutational signature and pathway analyses, and derive measures of bTMB and bCNB. Briefly, cell-free

DNA (cfDNA) extracted from patient plasma samples and germline DNA extracted from peripheral blood mononuclear cells (PBMC) were processed and subjected to library construction. The resulting DNA libraries were sequenced by PredicineCNB LP-WGS at 5x coverage or further enriched by hybrid capture for sequencing with PredicineWES+, a combination assay designed to sequence the entire exome with sequencing depth at 2,500× [1% level of detection (LOD)] along with boosted sequencing of 600 cancer genes covered by the PredicineATLAS targeted panel with sequencing depth at 20,000× (0.25% LOD; Supplementary Table S1). PredicineWES+ sequencing data were used to generate the landscape of genomic alterations including single-nucleotide variants (SNV), insertions and deletions (indels), CNVs, and gene fusions, to derive bTMB scores reporting the total number of somatic mutations detected per megabase of DNA and analyze mutational signatures and oncogenic signaling pathway involvement (Supplementary Materials and Methods). bTMB scores were also derived from sequencing data generated by analysis using the targeted 600-gene PredicineATLAS and 152-gene PredicineCARE panels to compare bTMB values generated by PredicineWES+ (41). PredicineCNB sequencing data were evaluated to generate bCNB scores representing a comprehensive genome-wide measure of CNV, including amplifications and deletions across the entire genome adapted from the previously developed ichorCNA method (Supplementary Materials and Methods; ref. 42).

Statistical analysis

Statistical associations among individual alterations, bTMB, and bCNB with clinical benefit rate (CBR), defined as the percentage of patients with a complete response, partial response, or stable disease lasting at least 24 weeks by RECIST (version 1.1), were analyzed using Wilcoxon and Kruskal–Wallis tests. Frequencies of alterations across patient subgroups were compared using the Fisher exact test. Comparison of the frequencies of alterations across patient subgroups at baseline and clinical progression timepoints was performed using McNemar test. The degree of association between variables was evaluated with Spearman Rank correlation coefficient or Pearson correlation coefficient. The Kaplan–Meier (K–M) method was applied to estimate empirical survival probabilities with K–M curves used to illustrate survival, and the log-rank test was utilized to compare differences in survival. HRs and 95% confidence intervals (95% CI) were estimated from univariate Cox proportional hazards regression analysis. Different cutoffs were applied to bTMB for analysis of association with progression-free survival (PFS), including the unbiased cutoffs of median and third quartile, whereas optimal cutoffs were further explored on the basis of Harrell C-index in a Cox model setting for PFS and ROC analysis for clinical benefit. Changes in bCNB were assessed at serial timepoints and compared with concurrent assessment of clinical progression based on RECIST v1.1. *P* values were corrected for multiplicities using the *post hoc* Benjamini–Hochberg procedure across all variants.

Data availability

Summary sequencing data are supplied as a supplementary data file. All other deidentified data are available upon reasonable request from the corresponding author.

Results

Serial ctDNA samples analyzed from a prospective clinical trial

Serial ctDNA testing was performed retrospectively on samples collected from a prospective clinical trial of palbociclib in combination with ET (letrozole or fulvestrant; ref. 40). Two-hundred sixty-five samples from 51 evaluable patients with HR+/HER2− MBC were analyzed using Predicine liquid biopsy NGS platforms (**Fig. 1A**). At the time of data censoring, there were 29 patients who had progressed on study at a median follow-up time of 16.4 months (range 1.4–50.9). Only two (2/265) samples failed sequencing quality control and greater than 99% of samples were successfully sequenced. PredicineWES+ was performed on 78 plasma samples collected at baseline ($N = 50$) and clinical progression ($N = 28$), combining WES at 2,500× (1% LOD) and boosted sequencing (20,000×, LOD 0.25%) of 600 genes. PredicineCNB at 5× was performed for 216 plasma samples including all available baseline ($N = 51$), C1D15 ($N = 47$), C2D1 ($N = 51$), staging evaluations prior to progression ($N = 38$), and clinical progression ($N = 29$) timepoints (**Fig. 1B** and **C**).

Clinical and pathologic characteristics of patients included in the study are summarized in Supplementary Table S2. The vast majority of patients were postmenopausal (84.3%) and received letrozole (72.6%) with the remaining patients receiving fulvestrant (27.5%). A total of 17 patients were *de novo* metastatic, 22 patients were classified as endocrine resistant, and 12 patients were endocrine sensitive based on ESMO 2020 criteria (43).

High baseline bTMB is associated with worse clinical outcomes

bTMB was evaluable for 50 patients at baseline (**Fig. 2A**). The median bTMB was 1.85 mutations per megabase pair (MBp) [interquartile range (IQR), 1.01–3.86; range 0.1–71.7]. Patients without clinical benefit who experienced disease progression within 6 months ($N = 10$) had a significantly higher bTMB compared with patients with clinical benefit [$N = 40$; median 8.90 mutations/MBp (IQR, 2.30–31.2) vs. median 1.63 (IQR, 0.69–2.83), Wilcoxon, $P = 0.012$; **Fig. 2B**]. Patients with an *ESR1* mutation at baseline ($N = 8$) also had significantly higher baseline bTMB (Wilcoxon, $P = 5.0 \times 10^{-4}$; **Fig. 2C**), and similar associations were found for baseline mutations in the *ARID1A, BSN, CDH1*, *DNAH10, DSP, MUC6, MUC16, PIK3CA*, and *USH2A* genes (Supplementary Fig. S1). Associations for *ESR1* ($P = 0.016$), *PIK3CA* ($P = 0.016$), *CDH1* ($P = 0.036$), and *USH2A* ($P = 0.032$) remained significant following false discovery rate (FDR) *P* value adjustment. When comparing patients based on the clinical classifications of *de novo*, endocrine-resistant, or endocrine-sensitive MBC, no significant difference in baseline bTMB was observed (**Fig. 2D**). However, the majority of patients with high bTMB at baseline were present in the endocrine-resistant cohort. ROC analysis was performed to determine the optimal cutpoint for bTMB in our dataset as 3.2 mutations/MBp with AUC 0.76 (Supplementary Fig. S2), which aligns closely to the 3.8 bTMB value that dichotomizes patients above and below the third quartile. Higher bTMB at baseline was significantly associated with worse PFS (median 13.8 months vs. 32.1 months) based on the median of the sample (HR, 2.62; 95% CI, 1.21–5.66; $P = 0.011$; **Fig. 2E**). A similarly significant difference between high and low bTMB was observed on the basis of the third quartile of the sample (median 6.5 months vs. 32.1 months; HR, 4.87; 95% CI, 2.19–10.81; $P = 2.27 \times 10^{-5}$; **Fig. 2F**) and at a cutoff of 10 mutations/MBp (median 3.8 months vs. 22.3 months; HR, 7.15; 95% CI, 2.82–18.13; $P = 1.94 \times 10^{-6}$; **Fig. 2G**). When assessing survival in the endocrine-resistant cohort, a significant difference was observed with high baseline bTMB significantly associated with worse PFS (median 6.5 months vs. 22.3 months; HR, 4.76; 95% CI, 1.52–14.97; $P = 0.004$; **Fig. 2H**). In summary, high baseline bTMB was significantly associated with lack of clinical benefit and shorter PFS as measured using multiple previously established and experimentally determined cutoff points.

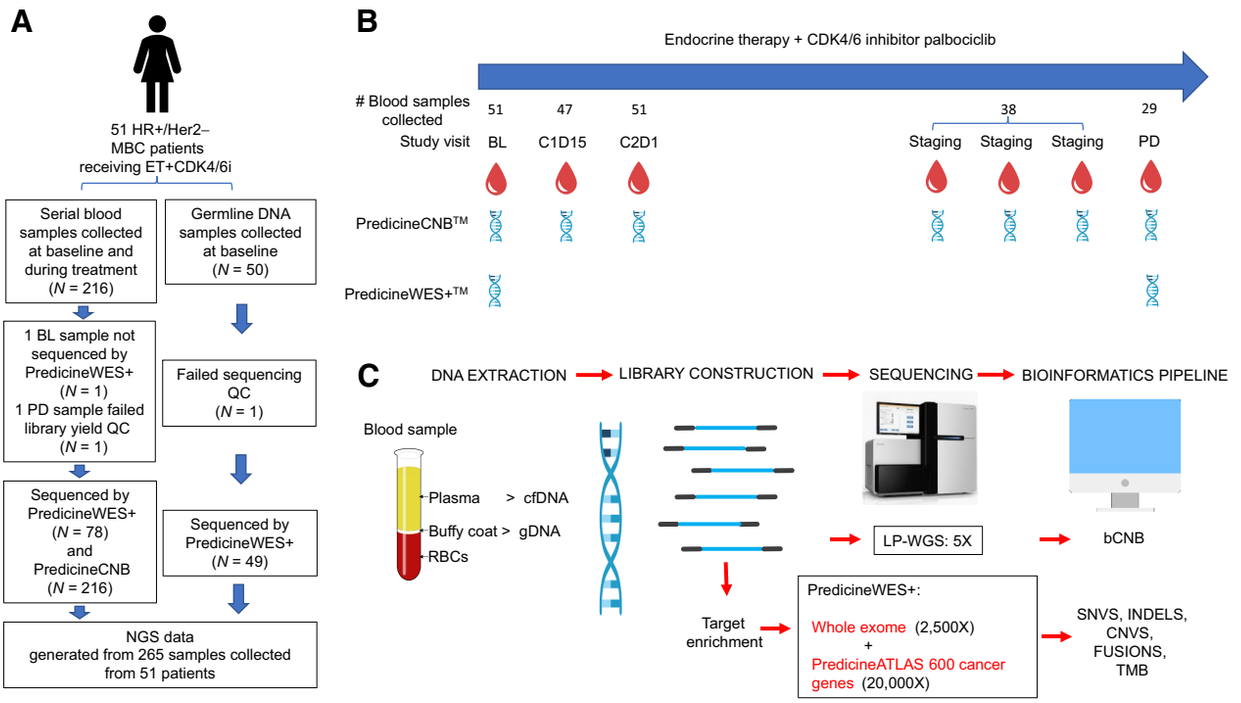

Figure 1.

Overview of study design. **A,** Study schema. **B,** Sample collection timepoints for PredicineCNB and PredicineWES+ profiling. **C,** Next-generation sequencing process. BL, baseline; C1D15, cycle 1 day 15; C2D1, cycle 2 day 1; cfDNA, cell-free DNA; gDNA, genomic DNA; PD, progressive disease; QC, quality control.

bTMB scores generated from targeted sequencing panels and WES are highly correlated

bTMB levels generated from 50 baseline samples using the PredicineWES+ assay were compared with values obtained using the targeted 600-gene PredicineATLAS and the 152-gene PredicineCARE sequencing assays. bTMB values obtained by PredicineWES+ were highly correlated with levels derived from PredicineATLAS (R = 0.98; **Fig. 3**) and PredicineCARE (R = 0.93; Supplementary Fig. S3; Spearman rank test). Together, these comparisons suggest that accurate bTMB scores can also be generated from fixed NGS gene panels of smaller size.

High baseline cfDNA and tumor fraction levels are associated with poor clinical outcomes

High cfDNA yield was associated with significantly shorter PFS based on the median (HR, 2.36; 95% CI, 1.12–4.98; P = 0.021) and third quartile (HR, 2.96; 95% CI, 1.34–6.54; P = 0.006) cutoffs of the samples (Supplementary Fig. S4). In the endocrine-resistant cohort, high cfDNA yield was also associated with significantly shorter PFS based on the median (HR, 3.45; 95% CI, 1.18–10.14; P = 0.017), and third quartile (HR, 4.35; 95% CI, 1.29–14.63; P = 0.01) cutoffs. Using a 10% cutoff point to dichotomize patients, shorter PFS was significantly associated with high ichorCNA-derived TF (HR, 2.28; 95% CI, 1.09–4.75; P = 0.024), but not high ctDNA fraction (HR, 1.85; 95% CI, 0.88–3.88; P = 0.099; Supplementary Fig. S5). Endocrine sensitivity versus endocrine resistance and site of disease at clinical presentation (e.g., bone or visceral) did not predict CBR or PFS for patients treated with palbociclib in combination with ET (Supplementary Table S2). Similarly, there was no significant association between sites of metastatic spread and bTMB at baseline (Supplementary Fig. S6). In summary, high baseline cfDNA yield and tumor fraction levels, but not commonly assessed clinicopathologic features, were associated with poor clinical outcomes.

Dominant APOBEC mutational signatures are present exclusively in high bTMB patients.

Off-target activity of the APOBEC family of mutator enzymes can generate somatic mutations across the genome leading to distinct mutational signatures that have been associated with the development and progression of multiple cancers (21, 44, 45). To assess the contribution of these mutational signatures to the genomic landscape of high versus low bTMB patients in this cohort, sequencing data obtained by PredicineWES+ from 50 patients at baseline were evaluated for single base substitution (SBS) patterns, which were compared against the 94 curated reference SBS mutational signatures available in the COSMIC database (Supplementary Materials and Methods). Dominant APOBEC signatures (SBS2 and SBS13) were identified exclusively in the patients with high bTMB, whereas the two other dominant mutational signatures detected, MMRd (mismatch repair deficiency, SBS6) and a signature of unknown etiology (SBS5) were observed across high and low bTMB groups (**Fig. 4A**). In contrast to another report, dominant POLE (defective DNA polymerase epsilon) and HRD (homologous repair deficiency) signatures were not observed (29). Dominant APOBEC signatures were detected in 5 of 13 (38.5%) patients with high bTMB versus 0/37 (0%) patients with low bTMB (P = 0.0006, Fisher exact test; **Fig. 4B**). The median bTMB score was significantly higher in patients with dominant APOBEC signatures (34.8 MBp) versus

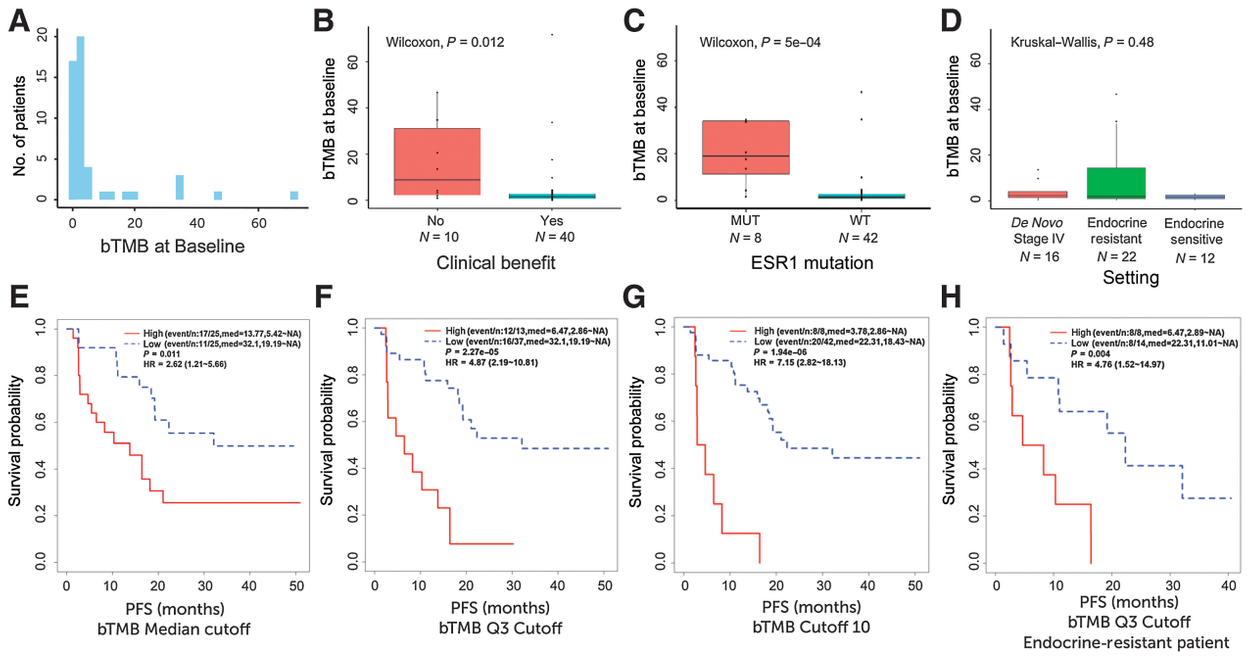

Figure 2.

High bTMB at baseline is associated with poor patient outcomes. **A,** Distribution of bTMB scores across 50 baseline patient samples sequenced by PredicineWES+. High bTMB scores were significantly associated with (**B**) lack of clinical benefit (CB) defined as progressive disease (PD) within 6 months and (**C**) the presence of *ESR1* mutations at baseline (Wilcoxon test; FDR $P = 0.016$). **D,** Clinical classification of endocrine resistance per ESMO 2020 guidelines did not predict bTMB, although there were a greater number of patients with high bTMB in the endocrine-resistant cohort (Kruskal–Wallis test). The association of high bTMB with significantly shorter PFS was observed using multiple cutoffs for bTMB including the median (**E**), third quartile (**F**), or bTMB scores of 10 mutations/megabase (**G**; log rank test). **H,** Within the endocrine-resistant cohort, high bTMB scores were significantly associated with shorter PFS (log-rank test).

other signatures (1.7 MBp; $P = 0.00048$, Wilcoxon rank sum test; **Fig. 4C**). Collectively, these findings support the identification of a subset of hypermutated HR+/HER2− patients with MBC.

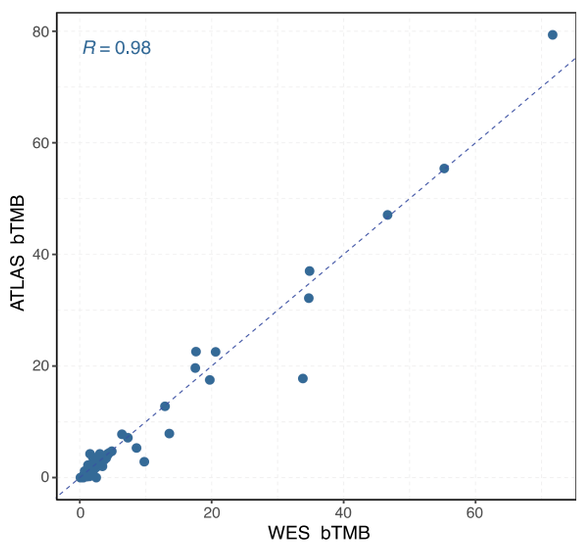

Figure 3.

High correlation between bTMB determined from WES and a 600-gene targeted sequencing panel. bTMB levels generated from 50 baseline samples using the PredicineWES+ and PredicineATLAS sequencing assays were highly correlated (Spearman rank test).

Specific oncogenic signaling pathways are more frequently altered in patients with high bTMB and high bCNB

To compare the relative proportion of alterations within key oncogenic signaling pathways in high versus low bTMB and bCNB patients, we compared the frequencies of alterations identified across breast cancer driver genes present in 12 previously described pathways (Supplementary Materials and Methods; refs. 33, 34, 46). Significantly higher frequencies of alterations (including SNVs and CNVs) were observed in patients with high versus low bTMB across breast cancer driver genes in the cell cycle ($P = 0.04$), DNA damage repair (DDR; $P = 0.02$), Hippo ($P = 0.009$), Notch ($P = 0.003$), PI3K ($P = 2.9 \times 10^{-05}$) and receptor tyrosine kinase (RTK)-RAS ($P = 0.005$) oncogenic signaling pathways (Fisher exact test; Supplementary Fig. S7). Following adjustment for FDR, associations remained significant for the Notch ($P = 0.03$), PI3K ($P = 2.6 \times 10^{-04}$), and RTK-RAS ($P = 0.03$) pathways. Significantly higher frequencies of alterations were also observed in high versus low bCNB patients in the cell cycle ($P = 0.009$), DDR ($P = 0.001$), Hippo ($P = 0.04$), Notch ($P = 0.04$), RTK-RAS ($P = 0.007$), and TP53 ($P = 0.002$) pathways (Fisher exact test; Supplementary Fig. S7). Following adjustment for FDR, associations remained significant for the DDR ($P = 0.01$), RTK-RAS ($P = 0.05$), and TP53 ($P = 0.02$) pathways. These findings suggest that several relevant signaling pathways, some of which have been implicated in driving ET and CDK4/6*i* resistance, are more frequently activated in hypermutated, genomically complex patients with breast cancer compared with patients with lower genomic complexity in this cohort.

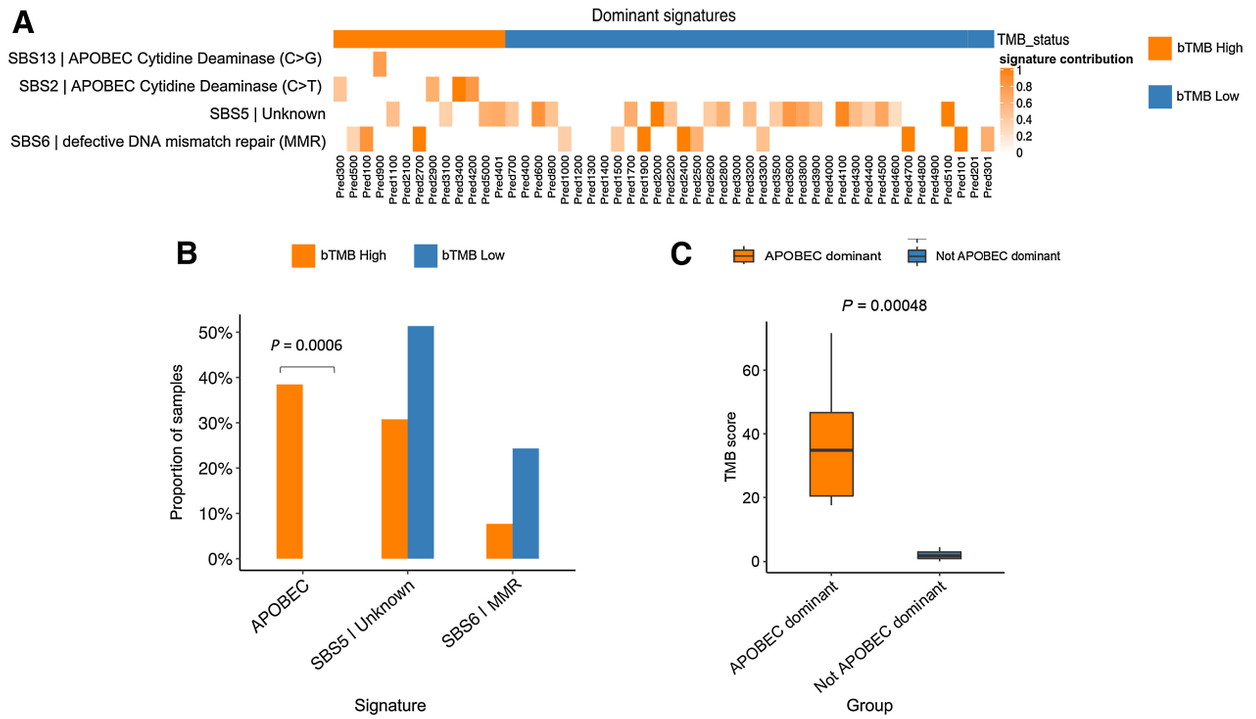

Figure 4.

High bTMB at baseline is associated with dominant APOBEC mutational signatures and upregulation of specific oncogenic signaling pathways. **A,** Dominant APOBEC mutational signatures (SBS2 and SBS13) were enriched in patients with high ($N = 13$) versus low ($N = 37$) bTMB scores (defined by upper quartile cutoff). Dominant SBS5 (unknown) and SBS6 (defective DNA mismatch repair signatures) were observed across patients with high and low bTMB scores. **B,** The proportion of dominant APOBEC signatures was significantly greater in patients with high bTMB scores versus low bTMB scores (Fisher exact test). **C,** The median bTMB score was significantly higher in patients with dominant APOBEC signatures versus other signatures (Wilcoxon rank sum test).

Comprehensive profiling extends detection of clinically relevant ctDNA alterations at baseline and detects enrichment of novel ctDNA alterations at progression

The PredicineWES+ assay was performed on 50 of 51 samples collected at baseline and 28 of 29 samples collected at progression. One of 51 baseline samples was sequenced using the PredicineATLAS assay instead of the PredicineWES+ assay, and one of the progression samples failed library yield quality control. The most frequently observed alterations across all 51 patients at baseline were *PIK3CA* (45%), *TP53* (31%), and *ESR1* (20%; Supplementary Fig. S8). Baseline alterations (SNVs and CNVs) in 17 genes were significantly associated with worse PFS, including *AURKA*, *AKT3, ATM, BRCA2, CCND1*, *CCNE2, DDR2*, *DSP, ESR1*, *MYC*, *MUC16, PIK3CA*, *PLCG1*, *RB1*, *RUNX1T1*, *USH2A*, and *ZFHX3* (Supplementary Table S3). All but three of these associations (*RB1, CCNE2*, and *USH2A*) remained significant following FDR *P* value adjustment. Five of these genes (*CCNE2, DSP, MUC16, PLCG1*, and *USH2A)* are not targeted by the PredicineATLAS panel and have not commonly been implicated in CDK4/6*i* and ET resistance, constituting novel resistance alterations detected by WES.

A comparison of the most frequently altered genes detected at progression versus baseline was made across all evaluable samples from patients who had progressed at the time of analysis (28/29; **Fig. 5A**). The most frequently observed alterations across all 28 patients at baseline were *PIK3CA* (54%), *TP53* (39%), *AKT3* (32%), *DDR2* (29%), *ATM* (29%), *AURKA* (25%) *ESR1* (25%), *BRCA2* (21%), and *EGFR* (21%), whereas at progression the most frequent alterations were *TP53* (50%), *PIK3CA* (43%), *RB1* (36%), *AURKA* (32%), *CCND1* (32%), *ESR1* (32%), *BRCA2* (29%), *ATM* (25%), and *MUC12* (25%). Alterations in *RB1* were significantly enriched at progression (36% vs. 14%; $P = 0.04$; McNemar test; **Fig. 5B**). Although no correction for multiplicity was performed due to the small sample size, this finding is consistent with prior reported observations (47). Nonsignificant enrichment at progression versus baseline was observed for a number of additional alterations previously implicated in CDK4/6*i* and/or ET resistance including AR (18% vs. 7%) *AURKA* (32% vs. 25%), *CCND1* (32% vs. 18%), *CDKN2A* (21% vs. 14%), *ESR1* (32% vs. 25%), *FGFR1* (18% vs. 14%), *PTEN* (21% vs. 11%), *MYC* (18% vs.14%), and *TP53* (50% vs. 39%). Nonsignificant enrichment of additional alterations not commonly implicated in CDK4/6*i* and ET resistance was also observed at progression, including *BRCA2* (29% vs. 21%), *CBL* (18% vs. 7%), *CDH1* (18% vs. 11%), *KMT2D* (18% vs. 14%), *MUC12* (25% vs. 14%), and *PREX2* (21% vs. 14%; **Fig. 5B**). Two of the enriched genes (*MUC12* and *PREX2*) were not targeted by the PredicineATLAS panel, representing novel alterations detected by WES. In contrast to increased levels of some individual variants, no significant difference was observed between the median bTMB or bCNB levels detected at baseline versus progression (Supplementary Fig. S9). In summary, extension of boosted WES sequencing to blood samples collected at baseline and progression led to the identification of additional candidate biomarkers of *de novo* and acquired resistance.

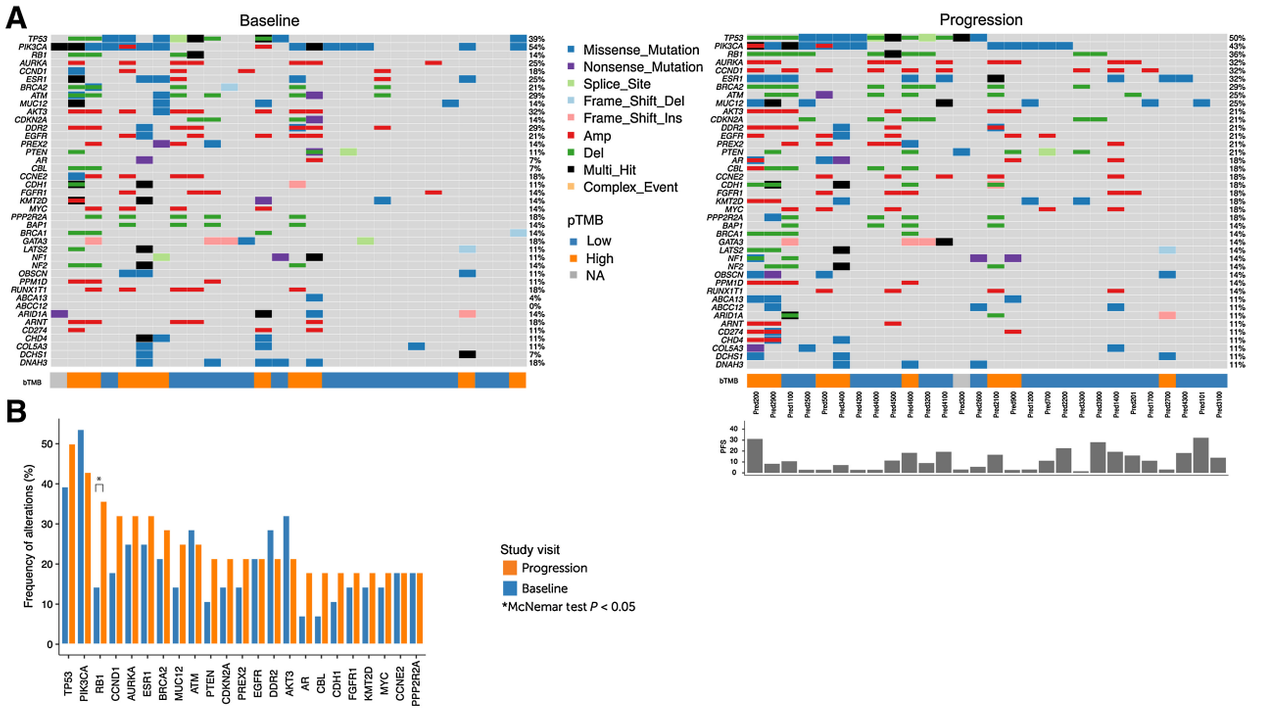

Figure 5.

Specific genomic alterations are enriched at disease progression. **A,** Heatmaps comparing the most frequently altered genes detected at progression versus baseline by PredicineWES+ ($N = 28$). Color coded bars below each heatmap indicate the level of bTMB, and the bar graph above the progression heatmap indicates PFS in months for each patient. Blue arrows denote patients with dominant APOBEC signatures. **B,** Enrichment at progression was observed for previously reported gene alterations implicated in resistance to endocrine and/or CDK4/6*i* therapy including a significantly higher frequency of *RB1* alterations ($P = 0.04$; McNemar test), and nonsignificant enrichment in *AR*, *AURKA*, *CCND1*, *CDKNKA*, *ESR1*, *FGFR*, *MYC*, *PTEN*, and *TP53* alterations. In addition, nonsignificant enrichment of gene alterations not commonly linked to ET + CDK4/6*i* resistance such as *BRCA2*, *CBL*, *CDH*, *KMT2D*, *MUC12*, and *PREX2* was observed at progression. Two of the enriched gene alterations (*MUC12* and *PREX2*) were not covered by the targeted PredicineATLAS panel and were detected only through comprehensive WES.

bCNB scores predict clinical outcomes and increase before radiographic detection of clinical progression

bCNB scores reflecting genomic-wide assessment of CNV were derived from LP-WGS data generated from all 51 baseline samples, 47 C1D15 samples, 51 C2D1 samples, 38 staging samples, and 29 progression samples (Supplementary Materials and Methods; **Fig. 1B**). The median bCNB score at baseline was 9.36 (IQR, 5.78–13.2). Baseline bCNB scores were significantly higher in patients who experienced progression within 6 months (without clinical benefit; Wilcoxon test, $P = 0.036$; **Fig. 6A**), and a high baseline bCNB score defined by a cutoff of 5.6 was significantly associated with shorter PFS (HR, 3.14; 95% CI, 1.34–7.74; $P = 0.009$; **Fig. 6B**). Baseline bCNB scores were also significantly correlated with baseline bTMB scores ($R = 0.68$; $P = 6.6 \times 10^{-08}$; Supplementary Fig. S10). Serial analysis of bCNB during treatment revealed decreases at C1D15 and/or C2D1 relative to baseline levels in 38 of 51 (74.5%) patients. Staging samples were collected concurrently with imaging studies performed every 3 months for assessment of tumor response. One to three timepoints (3 to 9 months) immediately prior to progression were available from 18 of the 29 patients who progressed. Analysis of these samples revealed increases (over previous nadir levels) that preceded imaging detection of progressive disease by at least 3 months in 12 of 18 (66.7%) patients, as shown in **Fig. 6C**. An increase in bCNB preceded imaging detection of progressive disease by 6 months in 5 patients, and by 9 months in 4 patients. Comparison of dynamic bCNB patterns using the orthogonal measure of ctDNA fraction from PredicineATLAS profiling in a subset of 4 patients revealed similar patterns with ctDNA fraction levels, with high average correlation observed across matched longitudinal profiles for individual patients ($R = 0.94$, Pearson correlation coefficient; Supplementary Fig. S11). Genome-wide plots of copy number changes and copy number levels for BRCA2 and RB1 at baseline and across treatment time points are shown for patient Pred200 in Supplementary Figs. S12 and S13, respectively, illustrating the copy number changes underlying the dynamic changes in bCNB levels shown in Supplementary Fig. S11. A high correlation was also observed between bCNB and ctDNA scores at baseline ($R = 0.84$), but not between bTMB and ctDNA scores at baseline ($R = 0.33$; Supplementary Fig. S14). Analysis of the relationship between bCNB scores and ichorCNA-derived TF levels in 216 samples collected over multiple treatment timepoints also revealed a strong correlation ($R = 0.76$; Supplementary Fig. S15). This analysis illustrates the high sensitivity of bCNB to detect tumor-associated copy number changes in plasma samples with low (<5%) tumor fraction. In summary, high baseline bCNB was significantly associated with poor clinical outcomes and increases in bCNB scores preceded radiographic detection of clinical progression in two thirds of the cases monitored over the course of treatment.

Discussion

In this study, we report for the first time a comprehensive ctDNA NGS analysis, encompassing a plasma-based boosted WES assay, LP-WGS, and a bioinformatics pipeline for determining bTMB and

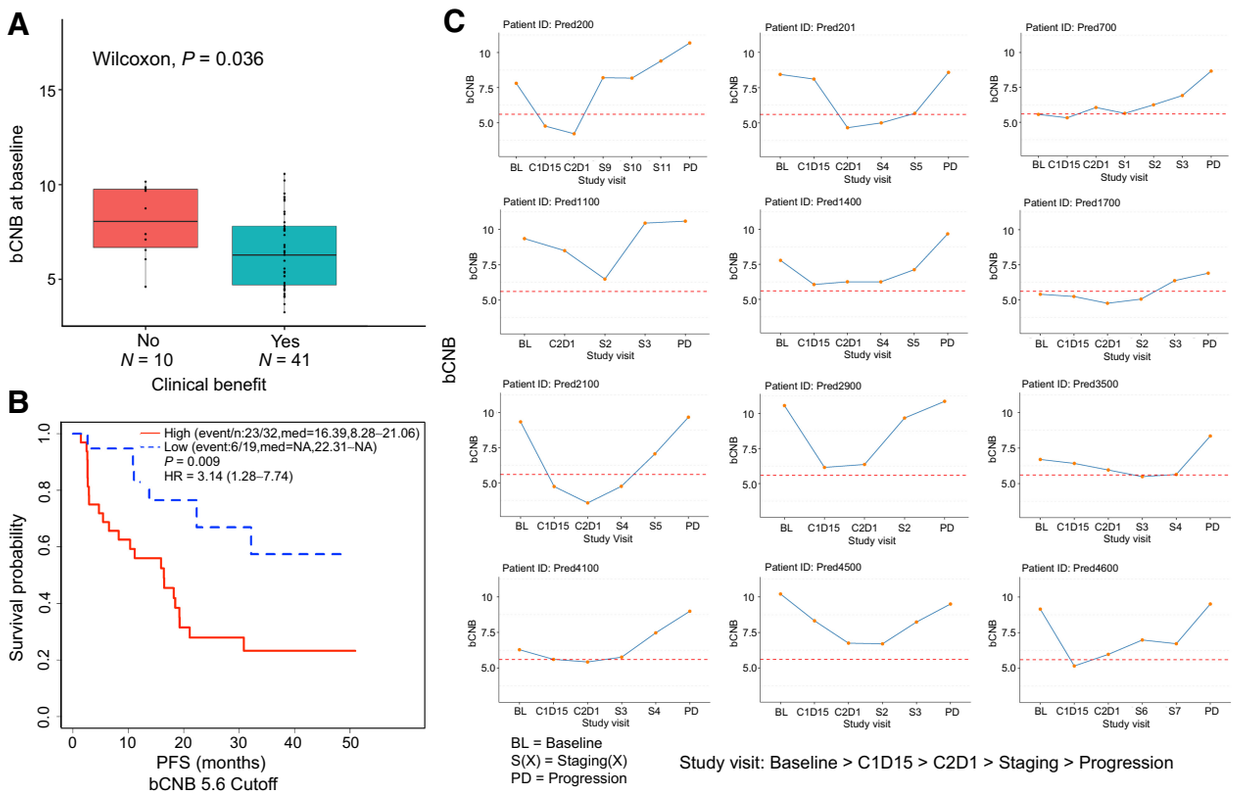

Figure 6.

bCNB scores predict patient outcomes and increase before radiographic detection of clinical progression. bCNB scores were generated for all 51 patients at baseline and during treatment via sequencing with PredicineCNB. **A,** Baseline bCNB scores were significantly higher in patients who experienced progression within 6 months (without clinical benefit; Wilcoxon test). **B,** High baseline bCNB scores were significantly associated with shorter PFS (log rank test). **C,** Serial analysis of bCNB during treatment revealed decreases at C1D15 and/or C2D1, followed by increases that preceded imaging detection of progressive disease in 12 of 18 (66.7%) patients for whom staging blood samples were analyzed.

bCNB to enable a genome-wide evaluation of novel resistance mechanisms in patients with HR+/HER2− MBC receiving ET in combination with CDK4/6*i*. Specifically, we identified a subset of patients, defined by hypermutation (high bTMB) and increased CNV (high bCNB), in association with poor outcomes who require novel therapeutic strategies. In addition, PredicineWES+ expanded the detection of genomic alterations associated with resistance at baseline and progression. We also demonstrated that dynamic changes in LP-WGS-derived bCNB scores over the course of treatment preceded radiographic response and clinical progression in a subset of patients, identifying potential utility for response monitoring. To our knowledge, our study using noninvasive blood-based sequencing represents the most comprehensive evaluation of genome-wide ctDNA in this patient population, resulting in the generation of biological insights and a potential therapeutic hypothesis to improve clinical outcomes.

Importantly, bTMB and bCNB were determined using one 8-mL tube of whole blood in all patients with evaluable samples, indicating the feasibility from a clinical application standpoint. As expected, median bTMB was relatively low in this cohort (less than 2 MBp), a finding that aligns with prior studies that evaluated tTMB in patients with breast cancer and particularly patients with HR+ MBC (29). On the basis of the observed association of high bTMB with lack of clinical benefit and shorter PFS, we demonstrate a novel stratification tool with treatment implications. Multiple prior studies have demonstrated tTMB as predictive of response for patients treated with ICI monotherapy in other tumor types (19). However, defining optimal cut points based on utilization of different sequencing platforms, bioinformatics techniques, and methods for determining tTMB has been challenging. Therefore, it appears that optimal tTMB thresholds may vary across different tumor types (48). Therefore, we did not use an *a priori* bTMB threshold in the outcome analysis. Instead, multiple bTMB thresholds including the median (1.9 MBp), third quartile (3.8 MBp), and FDA-approved threshold of 10 MBp in tissue, were significantly associated with PFS. These findings reinforce the consistency of defining a hypermutated, resistant subset of patients with higher bTMB.

Interestingly, the patients with high bTMB in our cohort were enriched for dominant APOBEC mutational signatures. The APOBEC family of DNA editing enzymes generate mutations during a variety of normal biologic processes including innate and adaptive immune responses (49). However, upregulated "off target" activity of APOBEC enzymes has been identified as a major source of somatic mutations in a number of cancers resulting in distinctive mutational signatures (21, 44, 45). APOBEC signatures have previously been observed in a variety of hypermutated malignancies and are associated with response to ICI (29, 30, 32, 50). The observed enrichment of these signatures in high versus low bTMB HR+/HER2− patients in this cohort further underscores the identification of a biomarker-defined

subset of patients who may benefit from the incorporation of ICI therapy. Our study also identified several oncogenic pathways (e.g., Notch, PI3K, and RTK-RAS) associated with high bTMB as potential drug targets.

Our data also demonstrate an overlap between patients with high bTMB and endocrine resistance defined by ESMO criteria. Although patients with clinically defined endocrine resistance had similar median bTMB compared with patients with *de novo* MBC or patients with endocrine-sensitive disease, a majority of high bTMB cases were present in the endocrine-resistant cohort at baseline. Importantly, bTMB scores stratified PFS in the subgroup of patients with clinically defined endocrine resistance. Moreover, patients with *ESR1* mutations at baseline had higher bTMB scores compared with patients with wild-type ESR1. Clinically defined endocrine resistance, sites of metastatic disease on imaging, and other pathologic variables did not stratify baseline patients with worse prognosis, further supporting the need for novel biomarkers for risk stratification. Collectively, these findings underscore the potential for bTMB scores to define a subgroup of patients unlikely to respond to standard first-line therapy with CDK4/6*i* and ET, and these findings warrant the exploration of alternative combination treatment strategies including ICI.

Our findings indicate that novel treatment strategies are needed for high bTMB and high bCNB patients at baseline. Although our data do not define an optimal treatment approach, patients with high tTMB do not appear to be innately resistant to all cytotoxic therapies (51). The association of high tTMB with response to ICI based on the tissue agnostic approval of pembrolizumab for patients with high tTMB (defined at a threshold above 10 mutations/MBp) suggests a potential treatment approach (52). In the TAPUR and NIMBUS studies, a subset of patients with MBC and high tTMB across subtypes were durable responders (31, 53). However, in other non-biomarker selected populations, there has been no improvement in outcomes when adding ICI to chemotherapy (54). For this reason, evaluating the potential of incorporating ICI for HR+/HER2− patients with high bTMB, either as monotherapy or in combination, is needed. Preclinical data suggest that CDK4/6*i* enhances T-cell activation, increases tumor infiltration, and may have a synergistic effect with ICI therapy (55). Although chemotherapy for patients with HR+/HER2− MBC is typically reserved for impending organ failure or endocrine refractory disease, the optimal use of chemotherapy in this biologically defined cohort is currently unknown, and these patients may benefit from earlier incorporation of cytotoxic therapy. In addition, the potential for bCNB to precede clinical detection of disease recurrence demonstrates the need for interventional studies to determine whether early switching of therapy based on molecular progression of disease, as opposed to imaging progression, may improve clinical outcomes.

Using a genome-wide approach also identified many individual resistance alterations, validating previously implicated mechanisms and yielding discovery of novel candidate genes. In keeping with previous studies, baseline alterations in *RB1* and other genes previously associated with *de novo* resistance to ET + CDK4/6*i* therapy were associated with shorter PFS, as were novel baseline alterations in *DSP*, *MUC16*, *PLCG1*, *USH2A*, and *ZFHX3*. Although median levels of bTMB and bCNB were not significantly increased at the time of clinical progression relative to baseline, we observed enrichment of individual alterations previously implicated in endocrine and/or CDK4/6*i* treatment resistance including *AR*, *AURKA*, *CCND1*, *CDKN2A*, *ESR1*, *FGFR1*, *MYC*, and *RB1* (47). We also observed enrichment of alterations in genes less commonly associated with ET and CDK4/6*i* treatment resistance, which encode a variety of oncogenic proteins, including *CBL*, a member of the RING finger ubiquitin ligase family that regulates RTK signaling (56–58); *KMT2D*, a methyltransferase involved in estrogen receptor recruitment and activation (59); *MUC12*, a glycosylated transmembrane protein in the mucin family implicated in the regulation of proliferation, invasion, and metastatic potential (60–62); and *PREX2*, a guanine nucleotide exchange factor that regulates cancer cell motility and invasion (63, 64). Many of these novel alterations are not covered by targeted sequencing panels, underscoring the value of the extended WES to identify diverse mechanisms of resistance.

Although WES extended the gold standard of TMB measurement to blood and led to the discovery of novel candidate resistance mechanisms in this study, the feasibility of applying it in clinical practice is limited at this time due to its high cost. However, we observed high correlation between bTMB measurements obtained by WES and targeted sequencing panels, illustrating the potential for measuring bTMB in the clinic using cost-effective targeted NGS panels. Furthermore, we demonstrated that bCNB, derived from more cost-effective PredicineCNB, illustrated a high degree of concordance with bTMB at baseline and was also associated with poor patient outcomes. We also demonstrated the potential of serial bCNB for monitoring dynamic changes in ctDNA levels during treatment. bCNB declined as early as 2 weeks after treatment initiation to provide an early signal of molecular response to therapy. In addition, when comparing concurrent imaging and bCNB assessment, an increase in bCNB preceded clinical progression of disease in two-thirds of patients. In the future, serial blood-based molecular assessments may serve as a surrogate for PFS, which is now standardly assessed via imaging.

There are several limitations to the study. First, some patients on study had not progressed, and therefore the landscape of alterations at progression may be less reflective of patients with long-term response to therapy. Second, our study utilized an alternative dosing regimen of palbociclib, and therefore it is unknown how this dosing may have affected the development of resistance alterations. Third, concurrent blood and tissue biopsies were not performed in most cases. In the future, we plan to externally validate our findings that high bTMB is associated with lack of response to ET + CDK4/6*i*, perform concurrent blood and tissue TMB testing to compare bTMB and tTMB, examine subclonal changes using serial WES on therapy, and evaluate novel treatment strategies for these patients in prospective interventional studies.

In summary, our study demonstrates the potential utility of blood-based bCNB and bTMB assessment in treatment decision making for patients with HR+/HER2− MBC. Furthermore, this study demonstrates the potential of utilizing whole-genome ctDNA analysis to comprehensively define the molecular mechanisms of baseline and serial resistance to CDK4/6*i* combined with ET for patients with HR+/HER2− MBC. This work defines a subset of patients at baseline with poor outcome to standard-of-care first-line therapy and also demonstrates a noninvasive approach for detecting early blood-based progression. Further work is needed to validate our findings and to define optimal treatments for these hypermutated, genomically complex patients, including the potential incorporation of early ICI and combination therapy to evaluate the clinical utility of bTMB and bCNB in this setting.

Authors' Disclosures

A.A. Davis reports other support from Pfizer, Inc. and Biotheranostics outside the submitted work. T. Zheng reports ownership of Predicine, Inc. stock. X. Dong reports employment with Predicine, Inc. and ownership of Predicine, Inc. stock. F.O. Ademuyiwa reports grants and personal fees from Pfizer, Gilead, and AbbVie; personal fees from AstraZeneca, Biotheranostics, Cardinal Health,

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Authors' Contributions

A.A. Davis: Conceptualization, formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **J. Luo:** Formal analysis, investigation, writing–review and editing. **T. Zheng:** Formal analysis, visualization. **C. Dai:** Formal analysis, methodology, writing–review and editing. **X. Dong:** Formal analysis, visualization, writing–review and editing. **L. Tan:** Project administration. **R. Suresh:** Writing–review and editing, enrolled patients onto the clinical trial. **F.O. Ademuyiwa:** Writing–review and editing, enrolled patients onto the clinical trial. **C. Rigden:** Writing–review and editing, enrolled patients onto the clinical trial. **T.P. Rearden:** Writing–review and editing, enrolled patients onto the clinical trial. **K. Clifton:** Writing–review and editing, enrolled patients onto the clinical trial. **K. Weilbaecher:** Writing–review and editing, enrolled patients onto the clinical trial. **A. Frith:** Writing–review and editing, enrolled patients onto the clinical trial. **P.K. Tandra:** Writing–review and editing, study coordinator for the clinical trial. **T. Summa:** Writing–review and editing, study coordinator for the clinical trial. **B. Haas:** Writing–review and editing, study coordinator for the clinical trial. **S. Thomas:** Project administration, writing–review and editing. **L.F. Hernandez-Aya:** Writing–review and editing, enrolled patients onto the clinical trial. **L.L. Peterson:** Writing–review and editing, enrolled patients onto the clinical trial. **X. Wang:** Supervision, methodology. **S.J. Luo:** Supervision, methodology. **K. Zhou:** Methodology. **P. Du:** Supervision, methodology, writing–review and editing. **S. Jia:** Conceptualization, writing–review and editing. **B.L. King:** Conceptualization, supervision, writing–original draft, writing–review and editing. **J. Krishnamurthy:** Conceptualization, project administration, writing–review and editing, helped design the original clinical trial. **C.X. Ma:** Conceptualization, resources, supervision, funding acquisition, investigation, writing–review and editing.

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Note

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