Supplemental Online Content

Stein A, Paschold L, Tintelnot J, et al. Efficacy of ipilimumab vs FOLFOX in combination with nivolumab and trastuzumab in patients with previously untreated *ERBB2*-positive esophagogastric adenocarcinoma: the AIO INTEGA randomized clinical trial. *JAMA Oncol*. Published online June 23, 2022. doi:10.1001/jamaoncol.2022.2228

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eMethods.

The tumor blocks for analysis of HER2, PD-L1, and EBV-association assessment were obtained at baseline. Blood was collected prior to first treatment, after the first treatment cycle (2-3 weeks), 8 weeks after treatment initiation (not analyzed in this manuscript) and at progression and/or end of treatment. Cell-free DNA (cfDNA) was isolated from blood plasma using QIAamp Circulating Nucleis Acid Kit (Qiagen, Hilden, Germany) and quantified using Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific, Waltham, USA) to determine cfDNA dynamics on treatment. Genomic DNA (gDNA) was isolated from tumor tissue via Maxwell® 16 FFPE Plus LEV DNA Purifcation Kit (Promega, Madison, USA). cfDNA and tumor gDNA were subjected to targeted next generation sequencing (NGS) at baseline and at disease progression.

Immunohistochemistry (IHC) and in situ hybridization (ISH). Central testing of Her2 status was performed using Bond Oracle HER2 IHC System (Leica Biosystems, Nussloch, Germany). Patients with Her2-Score 2+ were additionally tested for gene amplification with ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit (Zytomed Systems GmbH, Berlin, Germany) for in situ hybridization (ISH). PD-L1 was stained using antibody clone CAL 10 (Zytomed Systems GmbH, Berlin, Germany) which had been previously validated by collaborative ring trials at our central testing facility. Combined positive score (CPS) defines the ratio of PD-L1 positive cells (tumor, lymphocytes and macrophages) in relation to total tumor cells. EBV positivity was tested using EBER RNA chromogenic *in situ* hybridization (CISH) with ZytoFast EBV Probe PF29 (Zytomed Systems GmbH, Berlin, Germany). Expression of mismatch repair (MMR) proteins was assessed by immunohistochemistry using antibody clones MSH2 RED2 (Epitomics, Inc., Burlingame, California, USA), MSH6 EP49 (Epitomics, Inc., Burlingame, California, USA), MLH1 ES05 (Agilent, Santa Clara, California, USA), and PMS2 EP51 (Epitomics, Inc., Burlingame, California, USA).

Targeted gene sequencing. A QIAseq Targeted DNA Custom Panel (Qiagen, Hilden, Germany) was used for mutational analysis as described in [24,25]. Covered genes are listed in the eTable. The amplicon library was prepared according to the supplier's instructions using unique molecular identifiers (UMIs) and a DNA amount of 100 ng as input. NGS was performed as a dual indexed (8 nucleotides) paired-end run using NextSeq 500/550 High Output Kit v2.5 (300 Cycles) on an Illumina NextSeq instrument (Illumina, San Diego, USA) at a mean depth of 26 500 reads per base.

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Data analysis. The CLC workbench (Qiagen, Hilden, Germany) was used for data processing of fastq files and variant calling. Variants were filtered for minimum coverage of 10 UMIs, minimum count of 2 UMIs, minimal average base quality of 35 and minimum frequency of 1 % for cfDNA or 10 % for DNA from formalin-fixed paraffin-embedded tissue corresponding to the sensitivity threshold for this sequencing approach. Single nucleotide polymorphisms (SNPs) or mutations present in more than three patients were excluded. All analyses and data plotting of annotated variant files were performed using RStudio version 3.5.1., Graph Pad Prism version 8.3.1 and Adobe Illustrator version 24.1.1. Plotting and statistic tests of survival data were done with R package survminer[26].

Protein expression. The extracellular domain of Her2 (amino acid 1-638) was cloned as a C-terminal fusion with Fc into pcDNA3.1 as wild-type sequence or with point mutation His574Leu. HEK293T cells (DSMZ, Braunschweig, Germany) were transfected with pcDNA3.1_Her2wt-Fc and pcDNA3.1_Her2-H574L-Fc using polyethylenimine (PEI, Merck, Darmstadt, Germany) at a ratio of 3 µg PEI per 1 ng plasmid DNA. Culture medium was replaced and collected at day 1, 2, 3 and 6 after transfection. Supernatants were pooled and purified on a protein A sepharose column (Thermo Fisher Scientific, Waltham, USA). Protein identity was confirmed via Western blot on a PVDF membrane (Merck Millipore, Burlington, USA) using polyclonal anti-Her2 antibody clone D8F12 (1:5000, Cell Signaling Technology, Danvers, USA).

ELISA. Equal amounts of Her2-Fc wild-type and Her2_H574L-Fc (1-10 ng) were coated onto 96-well plates. Wells were blocked with 3 % (w/v) bovine serum albumin fraction V (BSA) in phosphate buffered saline (PBS) and washed with 0,5 % (w/v) BSA/ 0,05 % (v/v) Tween 20/ PBS. Trastuzumab binding was measured by incubating wells with 1:500 dilution of Herceptin/ PBS (Roche, Basel, Switzerland) for 1h at room temperature. Signals were generated by probing with goat anti-human kappa (1:1000, Southern Biotech, Birmingham, USA) and donkey anti-goat-HRP (1:2000, RD Systems, Minneapolis, USA). Signals were developed with 1-Step Ultra TMB ELISA Substrate and ELISA Stop Solution (Thermo Fisher Scientific, Waltham, USA). Absorption was measured at 450 nm. Wells without addition of trastuzumab were used as blank. Values were normalized to corresponding blank wells and measured as triplicates of duplicates.

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	All (n=88) ITT		HER2+ central (n=76)		CPS=0 (n=23)		CPS≥1 (n=59)		CPS≥5 (n=46)	
	Trast/Nivo/ Ipi	Trast/Nivo/ FOLFOX	Trast/Nivo/ Ipi	Trast/Nivo/ FOLFOX	Trast/Nivo/ Ipi	Trast/Nivo/ FOLFOX	Trast/Nivo/ Ipi	Trast/Nivo/ FOLFOX	Trast/Nivo/ Ipi	Trast/Nivo/ FOLFOX
	N=44	N=44	N=40	N=36	N=11	N=12	N=31	N=28	N=24	N=22
ORR	32%	56%	35%	63%	27%	50%	36%	63%	33%	67%
mPFS	3.2 mo	10.7 mo	3.4 mo	10.7 mo	3.2	11.4	2.2 mo	10.7 mo	2.2 mo	11 mo
PFSR@12	15%	37%	17%	36%	20%	50%	14%	33%	7%	38%
mDOR	5.8 mo	9.2 mo	na	na	na	na	na	na	na	na
mOS	16.4 mo	21.8 mo	16.4 mo	22.4 mo	25.2 mo	30.8 mo	16.4 mo	21.6 mo	12.5 mo	21.6 mo
OSR@12	57%	70%	58%	74%	78.8%	75%	54%	71%	53%	72%

eTable. Efficacy in intent to treat population (ITT) and subgroups

(Abbrev: ORR overall response rate, mPFS median Progression free survival, PFSR@12 progression free survival rate at 12 months, mDOR median duration of response, mOS median overall survival, OSR@12 overall survival rate at 12 months, mo months, na not applicable)

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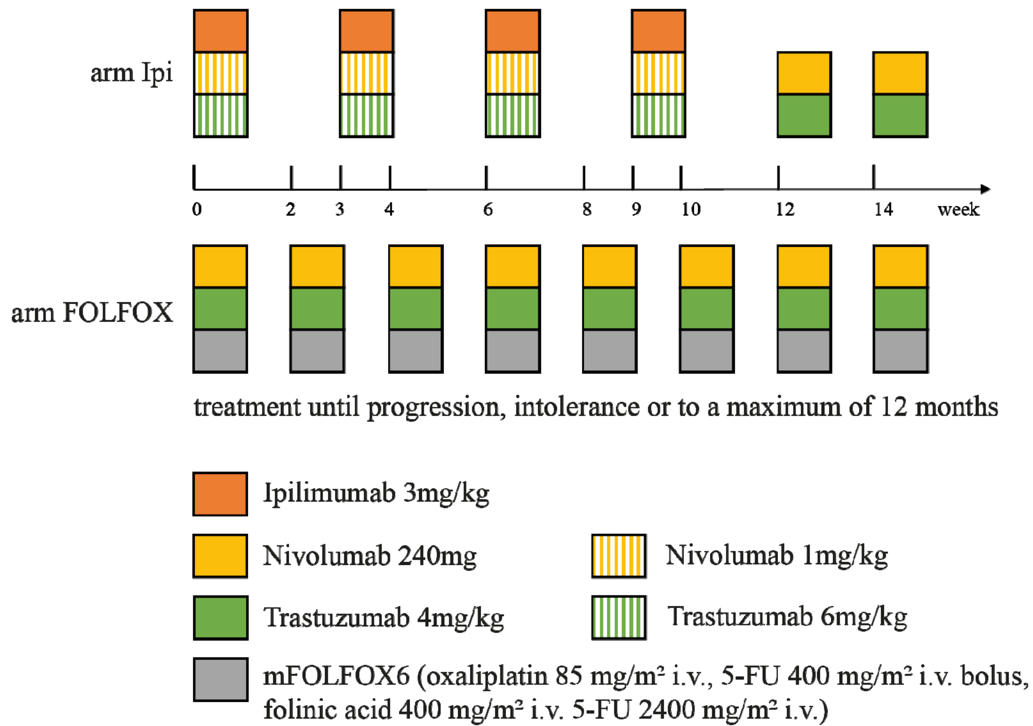

eFigure 1. Treatment regimen applied in the trial

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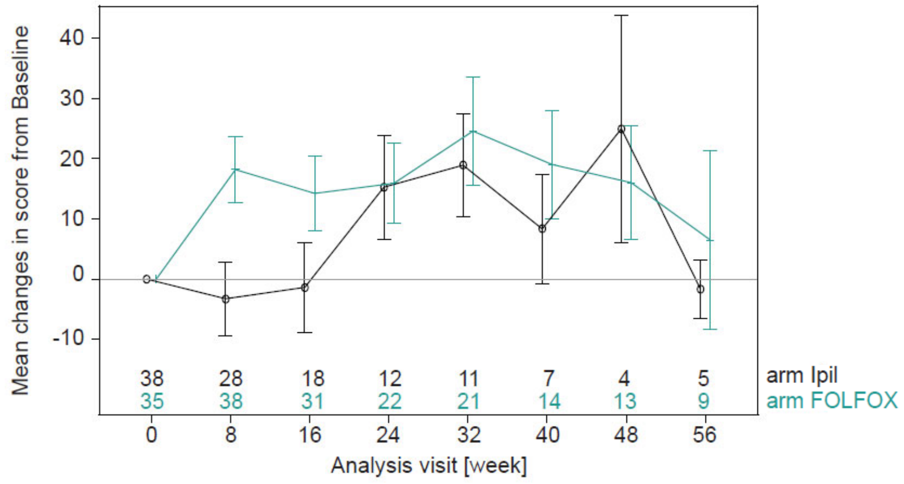

eFigure 2. Quality of life according to EORTC QLQ C30 Global Health Score. Mean +/- standard error is presented.

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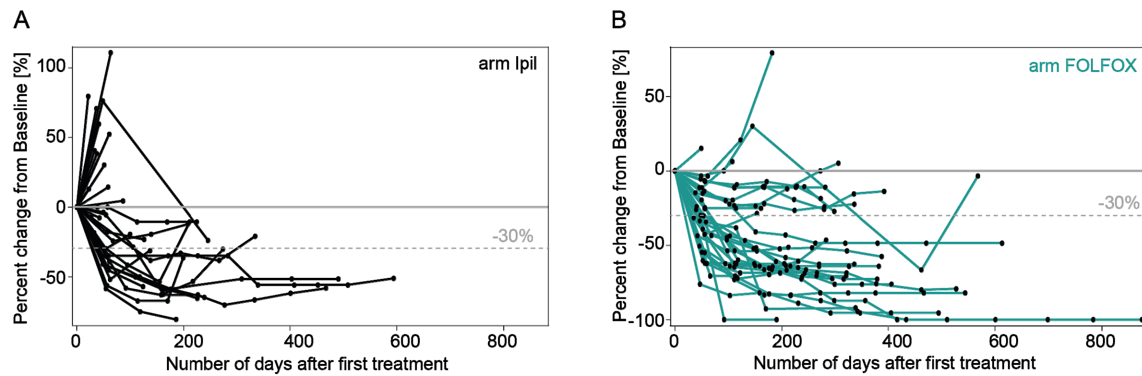

eFigure 3. The spider plots for the Ipi (A) and the FOLFOX arm (B) show responses in individual patients in the intent to treat population (two patients - one in each arm - were allowed to continue in the trial based on clinical response and local treatment applied beside RECIST progression)

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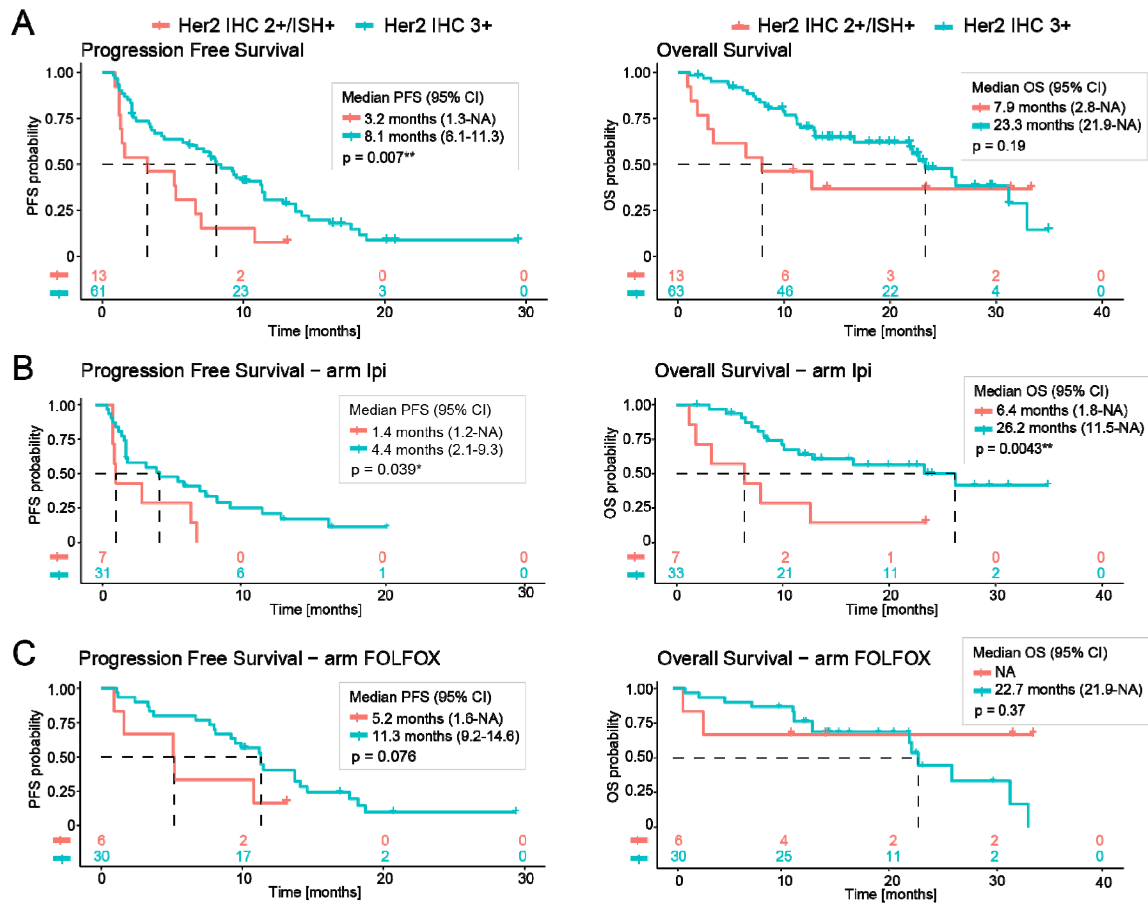

eFigure 4. Progression-free survival (PFS) and overall survival (OS) for patient subsets with different HER2 expression levels. Patients with Her2 score 3+ in immunohistochemistry staining (IHC) (n=63) are grouped versus patients with IHC score 2+ and confirmed DNA amplification of ERBB2 (ISH+) (n=13) according to central testing. Panel A shows PFS and OS for HER2 IHC3+ vs IHC2+ cases for all patients, panel B only of the Ipi arm and C only of the FOLFOX arm. The p-values were calculated using log-rank test. Median survival and 95% confidence interval (CI) are given in month.

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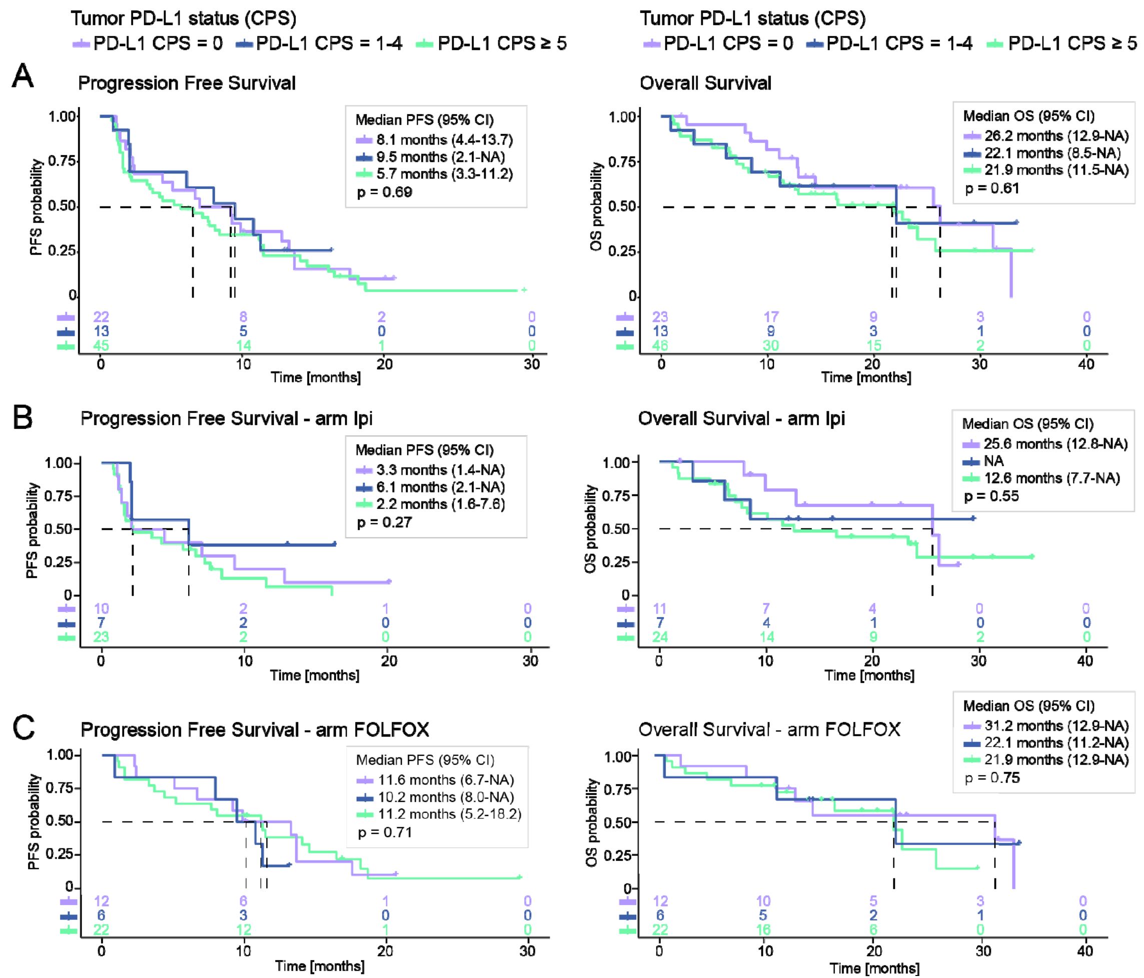

eFigure 5. Progression-free survival (PFS) and overall survival (OS) for patient subsets with different PD-L1 expression levels. Patients are grouped according to the level of PD-L1 combined positive score (CPS): CPS score =0 (n=23) versus score 1-4 (n=13) and score ≥5 (n=46). Panel A shows survival of all patients, panel B only of the Ipi arm and panel C only of the FOLFOX arm. The p-values were calculated using log-rank test. Median survival and 95% confidence interval (CI) are given in month.

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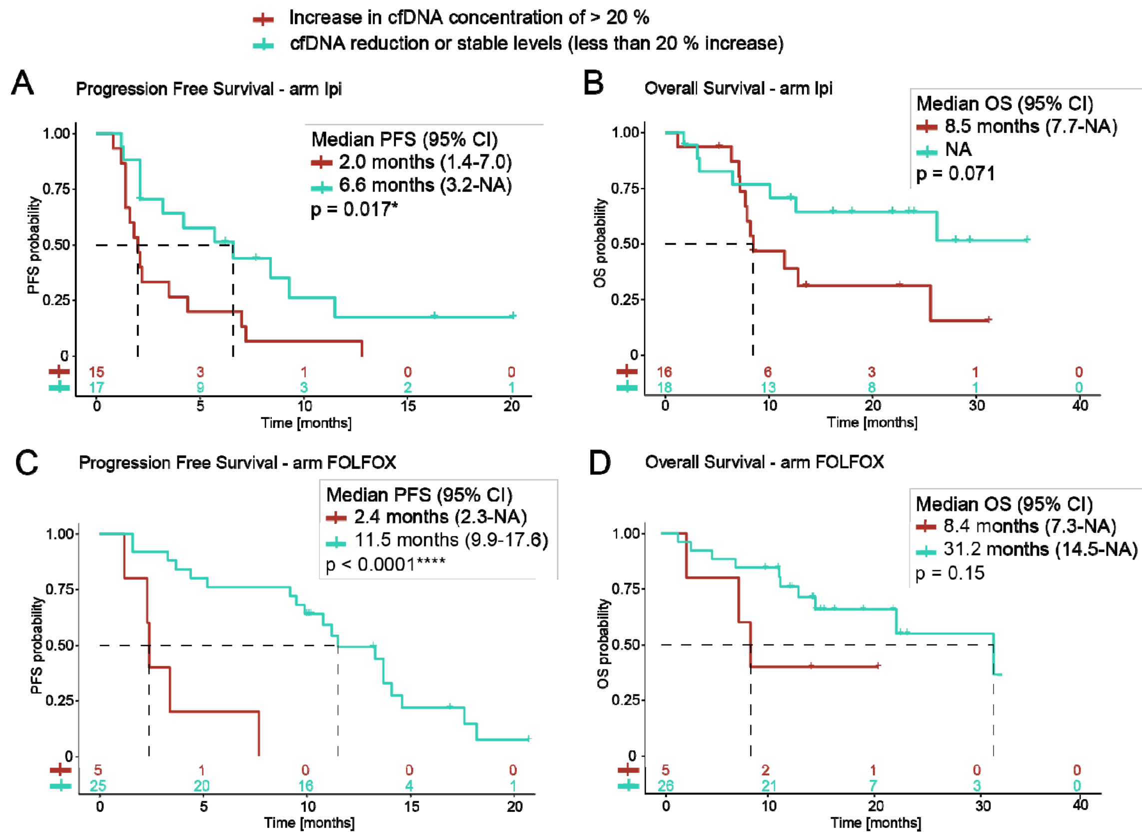

eFigure 6. cfDNA dynamics per treatment arm. Progression-free survival (PFS) and overall survival (OS) of patients according to the level of cfDNA in blood after the first treatment cycle (2-3 weeks) versus baseline levels are shown for the Ipi arm (A, B) and the FOLFOX arm (C, D). Patients with increase in cfDNA concentration of >20% were plotted versus patients showing cfDNA reduction or stability. Only patients with matched information about cfDNA concentration at baseline and after the first treatment cycle are shown (total n=65). Patients without an on-treatment sample available were excluded. P-values were calculated using log-rank test. Median survival and 95% confidence interval (CI) are given in months.

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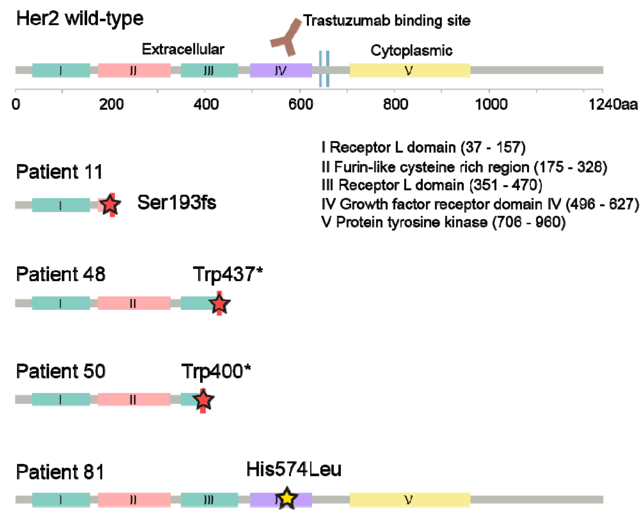

eFigure 7. Truncating and epitope escape mutations of HER2 associated with disease progression on trastuzumab-containing regimen. Red stars indicate mutation sites leading to a premature stop codon. The yellow star indicates a mutation leading to a single amino acid (aa) exchange.

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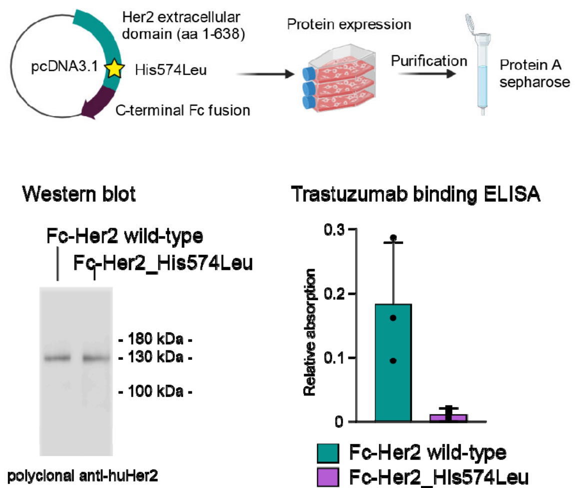

eFigure 8. Recombinant expression of Fc-Her2 wild-type and Fc-Her2-His574Leu protein. Schematic representation of recombinant expression of the extracellular domain (amino acid 1-638) of Her2 wild-type and variant as C-terminal fusion protein with Fc. Western blot analysis shows purified Fc-Her2 wild-type and Fc-Her2-His574Leu fractions using polyclonal anti-human-Her2 antibody. The fusion protein runs at an apparent molecular weight of ~130kDa. The bar graph shows the analysis of trastuzumab binding to Fc-HER2 proteins via ELISA. Values are measured as triplicates of duplicates. Bars indicate mean + SD.

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