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ORIGINAL REPORT

Association of Cell-Free DNA Tumor Fraction and Somatic Copy Number Alterations With Survival in Metastatic Triple-Negative Breast Cancer

Daniel G. Stover, Heather A. Parsons, Gavin Ha, Samuel S. Freeman, William T. Barry, Hao Guo, Atish D. Choudhury, Gregory Gydush, Sarah C. Reed, Justin Rhoades, Denisse Rotem, Melissa E. Hughes, Deborah A. Dillon, Ann H. Partridge, Nikhil Wagle, Ian E. Krop, Gad Getz, Todd R. Golub, J. Christopher Love, Eric P. Winer, Sara M. Tolaney, Nancy U. Lin, and Viktor A. Adalsteinsson

Author affiliations and support information (if applicable) appear at the end of this article.

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D.G.S., H.A.P., and G.H. contributed equally to this work.

V.A.A. and D.G.S. jointly directed this work.

Corresponding author: Viktor A. Adalsteinsson, PhD, Broad Institute of Harvard and MIT, 415 Main St, Cambridge, MA 02412; e-mail: viktor@broadinstitute.org.

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ABSTRACT

Purpose

Cell-free DNA (cfDNA) offers the potential for minimally invasive genome-wide profiling of tumor alterations without tumor biopsy and may be associated with patient prognosis. Triple-negative breast cancer (TNBC) is characterized by few mutations but extensive somatic copy number alterations (SCNAs), yet little is known regarding SCNAs in metastatic TNBC. We sought to evaluate SCNAs in metastatic TNBC exclusively via cfDNA and determine if cfDNA tumor fraction is associated with overall survival in metastatic TNBC.

Patients and Methods

In this retrospective cohort study, we identified 164 patients with biopsy-proven metastatic TNBC at a single tertiary care institution who received prior chemotherapy in the (neo)adjuvant or metastatic setting. We performed low-coverage genome-wide sequencing of cfDNA from plasma.

Results

Without prior knowledge of tumor mutations, we determined tumor fraction of cfDNA for 96.3% of patients and SCNAs for 63.9% of patients. Copy number profiles and percent genome altered were remarkably similar between metastatic and primary TNBCs. Certain SCNAs were more frequent in metastatic TNBCs relative to paired primary tumors and primary TNBCs in publicly available data sets The Cancer Genome Atlas and METABRIC, including chromosomal gains in drivers *NOTCH2*, *AKT2*, and *AKT3*. Prespecified cfDNA tumor fraction threshold of $\geq 10\%$ was associated with significantly worse metastatic survival (median, 6.4 *v* 15.9 months) and remained significant independent of clinicopathologic factors (hazard ratio, 2.14; 95% CI, 1.4 to 3.8; $P < .001$).

Conclusion

We present the largest genomic characterization of metastatic TNBC to our knowledge, exclusively from cfDNA. Evaluation of cfDNA tumor fraction was feasible for nearly all patients, and tumor fraction $\geq 10\%$ is associated with significantly worse survival in this large metastatic TNBC cohort. Specific SCNAs are enriched and prognostic in metastatic TNBC, with implications for metastasis, resistance, and novel therapeutic approaches.

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ASSOCIATED CONTENT

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Data Supplement
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INTRODUCTION

Triple-negative breast cancer (TNBC) makes up 10% to 15% of all breast cancers yet accounts for more than one third of breast cancer–related deaths.[1-5] TNBC is defined by lack of expression of therapeutic targets human epidermal growth factor receptor 2 (HER2) and estrogen receptor alpha (ERα), and chemotherapy remains the mainstay of treatment.[4,6,7] Extensive recent efforts have defined clinicopathologic, genomic, and transcriptomic features of primary TNBC (pTNBC).[2,5,8-18] pTNBC is defined by relatively few somatic single-nucleotide variants and indels (approximately one mutation per megabase).[2,18,19] However, pTNBC demonstrates frequent loss of *TP53* and genomic instability with widespread somatic copy number alterations (SCNAs), implicating a critical role of SCNAs in TNBC tumorigenesis.[2,9,10]

Although a few studies have begun to interrogate the genomic features of metastatic TNBC (mTNBC),[20-27] there have been no analyses of large cohorts of patients with mTNBC published to date.

Cell-free DNA (cfDNA) is shed into the circulation by both normal and malignant cells, and next-generation sequencing analysis of cfDNA offers minimally invasive genomic profiling of tumor alterations without tumor biopsy. Prior applications of cfDNA have focused on tracking specific mutations[28-33] or sequencing targeted panels of cancer-related genes.[22,34-38] Building on others' work demonstrating the feasibility of genome-wide copy number analysis from plasma in patients with cancer,[21,39,40] we developed an algorithm, ichorCNA,[38] to profile SCNAs and quantify tumor fraction (TFx) via low-coverage (0.1×) whole-genome sequencing of cfDNA, without the need for prior knowledge of tumor mutations. Here, we evaluate the association of cfDNA TFx with survival and use cfDNA as a comprehensive biopsy surrogate to study the genomics of a disease infrequently biopsied in clinical practice, identifying key SCNAs that are enriched and prognostic in mTNBC.

PATIENTS AND METHODS

Patient Identification and Clinicopathologic Data

Consecutive, nonoverlapping patients with metastatic biopsy-proven TNBC enrolled on ongoing clinical data and biospecimen banking protocols for metastatic breast cancer (DFCI#09-204, n = 97; and DFCI#05-246, n = 10) or collected as part of two clinical trials for patients with metastatic TNBC (DFCI#12-024 [ruxolitinib; ClinicalTrials.gov identifier: NCT01562873], n = 14; and DFCI#12-431 [cabozantinib; ClinicalTrials.gov identifier: NCT01738438[41]], n = 37) were identified for analyses. TNBC was defined as < 5% staining for estrogen receptor (ER) and progesterone receptor and human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) 0 to 1+ and/or HER2:Cep17 fluorescent in situ hybridization ratio < 2.0. Clinicopathologic data were abstracted from the medical record. Survival events were determined from medical record or Social Security Death Index. *BRCA1* or *BRCA2* germline mutation status was ascertained by medical record review for the receipt of Clinical Laboratory Improvement Amendments (CLIA)-approved germline testing. All patients received chemotherapy in the adjuvant, neoadjuvant, or metastatic setting before first blood draw used in analyses. Use of patients' clinicopathologic data were institutional review board approved, and all patients provided written consent.

Sample Processing and DNA Extraction

Venous blood samples were collected in EDTA (BD, Franklin Lakes, NJ), CellSave Preservative (Cell Search, Raritan, NJ), or Cell-Free DNA BCT (Streck, Omaha, NE) tubes.[42] Blood processing to component parts within 4 hours of collection, cell-free DNA extraction from plasma, and DNA quantification were performed as described previously.[38] For metastatic biopsy samples, > 50% tumor was confirmed via hematoxylin and eosin staining of fresh frozen samples and then DNA extracted using Qiagen AllPrep DNA kit (Qiagen, Germantown, MD).

Ultra-Low-Pass Whole-Genome Sequencing

Library construction of cfDNA was performed using the Kapa HyperPrep kit with custom adapters (IDT, Coralville, IA). Three to 20 ng of cfDNA input (median, 5 ng), or approximately 1,000 to 7,000 haploid genome equivalents, was used for ultra-low-pass whole-genome sequencing. Constructed sequencing libraries were pooled (2 μL of each × 96 per pool) and sequenced using 100-bp paired-end runs over 1 × lane on a HiSeq2500 (Illumina, San Diego, CA) to average genome-wide fold coverage of 0.1×. Segment copy number and TFx were derived via ichorCNA.[38] Samples were excluded if the median absolute deviation of copy ratios ($2^{\log2\ ratio}$) between adjacent bins, genome-wide, was > 0.20, suggesting poor-quality sequence data.

Identification of TNBC Samples in Publicly Available Data Sets

Patients with triple-negative breast cancer were identified in The Cancer Genome Atlas (TCGA[18]; n = 166) and METABRIC[1] (n = 277) on the basis of study-reported negative for the ER and progesterone receptor via IHC and HER2-receptor copy number diploid (GISTIC2.0 value of 0) or IHC 0 to 1. If ER status was not available, ER status was inferred from RNA expression data.[43]

Gene-Level Copy Number Analyses

GISTIC2.0[44,45] output was used for all gene-level copy number analyses. Segmented data files derived from ichorCNA for mTNBC cfDNA and publicly available segmented data for METABRIC[1] were purity and ploidy corrected, then input into GISTIC2.0[44,45] with amplification/deletion threshold $\log_2$ratio > 0.3, confidence level 0.99, and Q-value threshold 0.05. Genes were defined as gain (GISTIC value 1; corresponds to three copies) or amplification (GISTIC value 2; corresponds to four or more copies) versus diploid (GISTIC value 0). TCGA GISTIC2.0 copy number data were obtained from cBioPortal.[46,47]

Statistical Analyses and Data Visualization

All statistical analyses and data visualizations were performed in R version 3.3.1. Contrasts in patient and tumor characteristics were evaluated using Pearson's χ^2 tests. The association of TFx to continuous and categorical clinicopathologic factors was evaluated using Wilcoxon rank-sum and χ^2 test or analysis of variance, respectively. Correlation of cfDNA yield and TFx from independently processed same-day blood draw samples was calculated using interclass correlation coefficient. Performance of cfDNA relative to paired metastatic biopsy—including sensitivity and specificity with biopsy considered truth—was computed across 1-Mb bins. Correlation among bin-level copy number calls for all samples was calculated using Spearman correlation coefficient, and hierarchical clustering was performed using average linkage.

Comparison of Primary Versus Metastatic TNBC

For principal component analysis (PCA), gene-level cfDNA GISTIC copy number calls were projected onto the METABRIC TNBC PCA coordinate basis and visualized using ggbiplot.[48] Comparison in frequency of gain/amplification (*v* no gain) and loss/deletion (*v* no loss) between metastatic and primary samples was calculated using Fisher's exact test. All frequency calculations of copy number calls across the genome were multiple-testing corrected using Benjamini–Hochberg procedure for false discovery rate. Volcano plots were generated using ggplot2 package,[49] CoMut plots were visualized with GenVisR package,[50] and genome-wide significance plot using qqman package.[51]

Survival Analyses

All Kaplan-Meier plots were generated using packHV package.[52] For baseline clinicopathologic characteristics, survival was defined as time from metastatic diagnosis and significance evaluated by log-rank test. For cfDNA variables, including line of metastatic therapy at blood draw, first blood draw, and highest TFx blood draw, survival was defined as time from blood draw. Univariate and multivariable Cox proportional hazards models were calculated using the survival package.

RESULTS

mTNBC Cohort

We identified 506 plasma samples from 164 patients with biopsy-proven mTNBC collected between August 2010 and

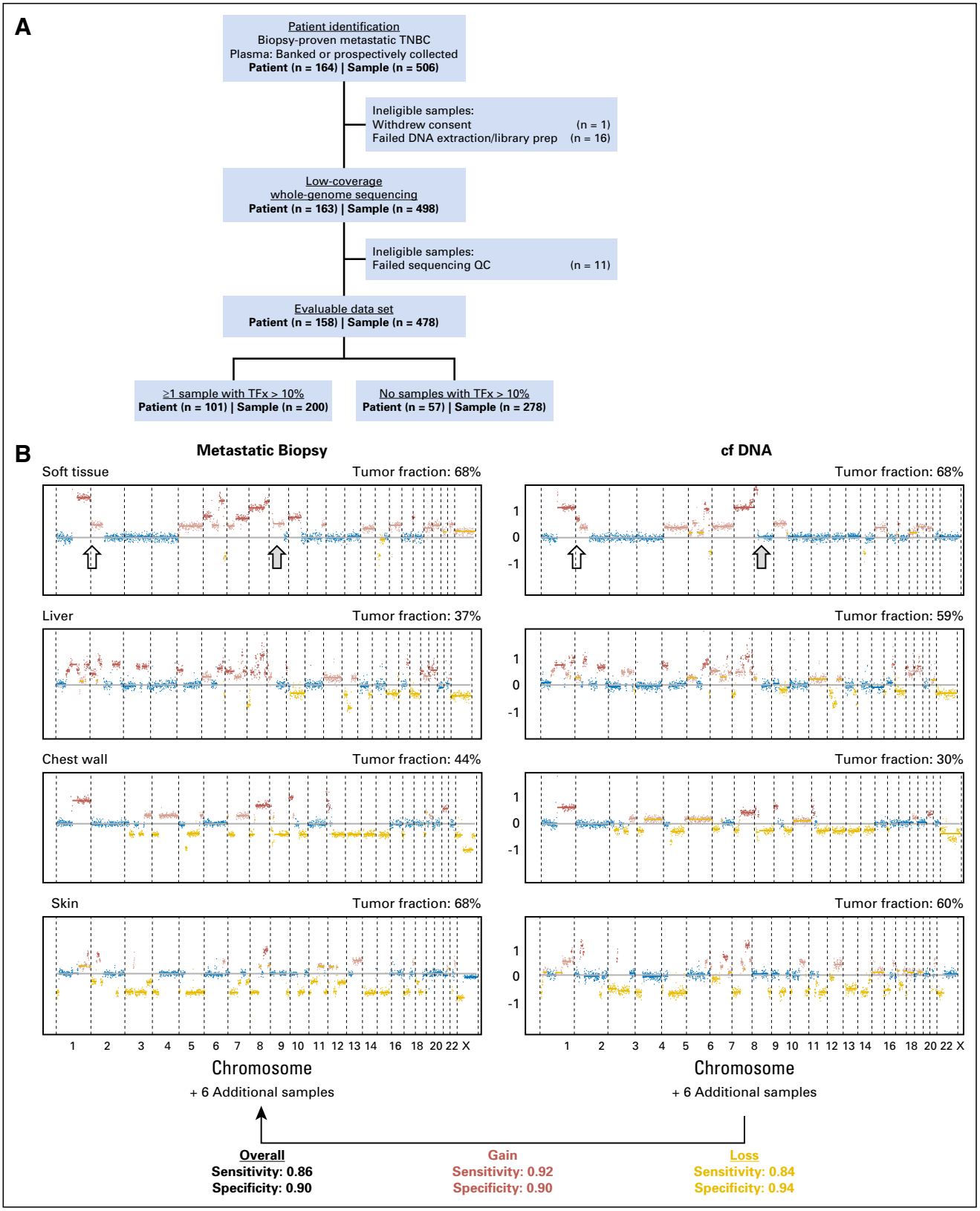

Fig 1. Genome-wide copy number profiles in cell-free DNA (cfDNA) are highly concordant with metastatic biopsy specimens. (A) REporting recommendations for tumor MARKer prognostic studies (REMARK) diagram. (B) Copy number plots of four representative pairs of metastatic biopsy (left panels) and cfDNA (right panels) with copy number (log2 ratio) indicated on the *y*-axis and chromosome on the *x*-axis. Sensitivity and specificity of tumor biopsy somatic copy number alterations detected in cfDNA (n = 10 pairs) are indicated for overall, gain, or loss. Examples of private somatic copy number alterations present in cfDNA but not metastatic biopsy (top panels, white arrow) and conversely metastatic biopsy but not cfDNA (top panels, gray arrow) are indicated. TFx, tumor fraction; TNBC, triple-negative breast cancer.

November 2016 under institutional review board–approved protocols at a single institution and abstracted detailed clinicopathologic information (Fig 1A; Table 1). All patients received chemotherapy before blood collection, with most patients having received neoadjuvant or adjuvant anthracycline and taxane-based chemotherapy. The median time to follow-up from metastatic diagnosis was 17 months (range, 0 to 82 months). Overall, this cohort reflects similar trends to other analyses of mTNBC, including worse prognosis for patients initially diagnosed with stage III relative to lower stage (I or II) disease and improved prognosis for patients with germline *BRCA1* or *BRCA2* mutations (Appendix Fig A1, online only).

Copy Number Is Highly Concordant With Metastatic Biopsy Secimens and Reflects Distinct Subsets of mTNBCs

Low-coverage whole-genome sequencing provided evaluable sequencing data for 478 (94.5%) samples that subsequently underwent copy number analysis and TFx determination via ichorCNA.[38] TFx could be determined for 158 of 164 patients (96.3%); 337 of 478 evaluable samples (70.5%) had detectable tumor DNA above the lower limit of detection (TFx ≥ 3%). One hundred one of 158 evaluable patients (63.9%) had at least one sample with TFx ≥ 10%, the prespecified proportion of tumor DNA adequate for high-confidence copy number calls on the basis of extensive prior benchmarking.[38] Patients with maximum TFx ≥ 10% had similar clinicopathologic characteristics relative to patients with maximum TFx < 10% (Table 1).

Table 1. Cohort Clinicopathologic Characteristics

Characteristic	All Patients (n = 164)	Tumor Fraction ≥ 10% (n = 101)	Tumor Fraction < 10% (n = 63)	*P*
Age at primary diagnosis, by decade				.30
< 40 years	34 (21)	26 (26)	8 (13)	
40-50 years	62 (38)	36 (36)	26 (41)	
50-60 years	45 (27)	27 (27)	18 (29)	
> 60 years	20 (12)	10 (10)	10 (16)	
Unknown	3 (2)	2 (2)	1 (2)	
Race				.49
White	147 (90)	93 (92)	54 (86)	
Nonwhite	13 (8)	6 (6)	7 (24)	
Unknown	4 (2)	2 (2)	2 (3)	
BRCA1/2 germline mutation status				.20
Mutant	24 (15)	17 (17)	7 (11)	
Wild type	112 (68)	69 (68)	43 (68)	
Unknown	28 (17)	15 (15)	13 (21)	
Primary receptor status				.24
HER2-positive	6 (4)	3 (3)	3 (5)	
HR-positive, HER2-negative	23 (14)	13 (13)	10 (16)	
Indeterminate	9 (5)	6 (6)	3 (5)	
HR-negative, HER2-negative	126 (77)	79 (78)	47 (75)	
Metastatic receptor status				1.0
HR-negative, HER2-negative	164 (100)	101 (100)	63 (100)	
AJCC stage at primary diagnosis				.80
I	22 (13)	14 (14)	8 (13)	
II	80 (49)	50 (50)	30 (48)	
III	43 (26)	25 (25)	18 (29)	
IV	16 (10)	11 (11)	5 (8)	
Unknown	3 (2)	1 (1)	2 (3)	
Adjuvant chemotherapy				.34
Anthracycline based	9 (5)	4 (4)	5 (8)	
Taxane based/other	11 (7)	9 (9)	2 (3)	
Anthracycline plus taxane based	119 (73)	74 (73)	45 (71)	
Metastatic at diagnosis/unknown	25 (15)	14 (14)	11 (18)	
Lines of metastatic therapy, median (range), No.	4 (1-11)	4 (1-11)	4 (1-9)	.76
Vital status				.04
Alive	51 (31)	25 (25)	26 (41)	
Deceased	113 (69)	76 (75)	37 (59)	

NOTE. Data presented as No. (%) unless otherwise noted.
Abbreviations: AJCC, American Joint Committee on Cancer Staging; HER2, human epidermal growth factor receptor 2; HR, hormone receptor.

We and others have demonstrated robust concordance of copy number and mutation between metastatic biopsy specimens and paired cfDNA.[38,53] As confirmation in this data set, we performed low-coverage sequencing of metastatic biopsy samples obtained at disease progression with concurrent plasma (range, 0 to 7 days from biopsy; n = 10 pairs). We compared copy number of 1-megabase segments across the genome using ichorCNA. Altered segments in the tumor biopsy specimen were detected in cfDNA with high sensitivity (0.86) and specificity (0.90), and, as anticipated, overlap was not identical,[22,54] with instances of private SCNAs present in cfDNA (Fig 1B).

TNBC is a heterogeneous disease comprising distinct subtypes.[8,55] To investigate patterns of chromosomal alterations, we compared genome-wide copy number profiles for all cfDNA samples with TFx ≥ 10%. Hierarchical clustering revealed two main copy number clusters, with cluster1 significantly enriched for patients with mTNBC whose primary receptor status was non-TNBC (χ^2 P = .007; Appendix Figs A2A-A2C, online only). We observed that the gene-level copy number profile of cluster2 tumors closely mirrors basal-like IntClust10 pTNBCs in METABRIC[1] (Appendix Figs A2D-A2E). Principal component analysis of METABRIC gene-level copy number data revealed high concordance of cfDNA cluster2 with basal-like METABRIC IntClust10 and cfDNA cluster1 with non-IntClust10 (nonbasal) pTNBCs (Appendix Fig A2F), although formal IntClust designation requires concurrent gene expression analysis.[1]

Copy Number Gains in Drivers NOTCH2, AKT2, *and* AKT3 *Are Enriched in mTNBCs Relative to pTNBCs*

We hypothesized that chemoresistant mTNBCs would be enriched for specific SCNAs relative to chemotherapy-naïve pTNBCs, including alterations potentially involved in drug resistance and/or metastasis. We determined gene-level SCNA status via GISTIC2.0[44,45] for the highest TFx (≥ 10%) cfDNA sample per patient with mTNBC (n = 101; Appendix Figs A3A and A3B, online only). We then identified 20 patients with mTNBC with at least one cfDNA sample with TFx ≥ 10% whose primary tumor underwent targeted panel sequencing[56] as part of clinical management. The median time between primary sample and metastatic cfDNA was 26 months (interquartile range, 11 to 38 months) with 18 of 20 primary tumors resected. We compared frequency of gain or loss for 25 cancer-related genes commonly altered in breast cancer between primary tumor panel sequencing and metastatic low-coverage cfDNA sequencing. Four genes demonstrated greater frequency of gain in mTNBC versus pTNBC samples (*NOTCH2* on 1p,

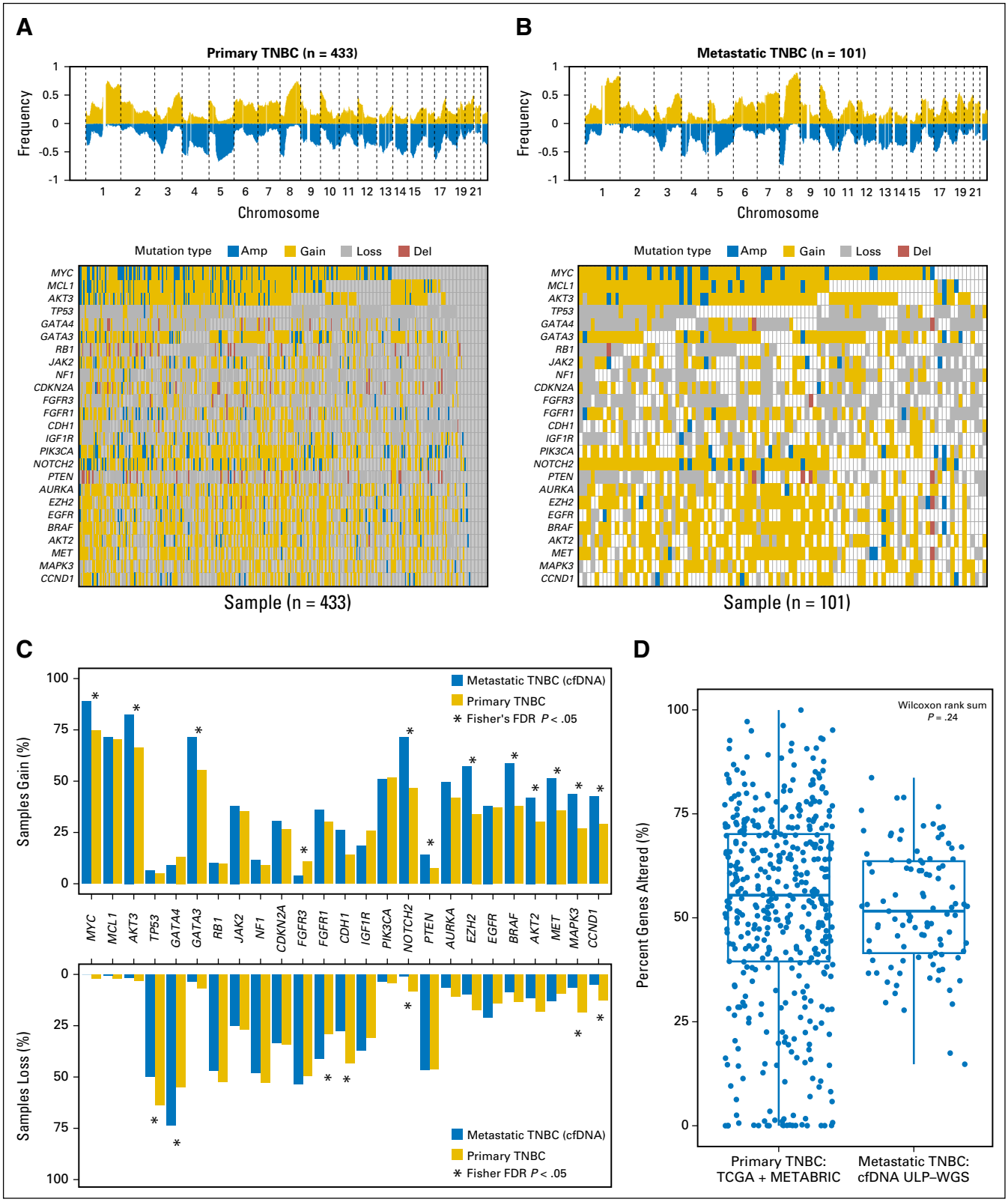

Fig 2. Metastatic triple-negative breast cancers (TNBCs) demonstrate enrichment of driver and targetable copy number alterations. (A) Gene-level copy number alterations in primary TNBCs from METABRIC and The Cancer Genome Atlas (TCGA), and (B) from metastatic TNBCs from cell-free DNA (cfDNA). The frequency of gene-level copy number gains (red) or losses (blue) across the genome (top panel) and per-sample copy number alteration for 25 breast cancer–related genes (bottom panel). (C) Percentage of samples with gain (top panel) or loss (bottom panel) for 25 breast cancer–related genes in primary (gold) versus chemoresistant metastatic TNBCs (blue). Genes with significant alteration in metastatic TNBC (Fisher's exact false discovery rate adjusted [FDR] $P < .05$) indicated by asterisk. (D) Percent of genes altered in primary TNBCs versus chemoresistant metastatic TNBCs. ULP-WGS, ultra-low-pass whole-genome sequencing.

AKT3 on 1q, *GATA3* on 10p, *AKT2* on 19q; Fisher's exact $P < .05$), whereas four genes demonstrated single copy loss more frequently in pTNBC than mTNBC (*CDKN2A* on 9p, *PTEN* on 10q, *RB1* on 13q, *NF1* on 17q; Fisher's exact $P < .05$; Appendix Figs A3C and A3D).

To evaluate SCNA differences in a large number of primary versus metastatic TNBCs, we identified pTNBCs in publicly available data sets METABRIC[1] and TCGA[18] (total, n = 433) and determined gene-level copy number status in both data sets via GISTIC2.0 to facilitate uniform comparison. Overall, altered regions were remarkably concordant between pTNBC and mTNBC (Figs 2A and 2B); however, mTNBCs demonstrated greater SCNA frequency of both commonly altered regions (1q, 7q, 8q) and less commonly altered regions (11q, 18q, 19p; Appendix Fig A3E). A subset of genes was altered more frequently in mTNBC relative to pTNBC, including high-frequency (> 50% of samples) gains in *MYC* (8q), *AKT3* (1q), *GATA3* (10p), *NOTCH2* (1p), *EZH2* (7q), *BRAF* (7q), and *MET* (7q; Fisher's exact, genome-wide false discovery rate (FDR) correction $P < .05$; Figs 2A-2C; Data Supplement). Four genes were enriched in mTNBC relative to pTNBC both in paired samples and across cohorts: gains in *GATA3* and drivers *NOTCH2*, *AKT2*, and *AKT3*. Interestingly, the genome-wide percentage of genes altered was not significantly increased in mTNBC relative to pTNBC, although there was greater heterogeneity among primary tumors (Fig 2D).

Chromosomal Gains of 18q11 and 19p13 Are Associated With Poor Survival in mTNBC

Little is known regarding genomic determinants of TNBC metastatic survival. Focusing on mTNBC-enriched SCNAs (Fig 3A), we calculated the Cox proportional hazard ratio of each gene for metastatic survival (Fig 3B; Data Supplement). Only a subset of mTNBC-enriched loci were prognostic in the metastatic setting. Unexpectedly, the loci most strongly associated with poor metastatic

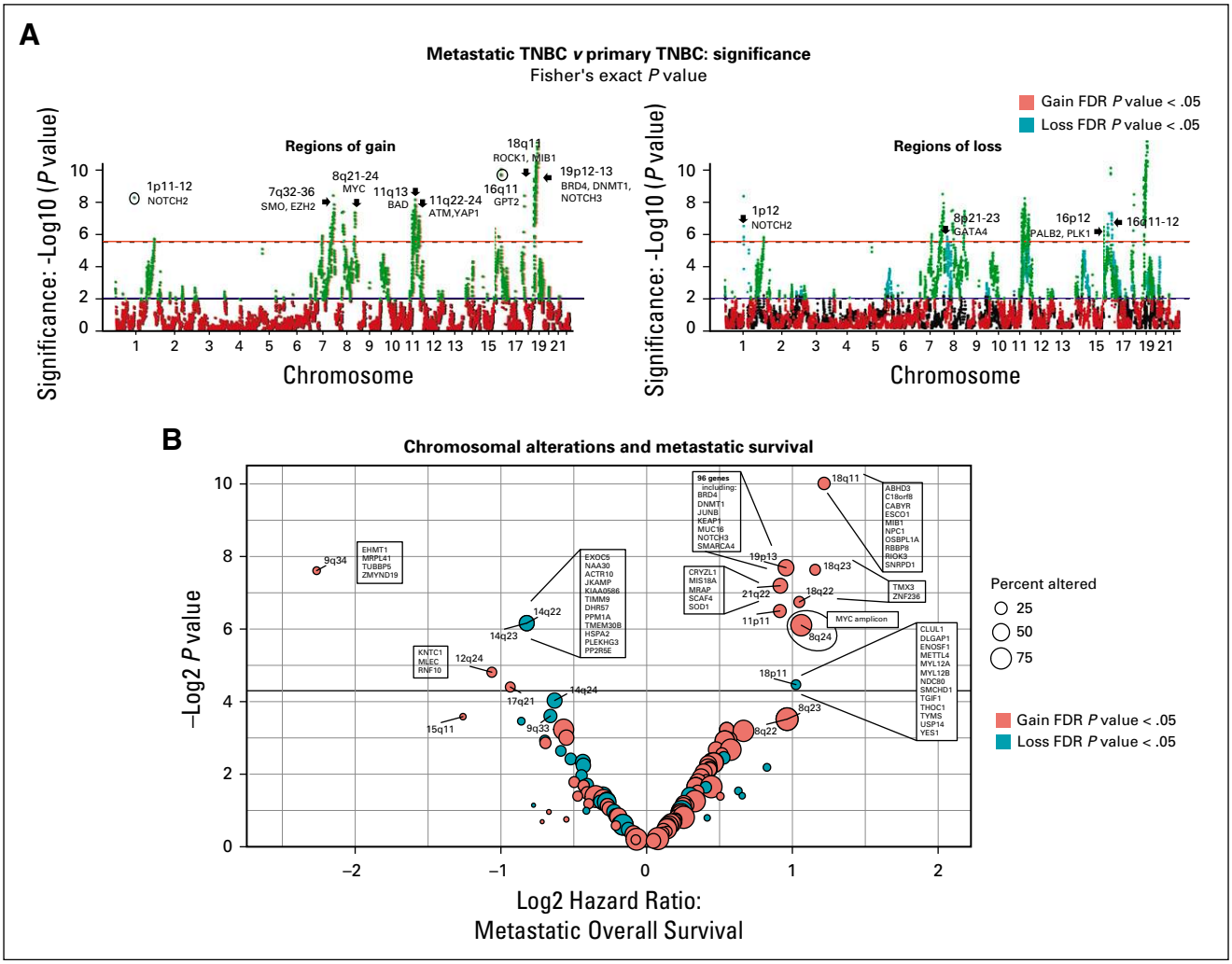

Fig 3. Prognostic copy number alterations in metastatic triple-negative breast cancer (mTNBC). (A) Significance (Fisher exact *P* value) of gene-level alteration of gain versus no gain (left panel) or loss versus no loss (right panel) across the genome. Associations with false discovery rate adjusted (FDR)-adjusted *P* value < .05 indicated in green (gain in light blue, loss in pink) above solid line. Gold line indicates nominal *P* value < .01, blue line indicates Bonferroni corrected $P < .05$ (nominal *P* value < .001). (B) Volcano plot of negative $\log_{10}$ hazard ratio for overall metastatic survival from highest tumor fraction blood draw by chromosomal cytoband versus hazard ratio significance. Only cytobands significantly gained (light blue) or lost (pink) in mTNBC relative to primary TNBC are plotted (Fisher exact FDR $P < .05$; corresponding segments in A). Size of individual point indicates the frequency altered among patients with mTNBC. Genes listed are those with expression likely altered by chromosomal alteration.

survival, 18q11 and 19p13, have never previously associated with TNBC survival. More than half of mTNBCs harbored gain/amplification of 18q11, 19p13, or both, significantly more frequent than in pTNBCs (χ^2 $P < .001$; Appendix Fig A4A, online only). Gain/amplification of both 18q11 and 19p13 was strongly associated with worse survival in univariate analyses and multivariable Cox proportional hazard models including clinicopathologic factors and TFx (hazard ratio, 3.30; 95% CI, 1.30 to 8.38; $P = .012$) and was also associated with poor prognosis in pTNBCs (log-rank $P = .038$; Fig A4B-A4E).

Tumor Fraction of cfDNA Is an Independent Prognostic Biomarker in mTNBC

Our approach offers a tumor fraction calculation on the basis of SCNAs detected in cfDNA without a priori knowledge of tumor mutation status.[38] To evaluate reproducibility, two plasma samples were drawn in a single venipuncture and fractionated in independent laboratories for 11 patients. Data showed high TFx concordance of paired samples (intraclass correlation coefficient = 0.984) and nearly identical copy number profiles despite variable cfDNA yield (Appendix Fig A5A-A5D, online only).

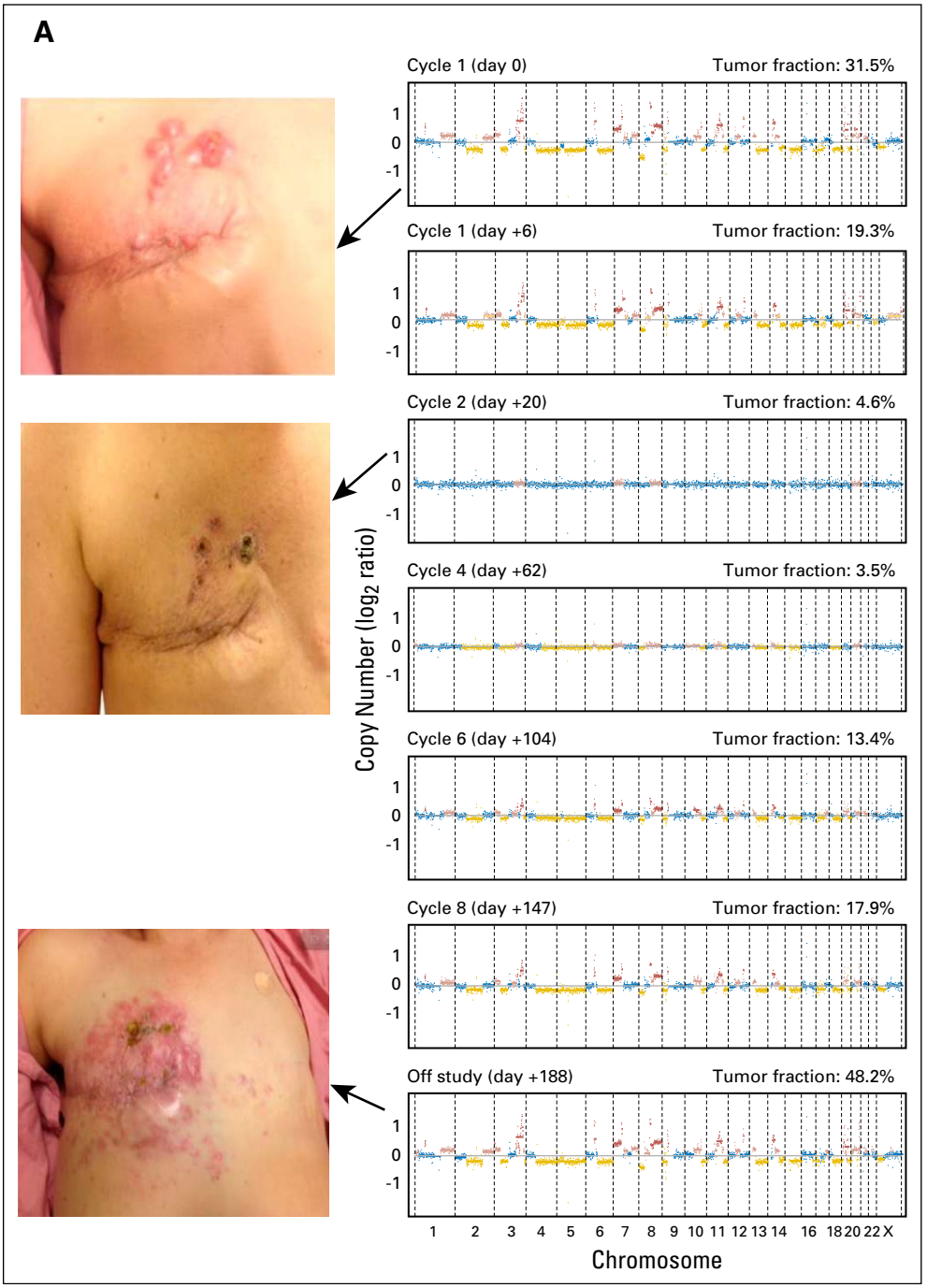

Fig 4. Cell-free DNA tumor fraction is an independent prognostic biomarker in metastatic triple-negative breast cancer. (A) Representative copy number plots and tumor fraction (TFx) demonstrating dynamic range of TFx within an individual patient whose chest wall disease initially responded then recurred on clinical trial of cabozantinib[41] with associated drop then rebound in TFx. (B) Scatter plot of individual sample TFx measurements for all samples (center), first blood sample collected per patient (left), and maximum TFx per patient (right). Boxplots indicate 25th to 75th percentiles, with median indicated by central line and whiskers representing 1.5 times interquartile range. (C) Kaplan-Meier curve of overall metastatic survival from first blood draw for patients with metastatic triple-negative breast cancer stratified by TFx of first blood draw. (D) Multivariable Cox proportional hazards model of overall metastatic survival from first blood draw. HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

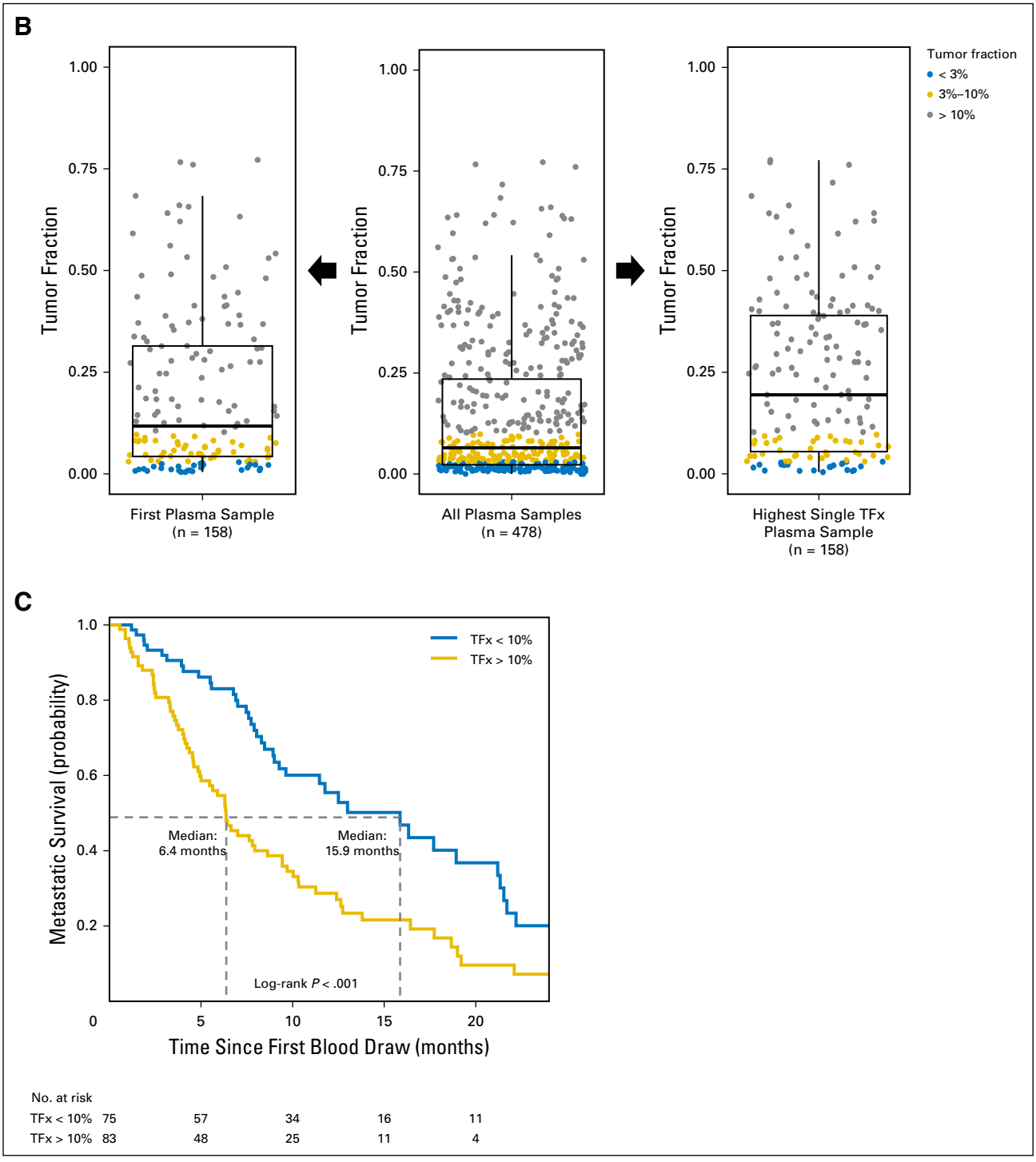

Fig 4. (Continued).

Tumor fraction measurement using ichorCNA has a broad dynamic range, both within individuals and among distinct patients. Within-patient TFx variability is illustrated by a single patient on a clinical trial of cabozantinib,[41] who demonstrated a TFx nadir of 3.5% while responding to therapy with a maximum TFx of 48.2% at progression (Fig 4A). Evaluating a first sentinel blood draw, this cohort demonstrated a diverse range of TFx from < 3% to 77.2% (Fig 4B). We hypothesized that metastases to more highly vascular organs could be associated with higher TFx, and indeed the presence of liver metastasis was associated with significantly higher TFx in both the sentinel draw and maximum TFx draw (Appendix Fig A6A-A6B, online only), remaining significant when adjusting for characteristics in a multivariate model (Appendix Fig A6A-A6B).

D

Multivariable Cox Proportional Hazards Model

Variables	Hazard Ratio	95% CI		*P*
		Lower	Upper	
Tumor fraction > 10%	2.14	1.40	3.28	< .001
BRCA status				
BRCA status unknown	ref	ref	ref	ref
BRCA wild-type	1.23	0.63	2.43	.546
BRCA mutant	0.75	0.29	1.97	.561
Primary receptor status				
Indeterminate	ref	ref	ref	ref
HR positive/HER2 negative	0.51	0.15	1.71	.275
HER2	0.90	0.20	4.12	.896
TNBC	1.08	0.38	3.03	.888
Primary stage at diagnosis				
Stage I	ref	ref	ref	ref
Stage II	0.74	0.39	1.41	.357
Stage III	1.36	0.70	2.66	.362
Stage IV	0.56	0.22	1.46	.236
Age at primary diagnosis (per decade > 40 years)	0.77	0.58	1.01	.058
Year sample collected	0.86	0.60	1.22	.400
Sample collection cohort	1.06	0.94	1.19	.342
Line of metastatic therapy at blood draw	1.12	0.97	1.28	.122
Number of samples per patient	1.00	1.00	1.01	.379

Fig 4. (Continued).

Allele fraction of tumor mutations detected in cfDNA is suggested to be a prognostic for metastatic breast cancer.[28] We evaluated prognostic association of TFx at a prespecified threshold ≥ 10% on the basis of > 2,400 tumor/normal in silico admixtures of varying sequencing coverage and tumor fractions that demonstrated optimal SCNA prediction performance at a tumor fraction ≥ 10%.[38] In this cohort, TFx ≥ 10% on a patient's first blood draw was associated with significantly shorter survival, median 6.4 months versus 15.9 months (log-rank $P < .001$; Fig 4C). TFx remained an independent prognostic factor in a multivariate Cox proportional hazards model (hazard ratio, 2.14; 95% CI, 1.40 to 3.28; $P < .001$; Fig 4D) and also in sensitivity analyses including only patients whose primary tumor was TNBC (n = 121) and with TFx as a continuous variable (Appendix Fig A6C-A6E).

DISCUSSION

We present the largest genomic characterization of mTNBC to our knowledge. Using a cfDNA-exclusive approach relevant for most patients with mTNBC, we demonstrate that TFx is a robust, minimally invasive independent prognostic biomarker in mTNBC. pTNBC and mTNBC exhibit remarkably similar copy number profiles, yet we identified known cancer drivers among SCNAs enriched in mTNBC relative to pTNBC.

Allele fraction of known mutations detected in cfDNA is suggested to be prognostic but is dependent on knowledge of existing tumor mutations and has not been evaluated in a large cohort of mTNBCs.[28] Our approach evaluates TFx without a priori tumor mutation status and is evaluable in the vast majority of patients with mTNBC. We demonstrate that TFx is a genomic biomarker for mTNBC independent of standard clincopathologic characteristics in a large modern cohort. Patients with higher tumor fraction (TFx ≥ 10%) had significantly inferior survival but showed no significant differences in baseline characteristics relative to patients with lower TFx. Patients with higher TFx were more likely to have documented liver metastases, potentially associated with highly vascular organs or distinct features of TNBC that metastasizes to the liver. In support of further testing of this approach in clinical practice, we will be launching a prospective cohort study to further investigate mTNBC TFx dynamics while on therapy and subsequent association with response to standard or experimental therapies. Future efforts may allow minimally invasive analysis of clinically relevant mutational signatures, such as homologous recombination deficiency or microsatellite instability.

Several cancer types have been shown to evolve with progressive collection of mutations over time and on therapy.[2,34,53] It has been hypothesized that primary tumors with genomic instability such as TNBC will collect immense numbers of genomic alterations in the metastatic setting after chemotherapy. Surprisingly, we demonstrate no significant difference in percent genome altered and remarkably similar patterns of chromosomal alterations when comparing more than 100 mTNBCs with more than 400 pTNBCs. This suggests that large-scale chromosomal events are rare in metastatic development and supports prior work demonstrating that most SCNAs occur early in tumorigenesis in TNBCs.[57,58]

Despite few large-scale SCNA changes between primary and metastatic tumors, we identify certain loci enriched in mTNBCs relative to paired primary and/or large cohorts of pTNBCs. We identify a novel association of 18q11 and 19p13 gains with metastatic survival that is independent of both clinicopathologic factors as well as TFx. Gain or amplification of both regions identifies a subset of TNBC rapid progressors with remarkably poor survival in the metastatic and also the primary setting. Both 18q11[59] and 19p13[60,61] include known breast cancer risk loci.[59,60] 19p13 is associated with increased breast cancer risk, specifically among *BRCA1* mutation carriers,[60] and associated specifically with ER-negative[61] and TNBC[60] in the general population. An assessment of focal events, recently shown to be a driving force in prostate cancer,[54] might lead to identification of additional prognostic SCNAs.

Our study involved a modern cohort representing current standard treatment approaches, with 86% of patients without distant metastasis at diagnosis having received anthracycline and taxane-based (neo)adjuvant chemotherapy. Over the first 20 months from metastatic diagnosis, patients initially diagnosed with stage III disease are more likely to die as a result of their disease relative to patients diagnosed with stage I or II or de novo metastatic disease, supporting prior epidemiologic studies.[5] Patients with germline *BRCA1*/2 mutations have improved prognosis. The patients in our cohort were relatively young, with more than half of the patients' primary diagnoses before age 50 years, primarily wild-type for germline *BRCA1*/2 (15% with documented mutation), and most patients were white, an important limitation of this study.

In summary, we illustrate a framework for minimally invasive genomic characterization of metastatic cancer and subsequent integration with clinicopathologic data and patient outcomes. This analysis provides the most comprehensive genomic profile of metastatic TNBC SCNAs to date, to our knowledge, and suggests that determining cfDNA TFx via a blood test provides important prognostic information beyond standard clinicopathologic factors. This approach has the potential to reveal clinically useful biomarkers while identifying unique genomic features of metastatic cancer and may advance our understanding of metastasis, drug resistance, and novel therapeutic targets.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

AUTHOR CONTRIBUTIONS

Conception and design: Daniel G. Stover, Heather A. Parsons, Gavin Ha, Atish D. Choudhury, Ann H. Partridge, Ian E. Krop, Todd R. Golub, J. Christopher Love, Eric P. Winer, Sara M. Tolaney, Nancy U. Lin, Viktor A. Adalsteinsson
Provision of study materials or patients: Nancy U. Lin
Collection and assembly of data: Daniel G. Stover, Heather A. Parsons, Gavin Ha, Samuel S. Freeman, Gregory Gydush, Sarah C. Reed, Denisse Rotem, Melissa E. Hughes, Deborah A. Dillon, Nikhil Wagle, Ian E. Krop, Sara M. Tolaney, Nancy U. Lin, Viktor A. Adalsteinsson
Data analysis and interpretation: Daniel G. Stover, Heather A. Parsons, Gavin Ha, Samuel S. Freeman, William T. Barry, Hao Guo, Atish D. Choudhury, Sarah C. Reed, Justin Rhoades, Nikhil Wagle, Gad Getz, Todd R. Golub, J. Christopher Love, Eric P. Winer, Sara M. Tolaney, Nancy U. Lin, Viktor A. Adalsteinsson
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

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Affiliations

Daniel G. Stover, Ohio State University Comprehensive Cancer Center, Columbus, OH; **Heather A. Parsons**, **Gavin Ha**, **William T. Barry**, **Hao Guo**, **Atish D. Choudhury**, **Melissa E. Hughes**, **Deborah A. Dillon**, **Ann H. Partridge**, **Nikhil Wagle**, **Ian E. Krop**, **Todd R. Golub**, **Eric P. Winer**, **Sara M. Tolaney**, and **Nancy U. Lin**, Dana-Farber Cancer Institute; **Gad Getz**, Massachusetts General Hospital, Boston; **Gavin Ha**, **Samuel S. Freeman**, **Atish D. Choudhury**, **Gregory Gydush**, **Sarah C. Reed**, **Justin Rhoades**, **Denisse Rotem**, **Nikhil Wagle**, **Gad Getz**, **Todd R. Golub**, and **Viktor A. Adalsteinsson**, Broad Institute of Harvard and Massachusetts Institute of Technology; and **J. Christopher Love**, Massachusetts Institute of Technology, Cambridge, MA.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Association of Cell-Free DNA Tumor Fraction and Somatic Copy Number Alterations With Survival in Metastatic Triple-Negative Breast Cancer

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Daniel G. Stover
No relationship to disclose

Heather A. Parsons
No relationship to disclose

Gavin Ha
Patents, Royalties, Other Intellectual Property: Broad Institute (Inst)

Samuel S. Freeman
Patents, Royalties, Other Intellectual Property: Broad Institute (Inst)

William T. Barry
Research Funding: Pfizer (Inst)

Hao Guo
No relationship to disclose

Atish D. Choudhury
Honoraria: Bayer HealthCare Pharmaceuticals, Astellas Pharma
Research Funding: Janssen Oncology (Inst)

Gregory Gydush
No relationship to disclose

Sarah C. Reed
No relationship to disclose

Justin Rhoades
No relationship to disclose

Denisse Rotem
Consulting or Advisory Role: Ecosystems Pharmaceuticals (I)
Patents, Royalties, Other Intellectual Property: Patent with the Broad Institute (I), patent with Dana-Farber (I)

Melissa E. Hughes
No relationship to disclose

Deborah A. Dillon
Consulting or Advisory Role: Oncology Analytics

Ann H. Partridge
No relationship to disclose

Nikhil Wagle
Stock or Other Ownership: Foundation Medicine
Consulting or Advisory Role: Novartis, Grail
Research Funding: Novartis, Merck

Ian E. Krop
Employment: AMAG Pharmaceuticals (I)
Leadership: AMAG Pharmaceuticals (I)
Stock or Other Ownership: AMAG Pharmaceuticals (I)
Consulting or Advisory Role: Genentech, Seattle Genetics
Research Funding: Genentech

Gad Getz
Research Funding: IBM (Inst), Bayer (Inst)
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Todd R. Golub
Consulting or Advisory Role: Foundation Medicine
Research Funding: Calico Life Sciences, Bayer HealthCare Pharmaceuticals
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J. Christopher Love
Research Funding: Janssen Oncology

Eric P. Winer
Research Funding: Genentech

Sara M. Tolaney
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Research Funding: Genentech, Array BioPharma, GlaxoSmithKline, Kadmon, Novartis

Viktor A. Adalsteinsson
Patents, Royalties, Other Intellectual Property: Broad Institute (Inst), Massachusetts Institute of Technology (Inst)

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Appendix

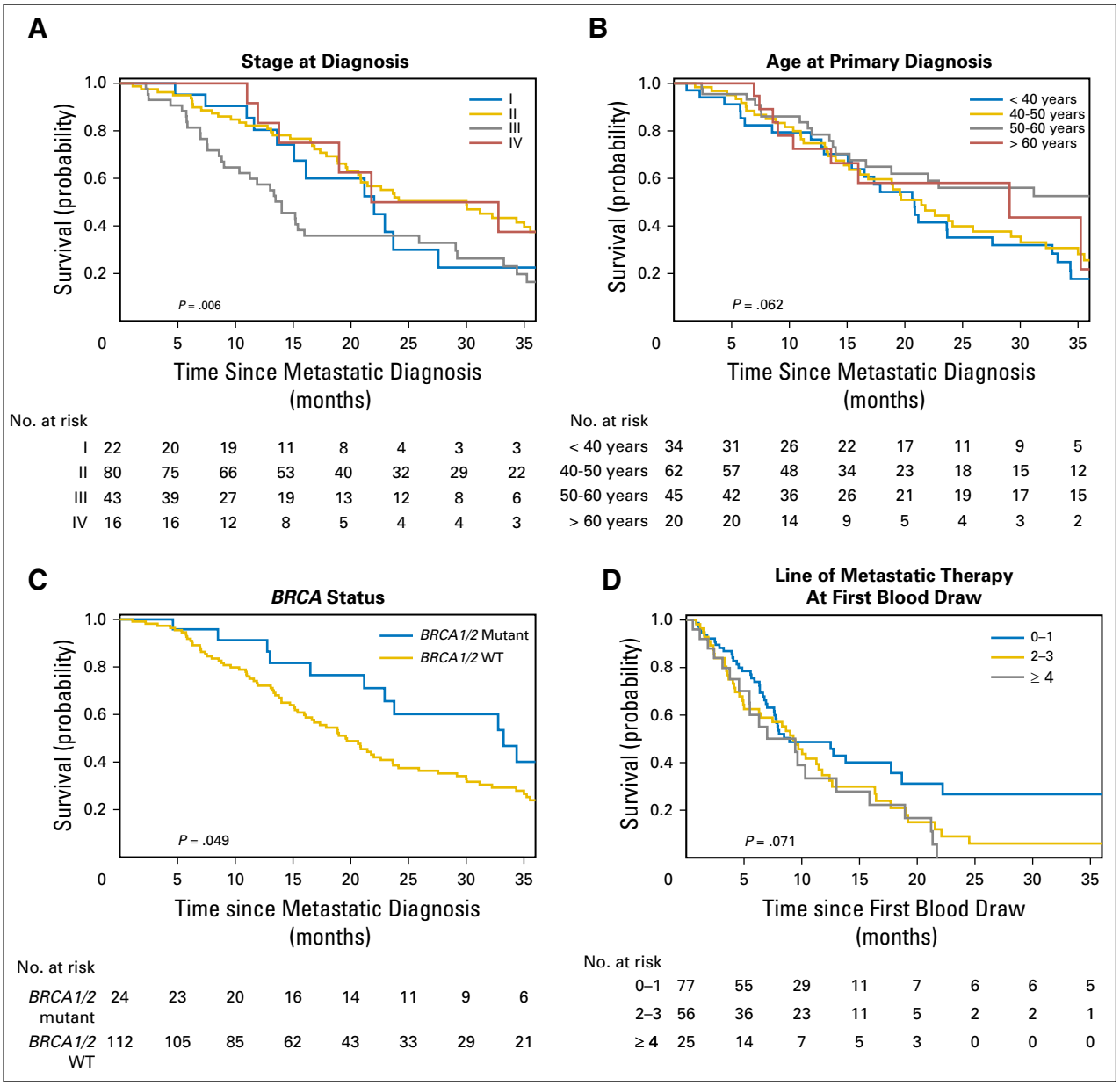
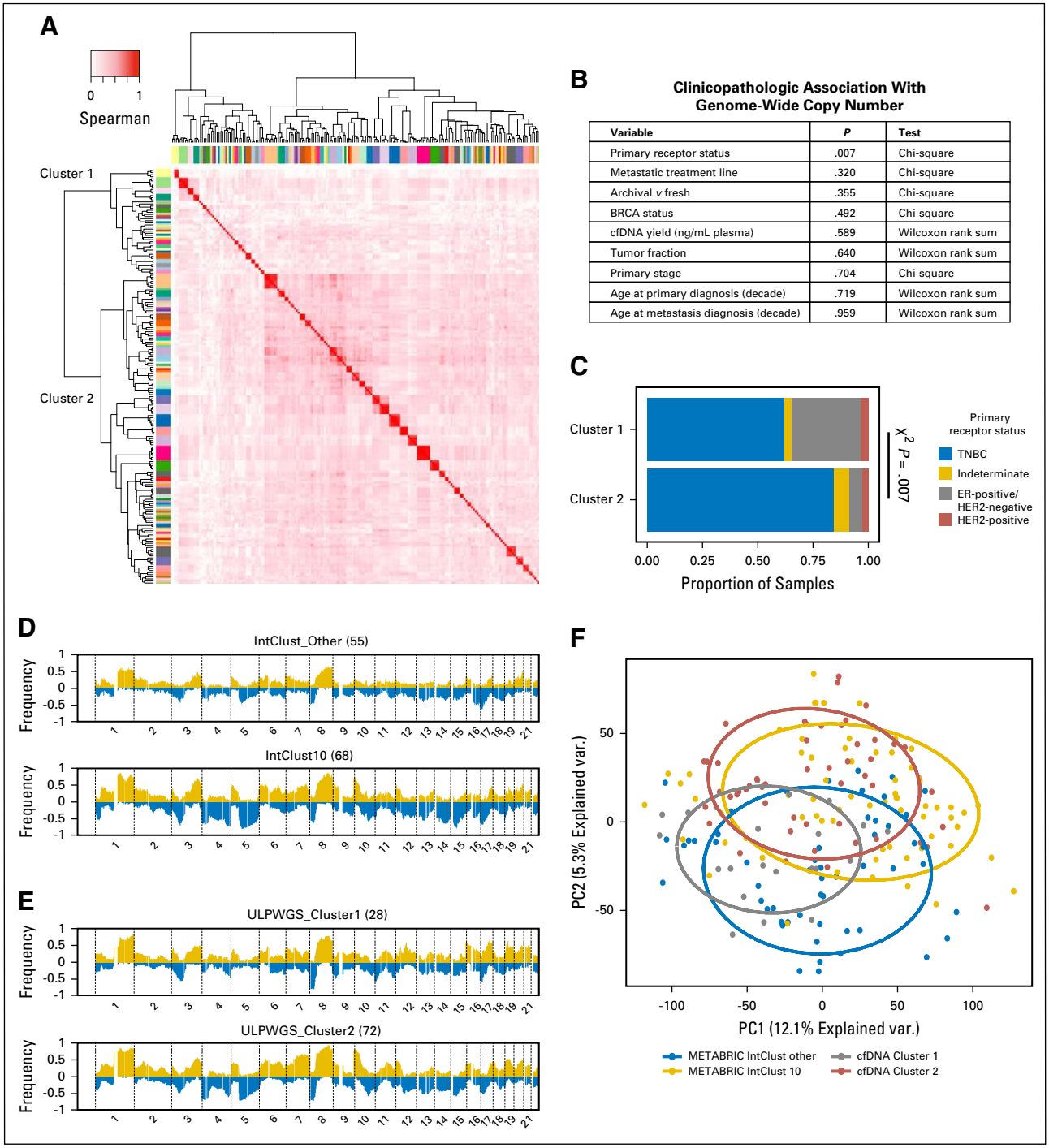


Fig A1. Metastatic triple-negative breast cancer cohort survival by baseline clinicopathologic characteristics. (A-C) Kaplan-Meier curves of survival from metastatic diagnosis stratified by stage at diagnosis (A), age at primary diagnosis by decade over 40 (B), and germline BRCA1/2 mutation status (C). (D) Kaplan-Meier curve of survival from first blood draw stratified by line of therapy in the metastatic setting. P-value indicates log-rank test.



Variable	P	Test
Primary receptor status	.007	Chi-square
Metastatic treatment line	.320	Chi-square
Archival *v* fresh	.355	Chi-square
BRCA status	.492	Chi-square
cfDNA yield (ng/mL plasma)	.589	Wilcoxon rank sum
Tumor fraction	.640	Wilcoxon rank sum
Primary stage	.704	Chi-square
Age at primary diagnosis (decade)	.719	Wilcoxon rank sum
Age at metastasis diagnosis (decade)	.959	Wilcoxon rank sum

Fig A2. Genome-wide copy number profiles across patients reveal high within-patient correlation and two distinct patterns of copy number alterations. (A) Unsupervised hierarchical clustering of correlation matrix of spearman rho for ichorCNA copy number call in 1 million base pair bins across the genome for all plasma samples with TFx 10%. Colors bars indicate unique patients. (B) Association of factors with metastatic TNBC Cluster. (C) Proportion of samples by primary receptor status of two largest clusters from. Chi-square test of independence on proportions indicated. (D) Gene-level copy number frequency plots of primary TNBCs from METABRIC, stratified by study-reported IntClust10 (bottom) versus other IntClust groups (top). (E) Gene-level copy number frequency plots of metastatic TNBCs from cfDNA, stratified by metastatic TNBC Cluster from Figure 1C. Gain and loss frequencies are shown in red and blue, respectively. Deletion frequencies are negated for comparison. (F) Principal component analysis (PCA) of gene-level copy number calls for METABRIC1 triple-negative breast cancers (n = 123) with projection of gene-level copy number calls for mTNBC from plasma (n = 101) onto the PCA coordinate basis. Samples were designated as basal-like IntClust10 versus other (non-basal) IntClust for METABRIC or Cluster1 versus Cluster2 for mTNBC.

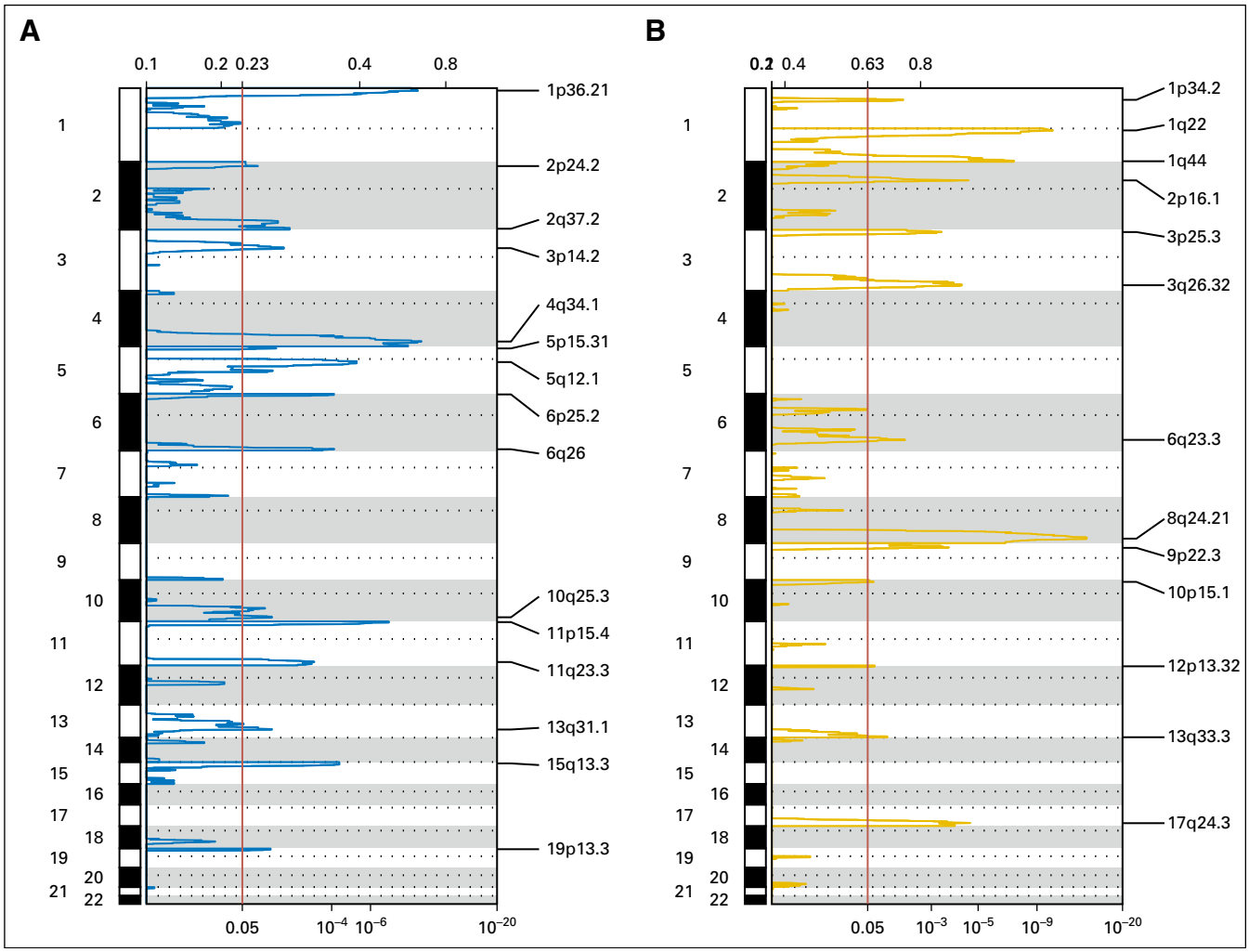


Fig A3. Copy number profiles of metastatic relative to primary TNBCs. (A-B) Regions of significant loss (A) and gain (B) from GISTIC2.0 analysis of metastatic TNBC copy number profiles derived from cfDNA. (C) Absolute difference in gene-level copy number alteration frequency across the genome in metastatic TNBC samples from cfDNA (n = 101) versus primary TNBCs (TCGA + METABRIC; n = 433). (D-E) Frequency of copy number gain or loss (D) and per-sample gene-level comparison (E) for 25 breast cancer-related genes in 20 patients with paired primary tumor and metastatic cfDNA copy number data. Genes with significant alteration (Fisher exact $P < .05$) in metastatic samples indicated by dot.

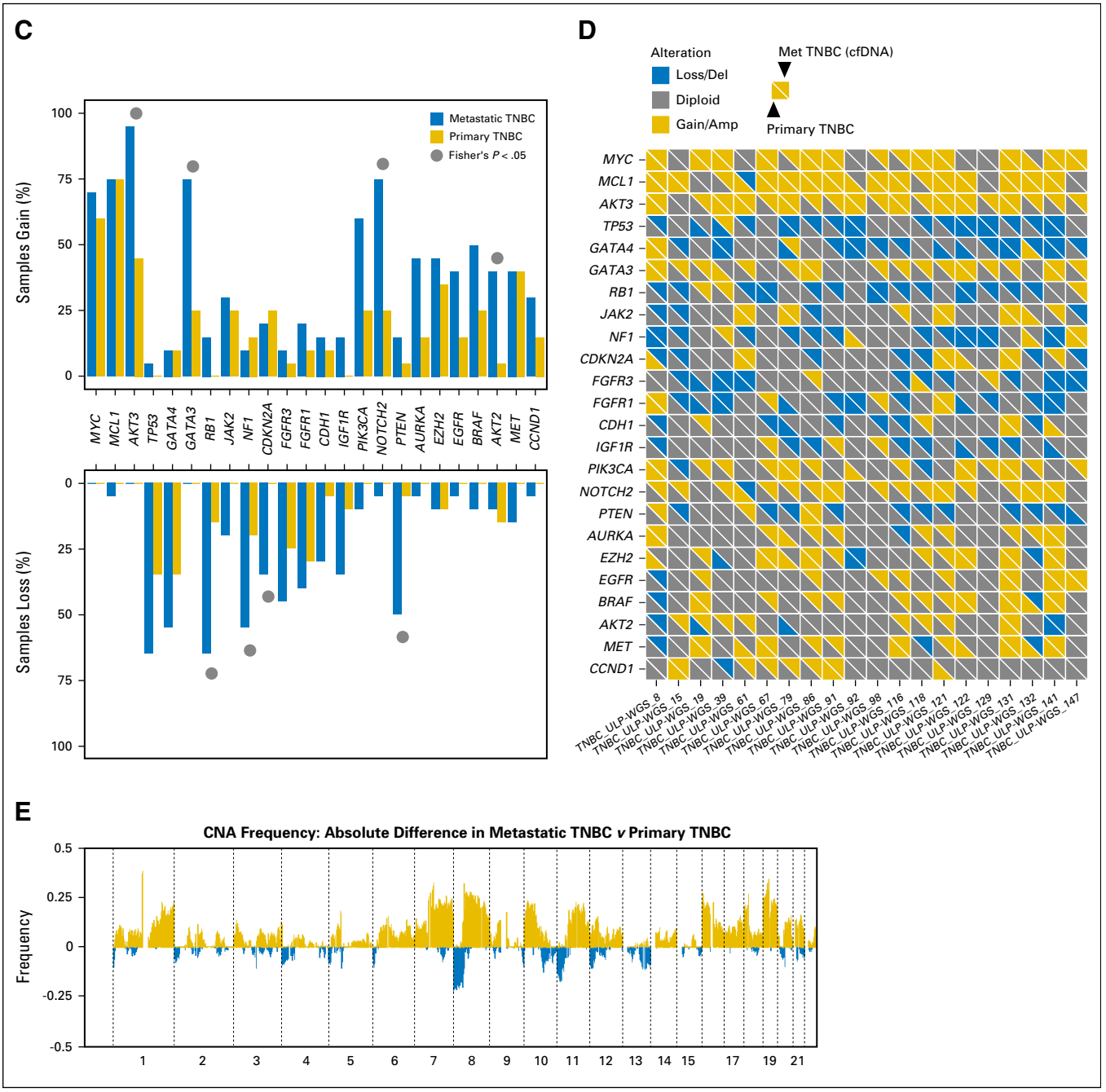
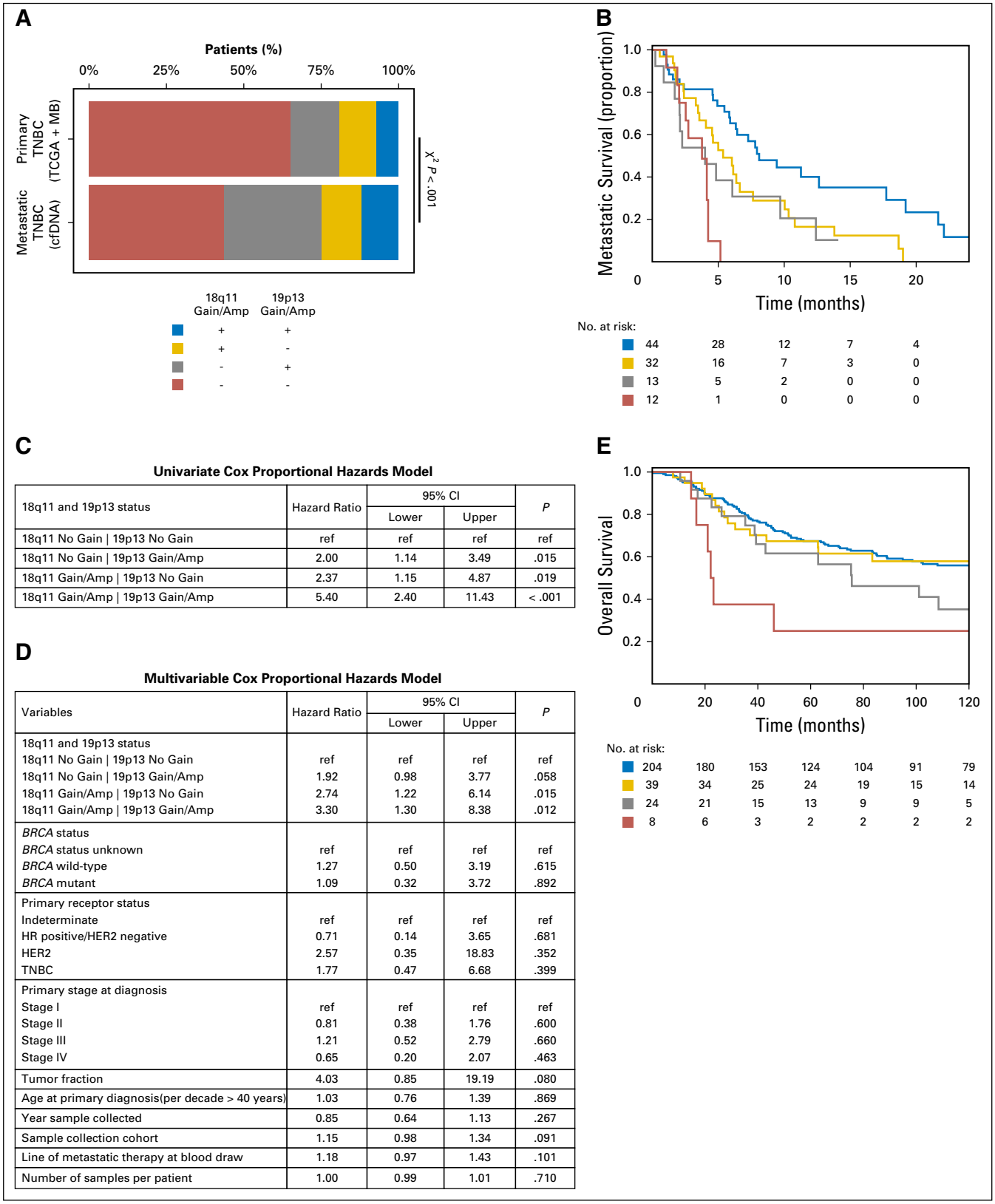


Fig A3. (Continued).



C

Univariate Cox Proportional Hazards Model

18q11 and 19p13 status	Hazard Ratio	95% CI		*P*
		Lower	Upper	
18q11 No Gain | 19p13 No Gain	ref	ref	ref	ref
18q11 No Gain | 19p13 Gain/Amp	2.00	1.14	3.49	.015
18q11 Gain/Amp | 19p13 No Gain	2.37	1.15	4.87	.019
18q11 Gain/Amp | 19p13 Gain/Amp	5.40	2.40	11.43	< .001

D

Multivariable Cox Proportional Hazards Model

Variables	Hazard Ratio	95% CI		*P*
		Lower	Upper	
18q11 and 19p13 status				
18q11 No Gain | 19p13 No Gain	ref	ref	ref	ref
18q11 No Gain | 19p13 Gain/Amp	1.92	0.98	3.77	.058
18q11 Gain/Amp | 19p13 No Gain	2.74	1.22	6.14	.015
18q11 Gain/Amp | 19p13 Gain/Amp	3.30	1.30	8.38	.012
BRCA status				
BRCA status unknown	ref	ref	ref	ref
BRCA wild-type	1.27	0.50	3.19	.615
BRCA mutant	1.09	0.32	3.72	.892
Primary receptor status				
Indeterminate	ref	ref	ref	ref
HR positive/HER2 negative	0.71	0.14	3.65	.681
HER2	2.57	0.35	18.83	.352
TNBC	1.77	0.47	6.68	.399
Primary stage at diagnosis				
Stage I	ref	ref	ref	ref
Stage II	0.81	0.38	1.76	.600
Stage III	1.21	0.52	2.79	.660
Stage IV	0.65	0.20	2.07	.463
Tumor fraction	4.03	0.85	19.19	.080
Age at primary diagnosis(per decade > 40 years)	1.03	0.76	1.39	.869
Year sample collected	0.85	0.64	1.13	.267
Sample collection cohort	1.15	0.98	1.34	.091
Line of metastatic therapy at blood draw	1.18	0.97	1.43	.101
Number of samples per patient	1.00	0.99	1.01	.710

Fig A4. Chromosomal gains of 18q11 and 19p13 are associated with poor survival in metastatic TNBC. (A) Proportion of patients with primary TNBC (TCGA + METABRIC, total n = 433) or metastatic TNBC (n = 101) with gain or amplification of 18q11, 19p13, or both. (B) Kaplan-Meier curve of overall metastatic survival from highest TFx blood draw for metastatic triple-negative breast cancer patients stratified by gain or amplification of 18q11, 19p13, or both. (C-D) Univariate (C) and multivariable (D) Cox proportional hazards model of overall metastatic survival from highest TFx blood draw. (E) Kaplan-Meier curve of overall survival for primary triple-negative breast cancer patients in METABRIC dataset stratified by gain or amplification of 18q11, 19p13, or both.

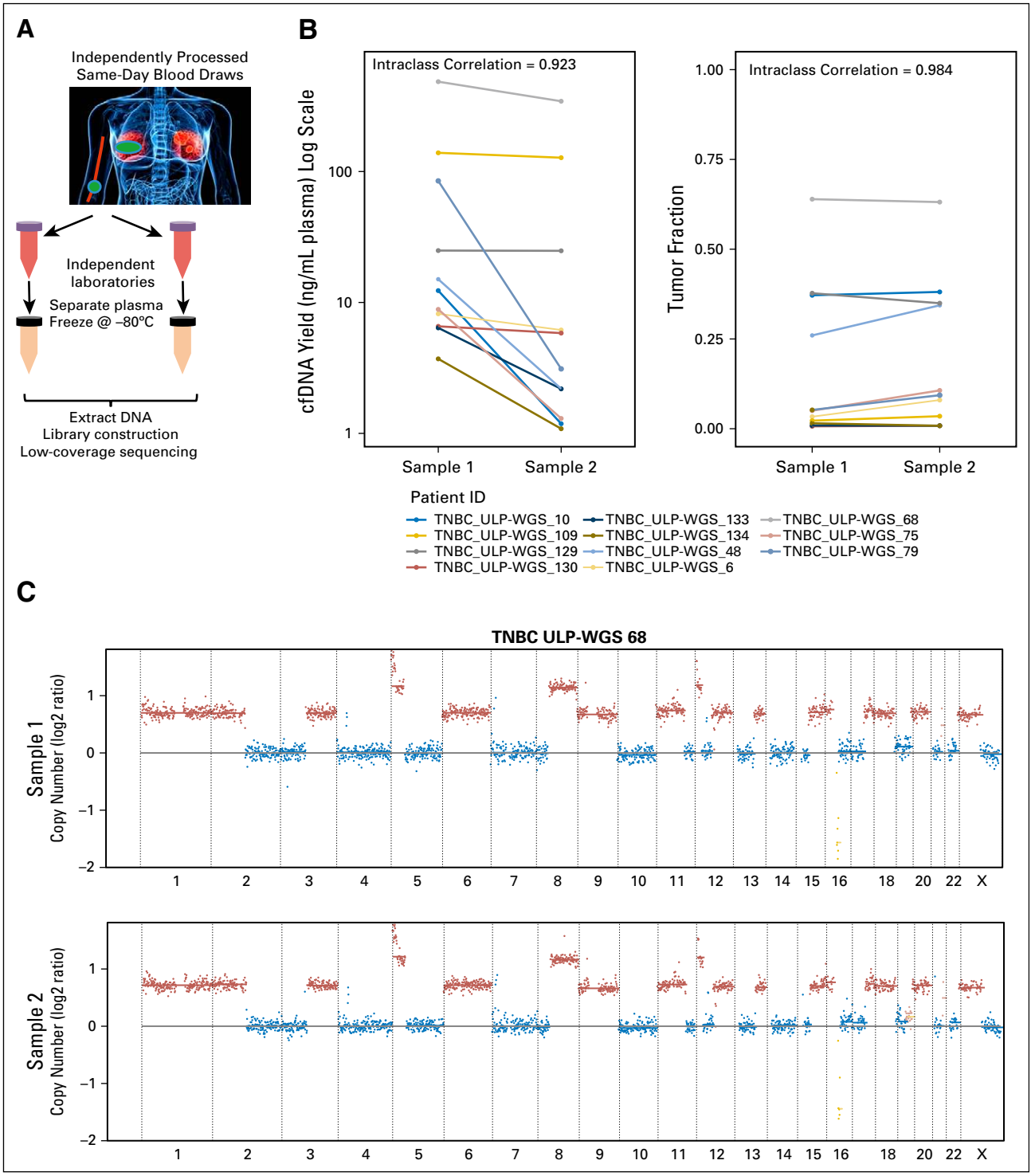


Fig A5. Tumor fraction and copy number profiles are reproducible from independent blood draws. (A) Schematic of independently processed same-day blood draws. Two separate blood tubes from a single venipuncture had plasma separated and were frozen in independent laboratories. Equivalent volumes of plasma then underwent DNA extraction, library construction, low-coverage sequencing, and TFx calculation via ichorCNA. (B) Total cell-free DNA yield (ng per mL plasma; left panel) and TFx (right panel) per patient. (C-D) Representative ichorCNA copy number plots from same-day blood draws for the two patients with the highest detected TFx.

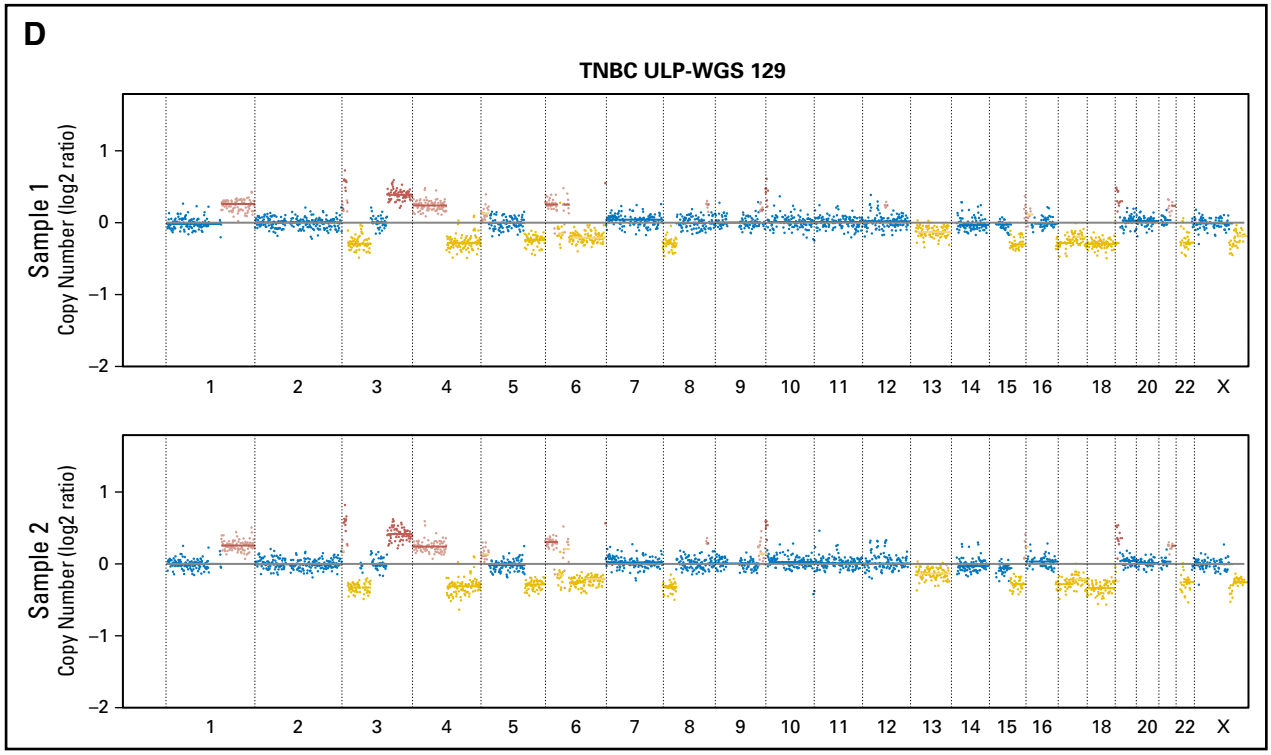


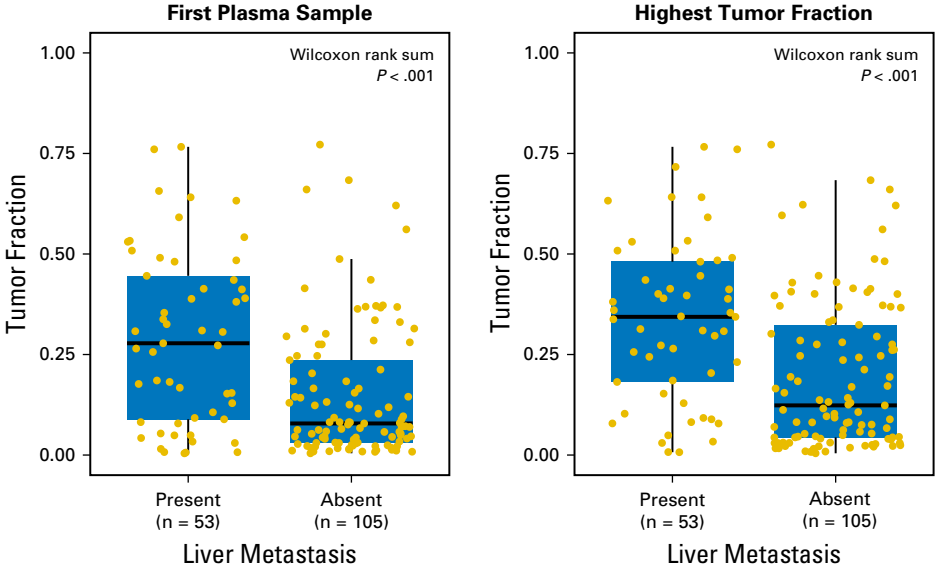
Fig A5. (Continued).

A

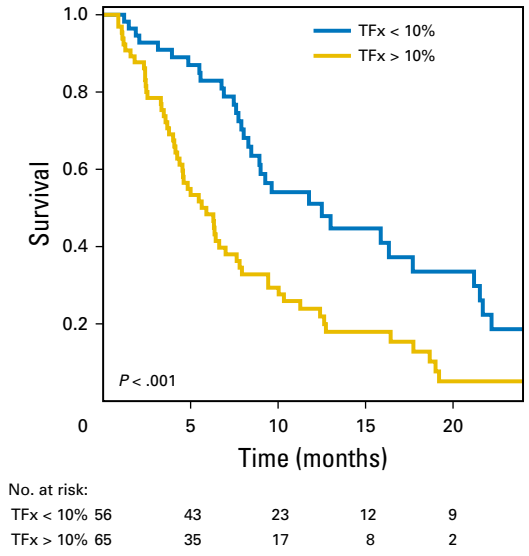
Tumor Fraction: Multiple Linear Regression

	Coefficient	SE	t value	*P*
Liver metastasis present	0.08	0.02	5.18	< .001
Year sample collected	–0.03	0.01	–4.44	< .001
Sample collection cohort	–0.01	0.00	–2.56	.011
BRCA status	–0.02	0.01	–1.73	.084
Line of metastatic treatment	4.28E-03	0.01	0.84	.400
No. samples per patient	7.44E-05	1.92E-04	0.39	.699

B



C



No. at risk:

TFx < 10%	56	43	23	12	9
TFx > 10%	65	35	17	8	2

Fig A6. Tumor fraction is associated with liver metastasis and metastatic survival in patients with primary TNBC. (A) Multiple linear regression of TFx with covariates. (B) TFx by presence or absence of liver metastasis in first blood sample collected per patient (left) or maximum TFx per patient (right). Boxplots indicate 25th-75th percentiles with median indicated by central line and whiskers representing 1.5 times interquartile range. (C) Kaplan-Meier curve of survival from first blood draw stratified by TFx above versus below 10% for only those patients with primary TNBC. (D) Multivariable Cox proportional hazards model of TFx above versus below 10% for only those patients with primary TNBC. (E) Multivariable Cox proportional hazards model of TFx as a continuous variable. Hazard ratio for TFx reported based on increments of 10%.

D

Variables	Hazard Ratio	95% CI		*P*
		Lower	Upper	
Tumor fraction > 10%	2.21	1.38	3.52	< .001
BRCA status				
BRCA status unknown	ref	ref	ref	ref
BRCA wild-type	1.45	0.66	3.17	.351
BRCA mutant	0.91	0.31	2.66	.861
Primary stage at diagnosis				
Stage I	ref	ref	ref	ref
Stage II	0.68	0.32	1.44	.316
Stage III	1.25	0.58	2.69	.569
Stage IV	0.56	0.20	1.62	.289
Age at primary diagnosis (per decade > 40 years)	0.85	0.64	1.13	.271
Year sample collected	0.72	0.47	1.10	.130
Sample collection cohort	1.08	0.95	1.22	.261
Line of metastatic therapy at blood draw	1.15	0.98	1.35	.086
Number of samples per patient	1.00	1.00	1.01	.198

E

Variables	Hazard Ratio	95% CI		*P*
		Lower	Upper	
Tumor fraction per 10% increase	1.24	1.12	1.38	< .001
BRCA status				
BRCA status unknown	ref	ref	ref	ref
BRCA wild-type	1.21	0.62	2.37	.579
BRCA mutant	1.00	0.38	2.58	.993
Primary receptor status				
Indeterminate	ref	ref	ref	ref
HR positive/HER2 negative	0.47	0.14	1.57	.222
HER2	0.86	0.19	3.89	.844
TNBC	0.92	0.33	2.55	.868
Primary stage at diagnosis				
Stage I	ref	ref	ref	ref
Stage II	0.67	0.35	1.28	.224
Stage III	1.31	0.67	2.54	.430
Stage IV	0.59	0.23	1.53	.279
Age at primary diagnosis (per decade > 40 years)	0.79	0.60	1.04	.090
Year sample collected	0.85	0.60	1.19	.341
Sample collection cohort	1.08	0.96	1.21	.187
Line of metastatic therapy at blood draw	1.10	0.96	1.27	.179
Number of samples per patient	1.00	1.00	1.01	.782

Fig A6. (Continued).