

## Enrollment

Patients with diagnosis of carcinoma of the breast with HER2 IHC staining score of 3+ or *ERBB2* amplification based on FISH (n=164)

## Pre-treatment Research Biopsy

Image-guided research biopsies performed in two different geographic areas of the same tumor (n=163)

Consent withdrawal (n=1)

## Allocation

Study population: patients received single arm neoadjuvant therapy of T-DM1+ Pertuzumab (n=163)

Consent withdrawal (n=1)  
Failed HER2 heterogeneity evaluation (n=5)

## Follow-up

Efficacy study population (n=157)

No available biopsies (n=28)

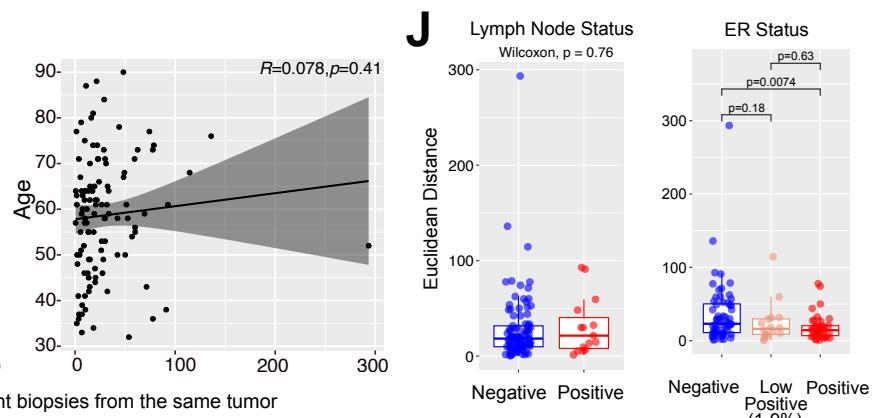
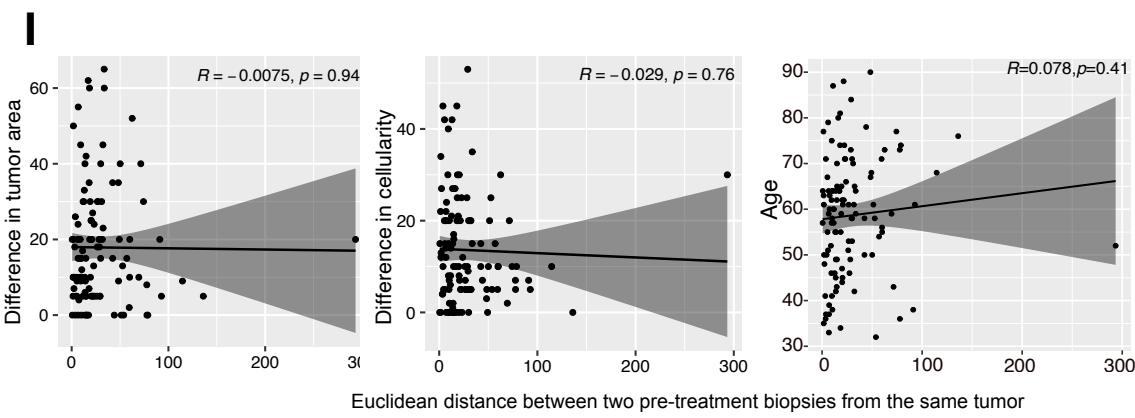
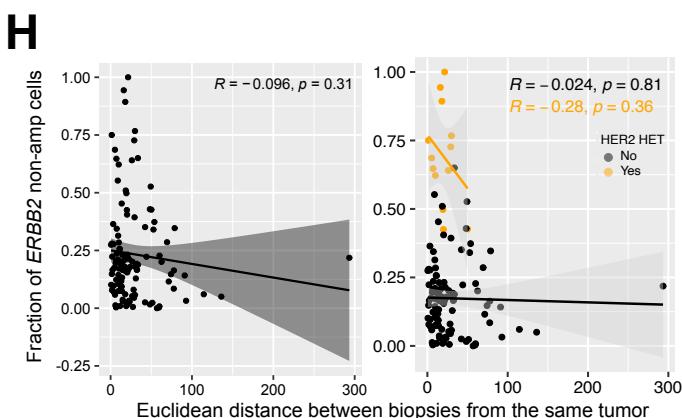
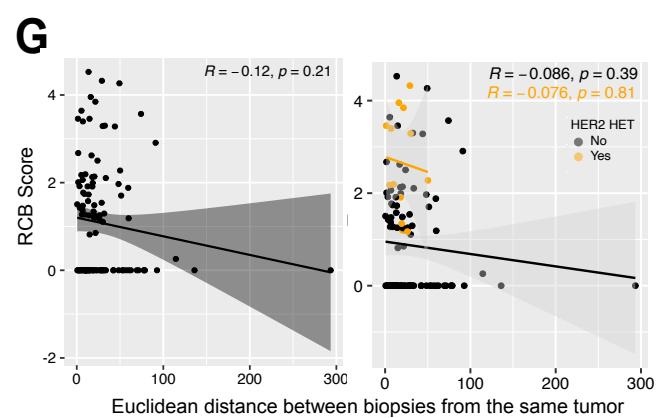
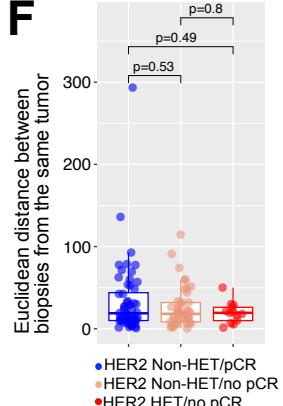
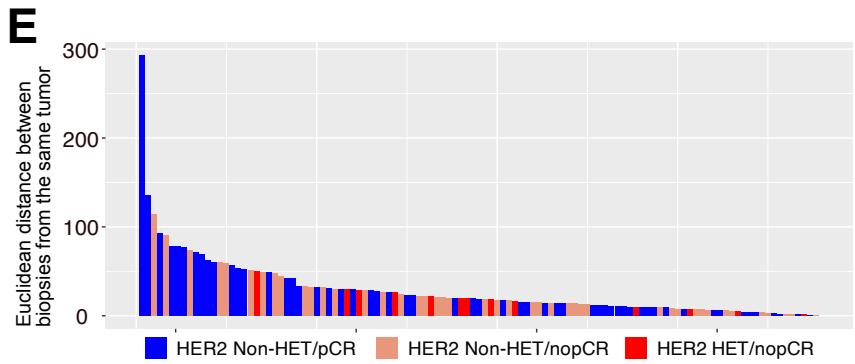
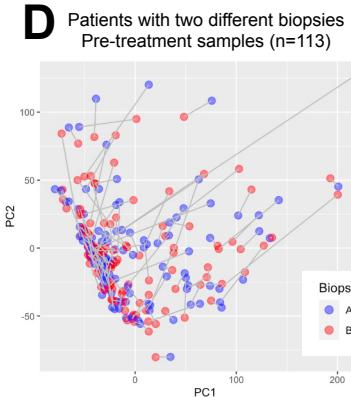
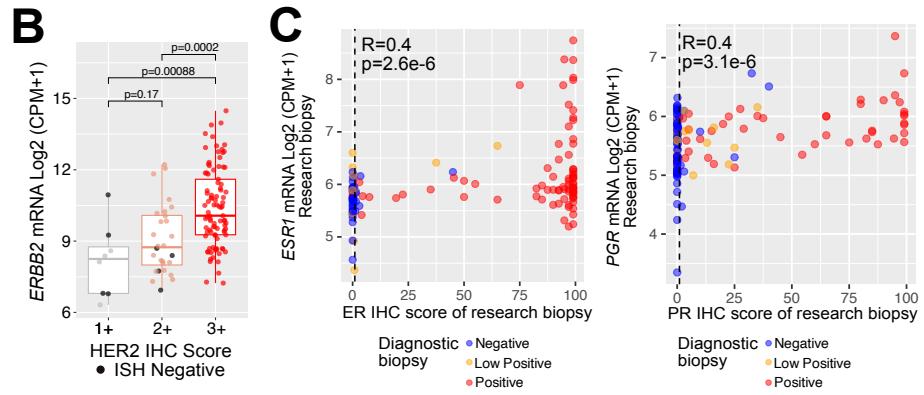
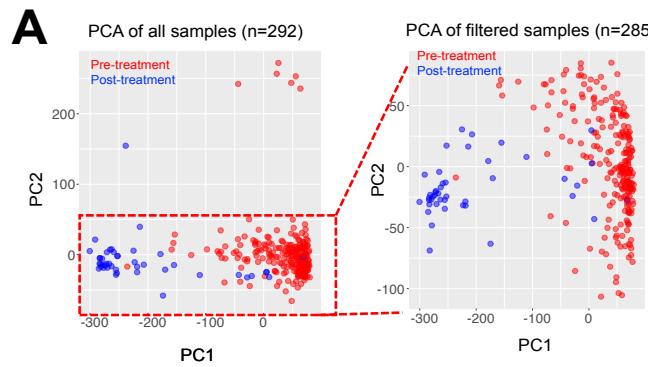
## Analysis

FFPE from research core biopsies available (292 biopsies from n=129 patients)

Exclude 7 biopsies from 7 patients due to failed QC

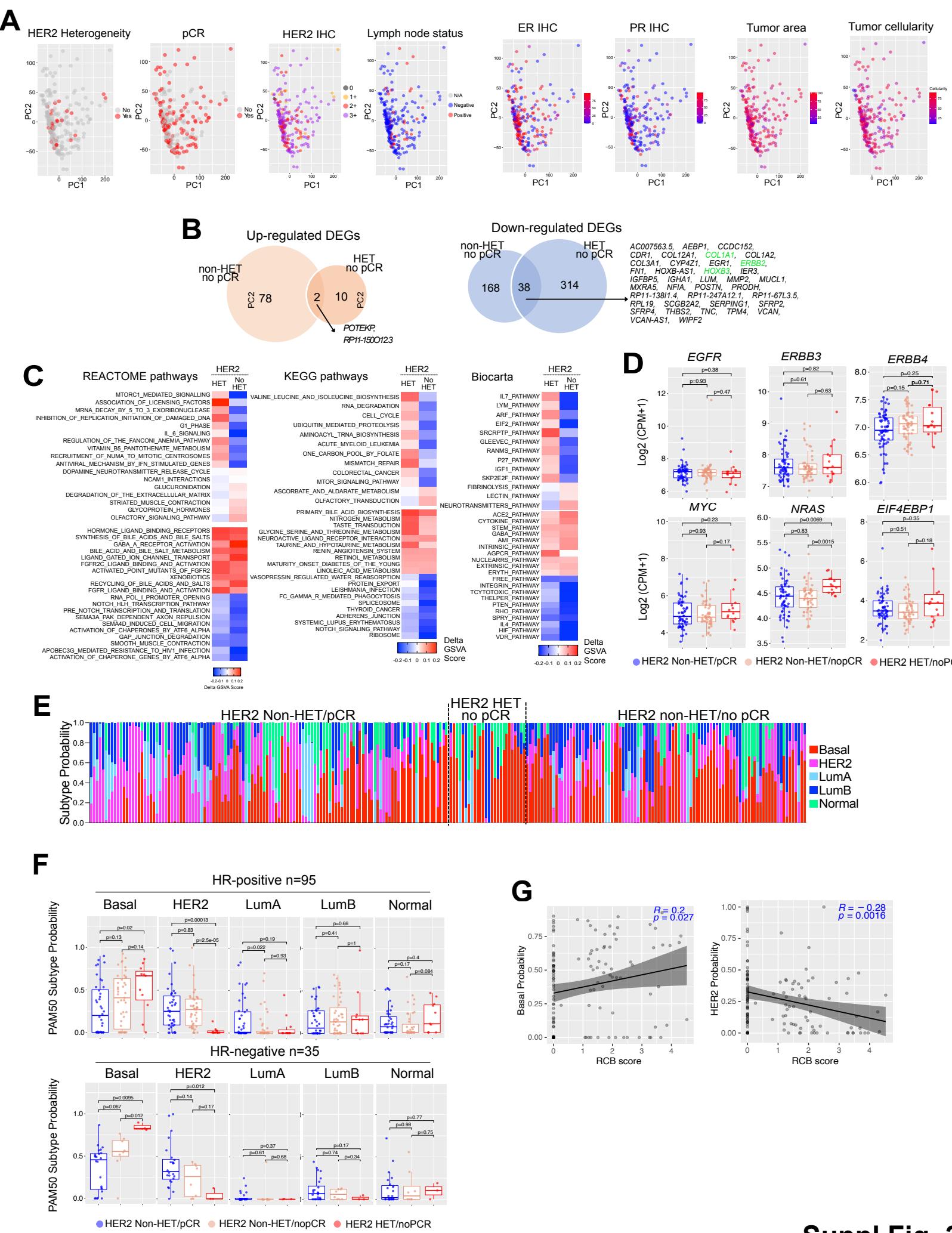
|                         |      |                            |
|-------------------------|------|----------------------------|
| HER2 Non-HET/<br>pCR    | Pre  | 120 biopsies (64 patients) |
|                         | Post | No Samples                 |
| HER2 Non-HET/<br>No pCR | Pre  | 95 biopsies (51 patients)  |
|                         | Post | 36 tumors (36 patients)    |
| HER2 HET/<br>No pCR     | Pre  | 27 biopsies (14 patients)  |
|                         | Post | 7 tumors (7 patients)      |

**Supplemental Figure 1. CONSORT diagram depicting study design.** Flow chart summarizing the study enrollment criteria, patient allocation, follow-up information, and analyses performed. Patients or sample excluded at different stages are indicated. Numbers of biopsies and patients in the three clinically distinct subgroups are listed.

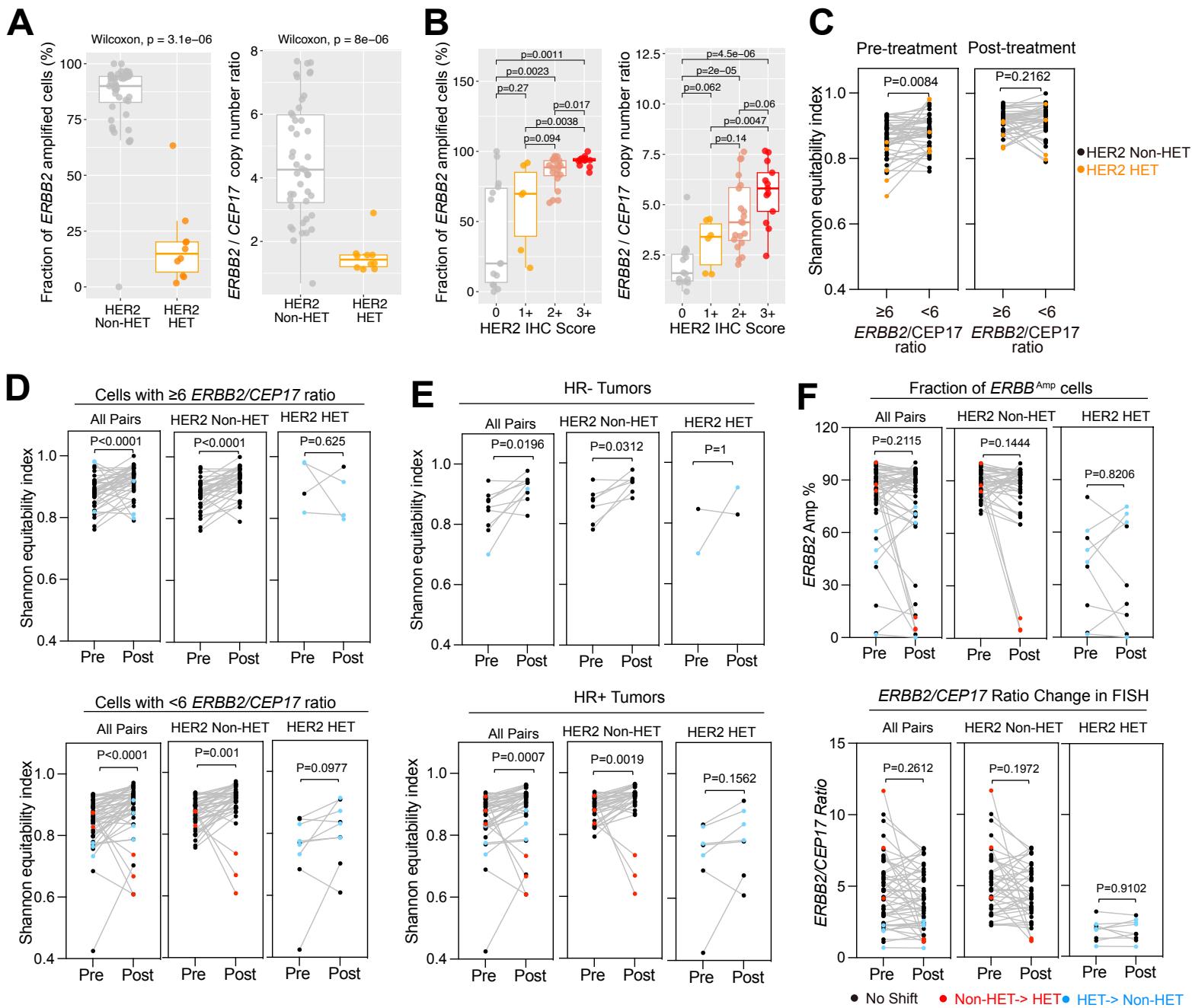


Suppl Fig. 2

**Supplemental Figure 2. Quality analyses of RNA-seq data and transcriptomic variation in intra-patient paired biopsies.** (A) PCA plot showing transcriptomic variation in all 292 samples. Seven samples identified as outliers were removed from subsequent analyses, the remaining 285 samples were replotted in the right panel. (B) Box plot showing *ERBB2* mRNA expression from RNA-seq among patients with different HER2 scores based on immunohistochemistry (IHC). Mean expression of two biopsies from the same patient was used. *ERBB2* ISH (in situ hybridization) negative cases are marked in the plot. (C) Scatter plots depicting Pearson correlation between mRNA (RNA-seq) and protein (IHC) levels for *ESR1* and *PGR* in pre-treatment samples assessed in research biopsies. Mean measurement of ER and PR IHC scores and mRNA expression from two pre-treatment biopsies of the same patient was used for plotting. Colors represent ER and PR IHC results of the diagnostic biopsy of the corresponding patients. (D) PCA plot showing transcriptomic variation of 113 intra-patient paired pre-treatment biopsies. Two biopsies from the same patients are connected with gray line. (E) Histogram illustrating the distance of each biopsy pairs in (D) and their indicated groups based on pCR and HER2 heterogeneity. (F) Box plots comparing transcriptomic distances in paired pre-treatment biopsies. (G,H) Scatter plots showing Pearson correlation between transcriptomic distance of intra-patient biopsy pairs and RCB scores (G) and HER2 negative ratios accessed by FISH (H) in all samples (left) or within HER2 HET and Non-HET cases (right). (I) Scatter plots showing the Pearson correlation between transcriptomic distances (x-axis) and differences in tumor areas (y-axis, left), tumor cell cellularity (y-axis, middle) or age (y-axis, right) in intra-patient biopsy pairs. (J) Box plots depicting transcriptomic distances between intra-patient biopsy pairs in patients with different nodal status and ER positivity. P values were calculated based on two tailed Mann-Whitney U test for B,F and J and Pearson correlation for C,G,H and I.

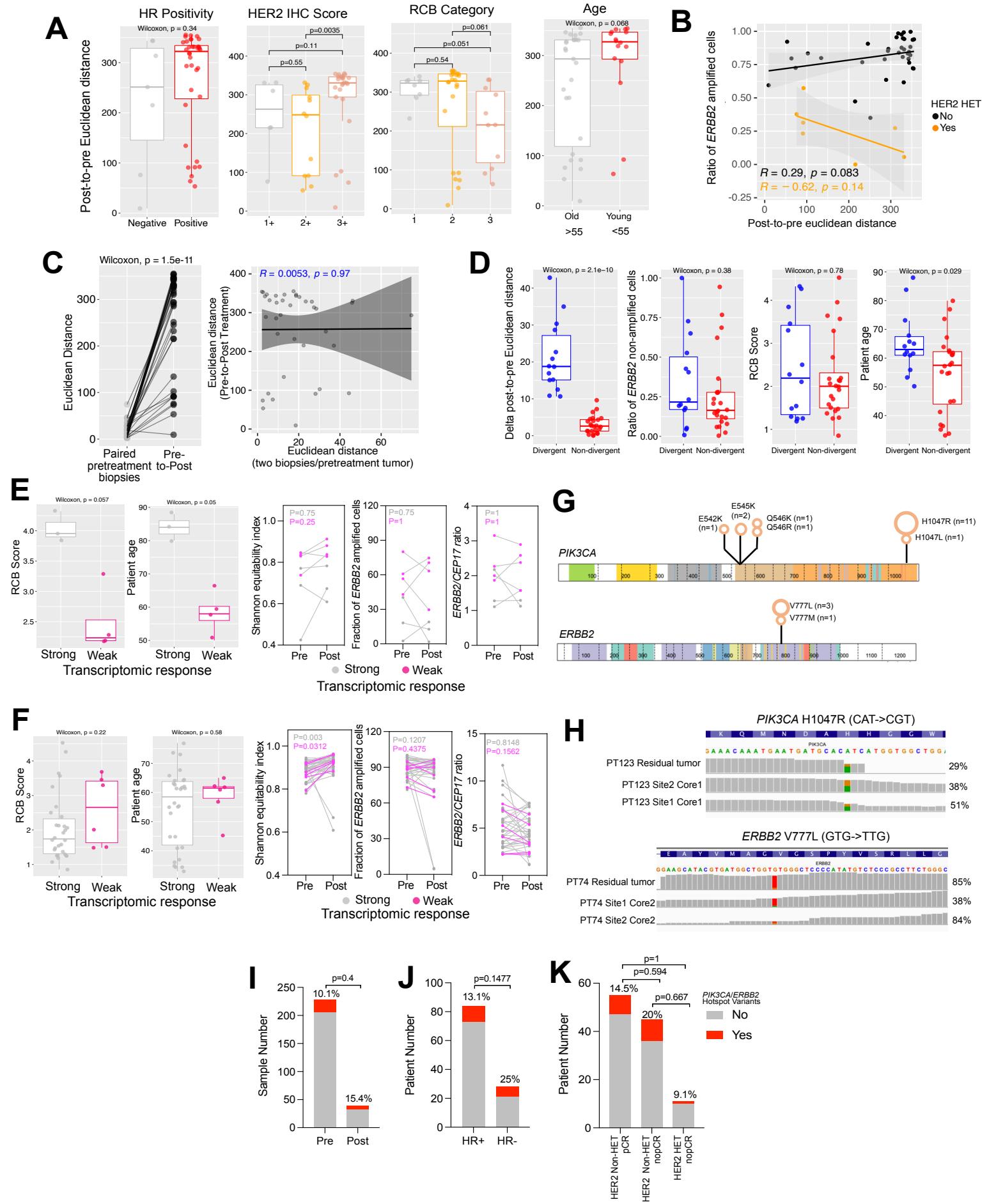


**Supplemental Figure 3. HER2 heterogeneity and treatment-associated differences in gene expression patterns.** **(A)** PCA plot depicting the distribution of 242 pretreatment samples separated by the indicated categorical and quantitative parameters. **(B)** Venn diagrams showing the overlap of DEGs between the indicated comparisons. Overlapping genes are listed. **(C)** Heatmaps showing top pathways differentially enriched in HER2 HET and Non-HET tumors normalized to pCR samples. Red and blue represents increased and decreased pathway enrichment compared to pCR samples. Color scale indicates magnitude of change. **(D)** Box plots showing expression level of ERBB gene family members and selected ERBB/PI3K pathway genes across the indicated groups using the mean of two biopsies from the same patient. **(F)** Stacked bar plot illustrating the PAM50 subtype probability among HER2 Non-HET/pCR, Non-HET/nopCR, and HET/nopCR samples. **(F)** Box plots depicting PAM50 subtype probability scores in the indicated groups separated by hormone receptor status using the mean score of two biopsies from the same patient. **(G)** Scatter plots showing the correlation between basal and HER2 subtype probability scores and RCB scores across all samples. P values were calculated based on two tailed Mann-Whitney U test in **D,F**, and Pearson correlation in **G**.



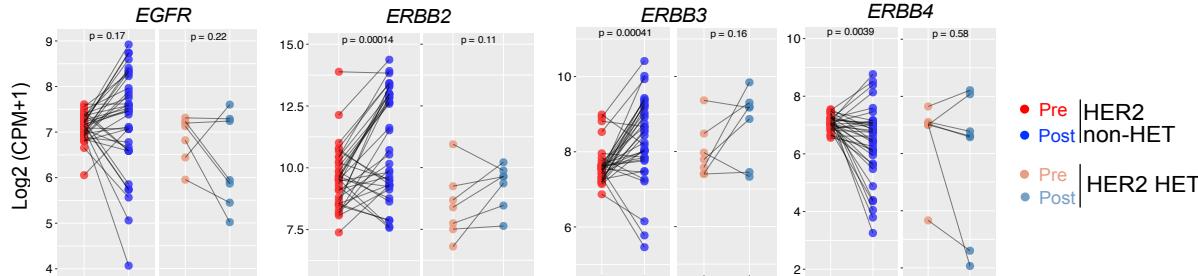
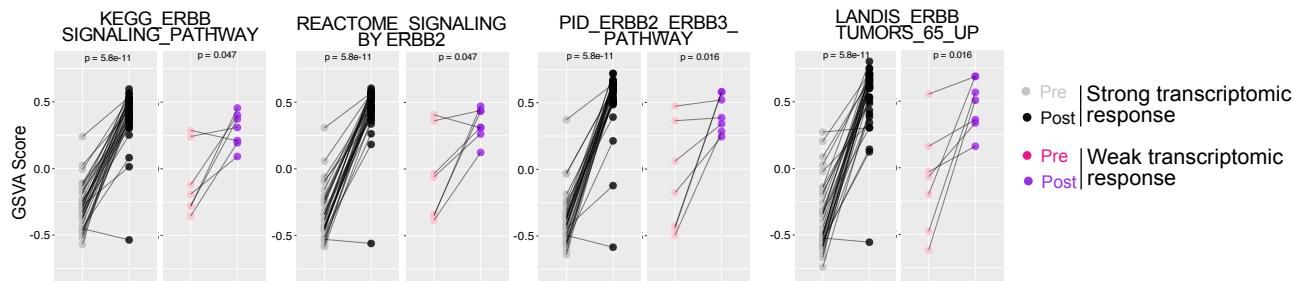
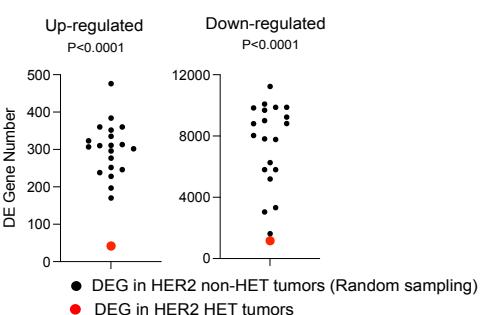
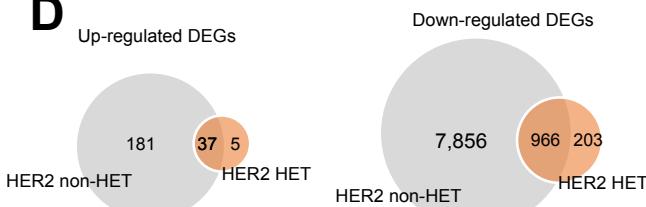
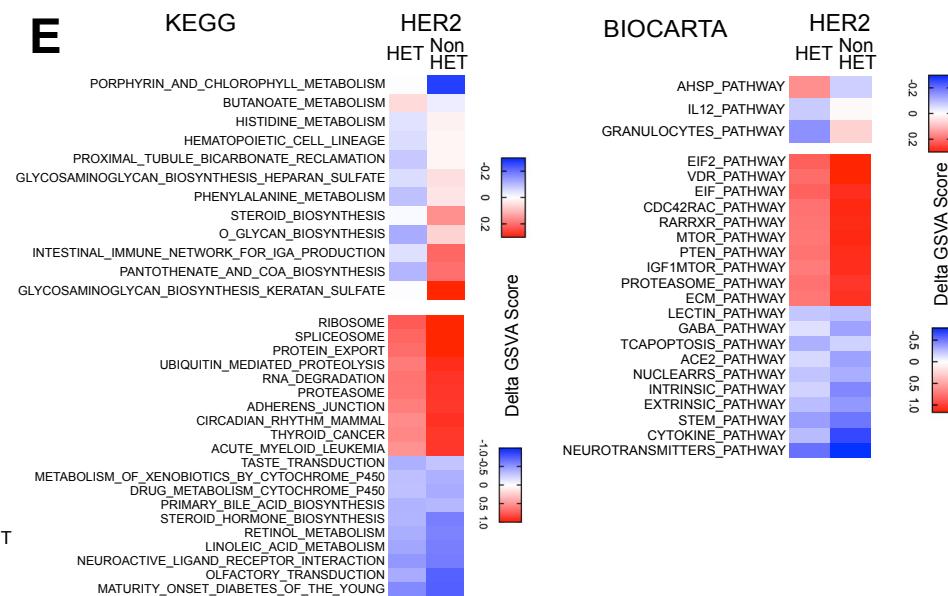
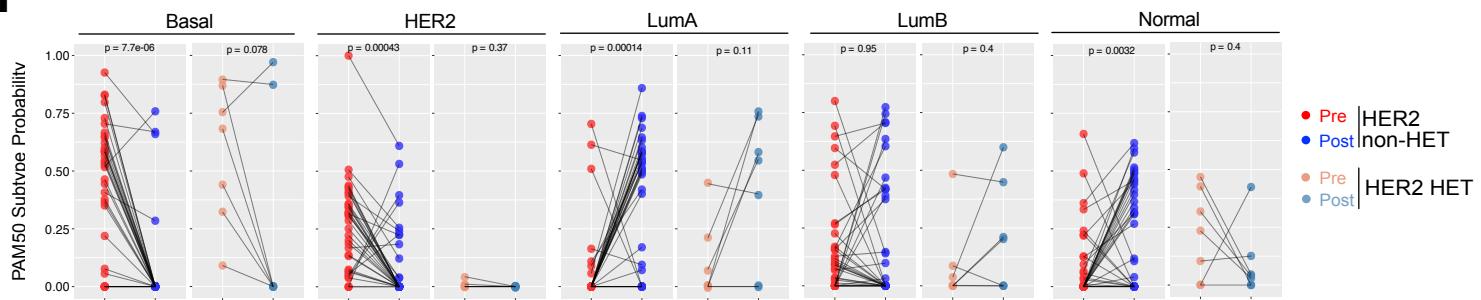
Suppl. Fig. 4

**Supplemental Figure 4. Treatment-induced HER2 heterogeneity alterations. (A)(B)** Box plots showing the relative proportion of *ERBB2* amplified cells and *ERBB2/CEP17* ratios in residual tumors classified as HER2 HET and Non-HET based on pretreatment biopsies **(A)** and with different HER2 IHC scores **(B)**. **(C)** Line plots depicting the Shannon's equitability index between tumor cell populations with the indicated *ERBB2/CEP17* ratios within the same tumor (connected by line) in pre- (left) and post-treatment (right) samples. **D-(F)** Line plots showing changes in Shannon's equitability index **(D)****E** the relative fraction of *ERBB2* amplified cells and in *ERBB2/CEP17* ratios **(F)** in paired pre- and post-treatment biopsies among all tumors or within samples classified as HER2 Non-HET and HER2 HET based on FISH on pre-treatment biopsies. Samples with a change in HER2 heterogeneity are highlighted in red and blue. Two-tailed Mann Whitney U test was used in **(A)** **(B)** and Wilcoxon matched-pairs signed rank test was used in **(C)** **(D)** **E** and **F**.



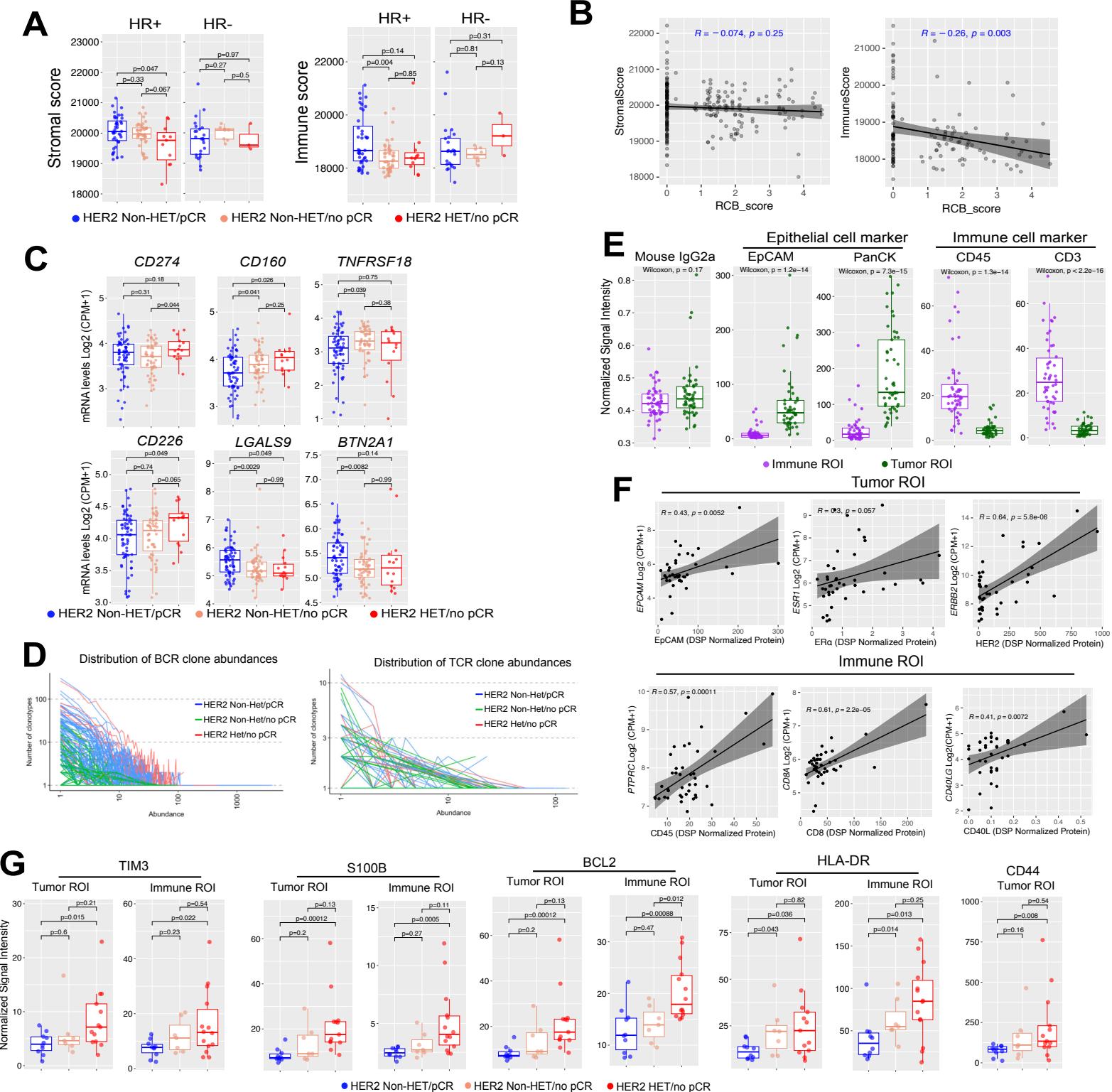
Suppl Fig 5

**Supplemental Figure 5. Treatment-induced differences in transcriptomic profiles.** **(A)** Box plots showing transcriptomic distances between pre- and post-treatment samples in the indicated patient groups, using the mean distance of two pre-treatment biopsies from the same patient for pre-treatment value. **(B)** Scatter plot illustrating Pearson correlation between transcriptomic distances between pre- and post-treatment samples and fraction of *ERBB2*<sup>amp</sup> cells in HET and non-HET cases. Values represent the mean of two biopsies from the same patient. **(C)** Line plot showing Euclidean distances between two pre-treatment biopsies from the same tumor and the corresponding pre-to-post treatment samples from the same patient (left panel). Scatter plot depicting the correlation of these two distances (right panel). **(D)** Box plots depicting differences in treatment-induced Euclidean distance between the two intra-patient biopsies, and differences in *ERBB2* amplified cell ratio, RCB scores, and patient age in biopsies with divergent or non-divergent responses. **(E,F)** Box plots (left) showing the comparisons of RCB score and patient age between strong and weak transcriptomic responders and line plots (right) showing changes of Shannon's equitability index, fraction of *ERBB2* amplified cells and *ERBB2*/CEP17 copy number ratios in paired pre- and post-treatment samples classified as strong and weak transcriptomic responders in HER2 HET (**E**) and Non-HET (**F**) tumors. **(G)** Schematic illustration of hotspot mutations detected in *PIK3CA* and *ERBB2*. **(H)** Screenshot of representative cases with *PIK3CA* H1047R and *ERBB2* V777L mutations across all the biopsies. **(I-K)** Stacked bar plots showing enrichment of *PIK3CA* and *ERBB2* mutations in the indicated subgroups. P values were calculated based on two tailed Mann-Whitney U test in **A,D** and left panels of **E,F**, two-tailed Wilcoxon matched-pairs signed rank test in the left panel of **C** and the right panels of **E** and **F**, Pearson correlation in the right panel of **B,C** and Two-tailed Fisher's Exact test in **I, J** and **K**.

**A****B****C****D****E****F**

**Supplemental Figure 6. Treatment-induced transcriptomic changes.** **(A,B)** Line plots showing the expression of ERBB gene family members in pre- and post-treatment tumors subdivided based on HER2 heterogeneity **(A)**, and enrichments scores of the four ERBB signaling signatures in the pre- and post-treatment tumors divided by transcriptomic response type **(B)**. Average measurements from the two pre-treatment biopsies of the same patient were used for the comparisons. **(C)** Dot plots depicting numbers of DEGs with higher or lower expression levels in post- compared to pre-treatment samples. Red and black dots represent DEGs in HER2 heterogenous and non-heterogeneous group, respectively, with the latter one taking 20 random sampling of the same numbers as HER2 heterogenous group to correct for differences in sample size. **(D)** Venn diagrams showing the overlap of DEGs up- and downregulated between post and pretreatment samples within HER2 heterogeneous and non-heterogeneous groups. **(E)** Heatmaps of top KEGG and BIOCARTA pathways differentially enriched between pre- and post-treatment samples from HER2 Non-HET and HET cases. Red and blue represents increased and decreased pathway enrichment in post-treatment samples compared to pre-treatment counterparts. Color scale indicates magnitude of change. **(F)** Line plots depicting PAM50 subtype probability scores in pre- and post-treatment tumors with the indicated HER2 heterogeneity status at patient level where the average scores from the two pre-treatment biopsies of the same patient were used for pre-treatment values. P values were calculated based on two-tailed Wilcoxon matched-pairs signed rank test in **A,B,F** and two-tailed one sample t test in **C**.

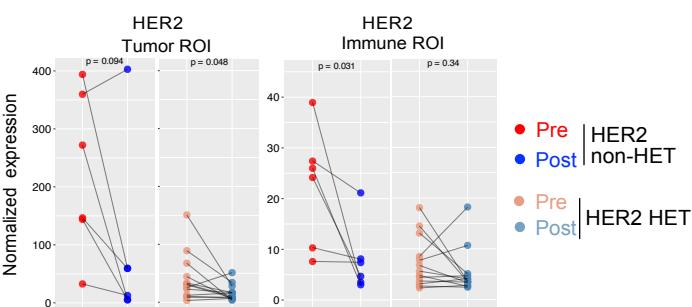
**Suppl Fig. 7**



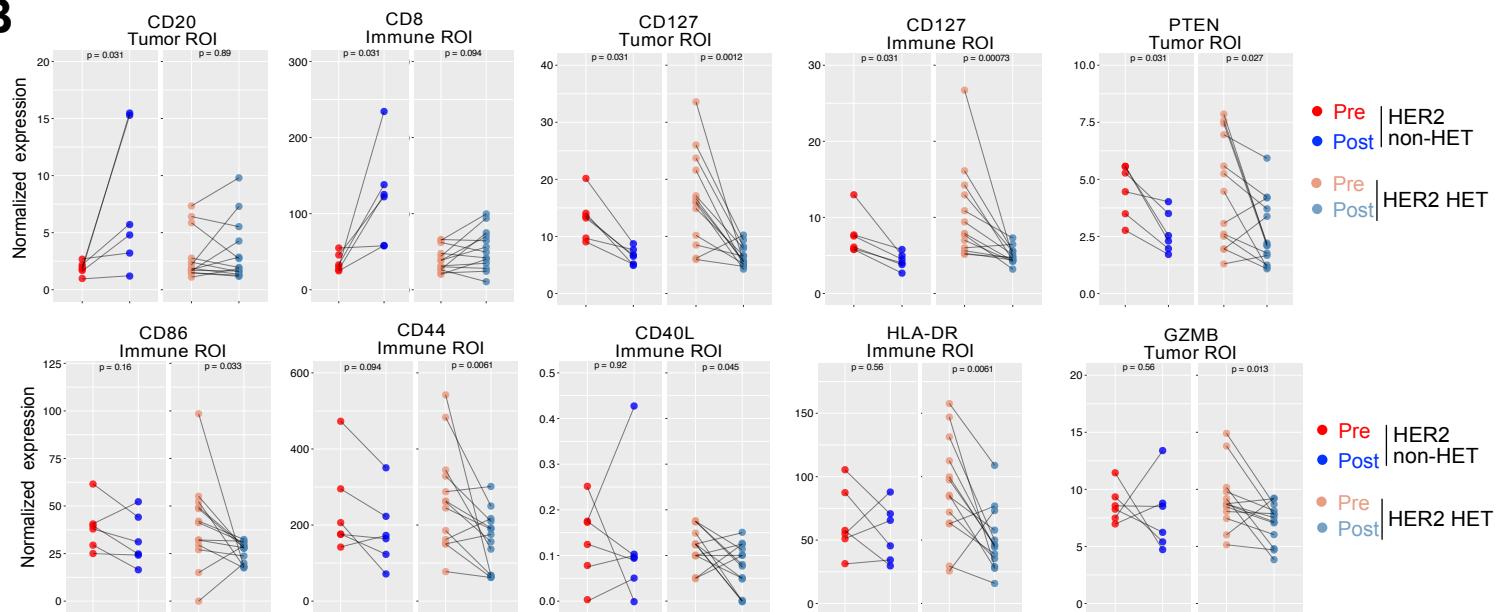
**Supplemental Figure 7. HER2 heterogeneity-associated differences in the immune microenvironment in pretreatment samples.** **(A)** Box plots depicting predicted overall stromal and immune scores in pre-treatment tumors within the indicated patient groups subgrouped by hormone receptor (HR) status. Mean scores from the two pre-treatment biopsies from the same patient were used. **(B)** Scatter plot showing the correlation of stromal and immune scores with RCB scores at patient level. **(C)** Box plots showing expression level of genes encoding immune checkpoint proteins among the indicated groups, where mean expression value from the two pre-treatment biopsies were calculated for pre-treatment value. **(D)** Line plots showing distribution of BCR (left panel) and TCR (right panel) clonotype abundances of each individual pre-treatment sample. **(E)** Box plots depicting the comparison DSP signal intensities of negative control, tumor and immune markers in tumor and immune ROIs in all the samples. **(F)** Scatter plots showing the Pearson correlation between mRNA (RNA-seq) and protein expression (DSP immune or tumor ROIs) of six targets. **(G)** Box plots showing the normalized expression of indicated targets from DSP among the three groups in pre-treatment samples. Tumor or immune ROI was labelled. P values were calculated based on two tailed Mann-Whitney U test for **A,C,E,G** and Pearson correlation in **B,F**.

**Suppl Fig. 8**

**A**



**B**



**Supplemental Figure 8. Treatment-induced changes in the immune environment. (A,B)** Line plots showing the normalized expression of HER2 (A) and other immune-related targets (B) from DSP profiling among the four subgroups based on HER2 heterogeneity and pre-/post-treatment samples from the same patient. Tumor or immune ROI are indicated. P values were calculated by two-tailed Wilcoxon matched-pairs signed rank test in all panels.

## **SUPPLEMENTAL TABLE LEGENDS**

**Supplemental Table 1. Inventory of profiled sample and metadata.** List of all 285 samples for RNA-seq profiling and their associated metadata including HER2 heterogeneity identification, pCR status, RCB categories, hormone receptor status, *ERBB2* FISH amplified cell percentage and HER2 IHC score. NE stands for “not evaluated”.

**Supplemental Table 2. Genes list of differential expression analysis output.** Lists of differentially expressed genes from the four comparisons: non-HET/nopCR vs non-HET/pCR (pre-treatment samples), HET/nopCR vs non-HET/pCR (pre-treatment samples), Pretreatment vs Posttreatment samples (non-HET/nopCR) and Pretreatment vs Posttreatment samples (HET/nopCR). Log2Fold change and adjusted p values are shown to each individual gene.

**Supplemental Table 3. HER2 immunohistochemistry score and heterogeneity shift.** List of 50 patients with their associated HER2 heterogeneity calling and HER2 immunohistochemistry score in pre-treatment biopsies and residual tumors. Patients showing shift of HER2 heterogeneity are indicated.

**Supplemental Table 4. Inventory of *PIK3CA* and *ERBB2* hotspot variants.** List of samples harboring *PIK3CA* or *ERBB2* hotspot variants called from RNA-seq. Allele frequencies are listed for each variant.

| Sample               | Patient | Variant gene  | <i>PI3KCA</i> exon21 | <i>PIK3CA</i> exon9 | <i>ERBB2</i> exon23 |
|----------------------|---------|---------------|----------------------|---------------------|---------------------|
| 101_site_1_core_2    | 101     | <i>PIK3CA</i> | H1047R (48%)         |                     |                     |
| 101_site_2_core_1    | 101     | <i>PIK3CA</i> | H1047R (14%)         |                     |                     |
| 109_site_1_core_2    | 109     | <i>PIK3CA</i> |                      | Q546K (20%)         |                     |
| 115_site_1_core_1    | 115     | <i>PIK3CA</i> | H1047R (50%)         |                     |                     |
| 115_site_2_core_2    | 115     | <i>PIK3CA</i> | H1047R (69%)         |                     |                     |
| 116_site_1_core_2    | 116     | <i>PIK3CA</i> | H1047R (23%)         |                     |                     |
| 120_site_1_core_1    | 120     | <i>PIK3CA</i> |                      | Q546R (31%)         |                     |
| 120_site_2_core_1    | 120     | <i>PIK3CA</i> |                      | Q546R (23%)         |                     |
| 123_Residual_Disease | 123     | <i>PIK3CA</i> | H1047R (29%)         |                     |                     |
| 123_site_1_core_1    | 123     | <i>PIK3CA</i> | H1047R (38%)         |                     |                     |
| 123_site_2_core_1    | 123     | <i>PIK3CA</i> | H1047R (51%)         |                     |                     |
| 124_site_2_core_1    | 124     | <i>PIK3CA</i> | H1047R (91%)         |                     |                     |
| 137_site_2_core_2    | 137     | <i>PIK3CA</i> |                      | E545K (55%)         |                     |
| 143_Residual_Disease | 143     | <i>PIK3CA</i> | H1047R (25%)         |                     |                     |
| 161_site_2_core_2    | 161     | <i>PIK3CA</i> | H1047R (33%)         |                     |                     |
| 27_site_2_core_1     | 27      | <i>PIK3CA</i> | H1047R (73%)         |                     |                     |
| 32_Residual_Disease  | 32      | <i>PIK3CA</i> | H1047L (33%)         |                     |                     |
| 43_site_1_core_2     | 43      | <i>PIK3CA</i> | H1047R (51%)         |                     |                     |
| 46_Residual_Disease  | 46      | <i>PIK3CA</i> | H1047R (33%)         |                     |                     |
| 48_site_1_core_3     | 48      | <i>ERBB2</i>  |                      |                     | V777M (25%)         |
| 68_site_1_core_2     | 68      | <i>PIK3CA</i> | H1047R (40%)         |                     |                     |
| 72_site_1_core_2     | 72      | <i>PIK3CA</i> |                      | E542K (35%)         |                     |
| 74_Residual_Disease  | 74      | <i>ERBB2</i>  |                      |                     | V777L (85%)         |
| 74_site_1_core_2     | 74      | <i>ERBB2</i>  |                      |                     | V777L (38%)         |
| 74_site_2_core_2     | 74      | <i>ERBB2</i>  |                      |                     | V777L (84%)         |
| 83_site_2_core_1     | 83      | <i>ERBB2</i>  |                      |                     | V777L (73%)         |
| 91_site_2_core_2     | 91      | <i>PIK3CA</i> |                      | E545K (21%)         |                     |
| 93_Residual_Disease  | 93      | <i>ERBB2</i>  |                      |                     | V777L (64%)         |
| 93_site_1_core_1     | 93      | <i>ERBB2</i>  |                      |                     | V777L (65%)         |

**Supplemental Table 5. Summary of key findings.** List of eight biomarker/features investigated in this study with their correlative summary including 1) association with pCR and if it is different in HET and non-HET groups; 2) difference in no pCR to pCR groups split by HER2 heterogeneity, and 3) T-DM1/P induced changes split by HER2 heterogeneity.

| Feature  | Method  | Relationship to pCR                            | Association with HER2 HET | Difference in no-pCR vs. pCR patients pre-treatment |  | T-DM1/P treatment-induced differences                |   |
|--|---------|--|---------------------------|---|--|--|---|
|  |         |  |                           | HER2 non-HET  | HER2 HET   | HER2 non-HET   | HER2 HET  |
| <b>HER2 heterogeneity</b>                        | FISH    | Only present in no pCR patients                | Yes                       | No difference                                       | Fewer <i>ERBB2</i> amplified cells               | Decreased by treatment                               | No significant difference                                     |
| <b><i>ERBB2</i> mRNA expression</b>              | RNA-seq | Lower in no pCR patients                       | Yes                       | Lower than in pCR patients                          | Lower than in pCR and non-HET/no pCR patients    | Increased by treatment                               | Same trend as HER2 non-HET tumors but no statistic difference |
| <b><i>ERBB2</i> signaling signature</b>          | RNA-seq | Lower in HER2 non-HET/no pCR patients          | Yes                       | Lower than in pCR patients                          | No significant difference                        | Increased by treatment                               | Increased by treatment  |
| <b>PAM50 subtype</b>                             | RNA-seq | Higher basal and lower HER2 in no pCR patients | Yes                       | Higher basal and lower HER2 than in pCR patients    | Higher basal and lower HER2 than in pCR patients | Decreased basal and HER2 and increased LumA features | Same trend as HER2 non-HET tumors but no statistic difference |
| <b>Treatment-induced transcriptomic response</b> | RNA-seq | Only analyzed in no pCR patients               | Yes                       | NA  | NA   | Strong transcriptomic response                       | Weak transcriptomic response                                  |
| <b>Stromal Score</b>                             | RNA-seq | Lower in HER2 HET/no pCR patients              | Yes                       | No significant difference                           | Lower than in pCR patients                       | Increased by treatment                               | No significant difference                                     |
| <b>Immune Score</b>                              | RNA-seq | Lower in HER2 non-HET/no pCR patients          | Yes                       | Lower than in pCR patients                          | No significant difference                        | Increased by treatment                               | No significant difference                                     |
| <b>BCR/TCR richness and diversity</b>            | RNA-seq | Lower in HER2 non-HET/no pCR patients          | Yes                       | Lower than in pCR patients                          | No significant difference                        | Increased by treatment                               | No significant difference                                     |