Plasma HER2 (ERBB2) copy number predicts response to HER2-targeted therapy in metastatic colorectal cancer

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Rebecca Nagy, Justin Odegaard and Richard Lanman: Employees and shareholders at Guardant Health, Inc.

Salvatore Siena: Advisory board member for Amgen, Bayer, BMS, Celgene, Incyte, Merck, Novartis, Roche, Seattle Genetics.

Alberto Bardelli: Advisory role, Guardant Health, Inc.

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Translational relevance:

ERBB2 (HER2) amplification is an emerging biomarker in colon cancer that confers sensitivity to combination anti-HER2 therapy and predicts resistance to anti-EGFR treatment. Measurement of HER2 copy number is typically performed using tissue obtained from surgical specimen or diagnostic biopsies, but with the advent of cell-free circulating tumor DNA (ctDNA) analysis, this information can also be obtained quickly and non-invasively when tissue is not available, while capturing the spatial and termporal tumor heterogeneity often present in treatment refractory patients. Herein, we present accurate determination of *ERBB2* copy number in ctDNA. We describe a clinically validated ctDNA assay as a reliable diagnostic of *ERBB2* copy number in plasma that predicted response rates to trastuzumab and lapatinib in a mCRC cohort similar to tissue-based HER2 protein expression. We also determined a plasma *ERBB2* copy number cutoff, corrected for tumor shedding, that is predictive of anti-HER2 treatment response.

Abstract

Purpose:

ERBB2 (HER2) amplification is an emerging biomarker in colon cancer, conferring sensitivity to combination anti-HER2 therapy. Measurement of HER2 copy number is typically performed using surgical specimens, but cell-free circulating tumor DNA (ctDNA) analysis may be a non-

invasive alternative. We determined the sensitivity of pCN for detecting *ERBB2* amplifications and whether plasma copy number (pCN) correlated with tissue-detected copy number. We also assessed response to HER2-targeted therapy based on pCN and suggest a pCN threshold predictive of response.

Experimental Design:

Forty-eight pre-treatment and progression plasma samples from 29 HER2-positive patients in the HERACLES A clinical trial were tested using the Guardant360™ cfDNA assay. We correlated *ERRB2* pCN with progression-free survival (PFS) and best objective response (BOR) and applied an adjustment method based on tumor DNA shedding using the maximum mutant allele fraction as a surrogate for tumor content to accurately determine the pCN threshold predictive of response.

Results:

47/48 samples had detectable ctDNA and 46/47 samples were *ERBB2*-amplified based on cfDNA (2.55-122 copies; 97.9% sensitivity (95% CI = 87.2-99.8%). An adjusted *ERBB2* pCN of ≥25.82 copies correlated with BOR and PFS (p=0.0347).

Conclusions:

cfDNA is a viable alternative to tissue-based genotyping in the metastatic setting. The cfDNA platform utilized correctly identified 28/29 (96.6%) of pre-treatment samples as *ERBB2*-amplified and predicted benefit from HER2-targeted therapy. In this study, an observed pCN of 2.4 and an adjusted pCN of 25.82 copies of *ERBB2* is proposed to select patients who will benefit from HER2-targeted therapy.

Introduction

Colon cancer is the 3rd most common cancer worldwide, and approximately 20% of patients present with metastatic disease (mCRC), which is associated with a poor prognosis and median overall survival (OS) of 24–30 months (1). Use of the anti-EGFR monoclonal antibodies cetuximab and panitumumab has improved progression-free survival (PFS) and OS in patients who are negative for *KRAS*, *NRAS* and *BRAF* mutations; however, these therapies are inevitably followed by disease progression (2).

Very few effective therapies remain for the majority of patients with mCRC tumors that have become resistant to cetuximab or panitumimab (3). Amplification of *ERBB2* (HER2) is an emerging biomarker present in 3-5% of genetically unselected mCRC and is enriched in *RAS/RAF/PIK3CA*-wildtype tumors (4,5). Several pre-clinical studies have also suggested that *ERBB2* copy number gain it is a negative predictor of response to anti-EGFR therapy (4,6–8).

Two studies have assessed the feasibility of targeting *ERBB2* amplification in mCRC patients. HERACLES A was an open-label, phase 2 trial of trastuzumab and lapatinib in chemotherapy and EGFR antibody-refractory, HER2-positive patients and showed an objective response rate (ORR) of 30% (95% CI 14–50) and a disease control rate (DCR) of 59% (95% CI 39%-78%) compared to a 41.9% DCR associated with standard-of-care therapy (9–11). More recently, the MyPathways open-label phase 2 basket trial showed a 38% (95% CI 23%-55%) ORR using pertuzumab and trastuzumab in the same population (12). These studies confirmed HER2 as an important driver of mCRC and a successfull therapeutic target in EGFR antibody- and chemotherapy-refractory disease.

HER2 over-expression and/or amplification can be assessed using a variety of tissue-based approaches, including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), PCR, or next generation sequencing (NGS). Non-invasive methods are also possible and

include assessment of HER2 over-expression in circulating tumor cells (CTCs) or measurement of *ERBB2* copy number via next generation sequencing (NGS) of cell-free DNA (cfDNA). Although, tissue copy number (tCN) appears to predict benefit from HER2 inhibition (10) no data exists regarding use of cfDNA to predict this benefit in mCRC.

Measurement of copy number using cfDNA is challenging due to the overwhelming excess of diploid leukocyte-derived DNA relative to the very small amount of tumor DNA in the cell-free compartment, even in individuals with metastatic disease who typically have a large tumor burden. A recent study of cfDNA analysis in >21,000 individuals with metastatic solid tumors showed a median mutant allele fraction of only ~0.4%, which is equivalent to 4 mutant molecules for every 1000 total (mutant and wild-type) molecules, and a median copy number for gene amplifications of 2.56 (13). Therefore, any cfDNA assay utilized for clinical genotyping must be highly sensitive while maintaining high specificity. Furthermore, if the observed copy number in the tumor is low to moderate, even a tumor shedding large amounts of cfDNA into circulation may not yield detectable elevations in plasma copy number (pCN). In this context, as the plasma copy number in plasma is driven not only by tissue copy number but also by the extent of tumor DNA shedding, distinguishing between genomic characteristics, tumor burden, tumor proclivity to shed DNA, and tumor volume changes during therapy remains daunting. Finally, clinical specificity can also be a challenge, as copy number gains in tumor cells can be the result of focal gene amplification, which is often a viable target for drug therapy, or of amplification of large portions of a chromosome, which is less likely to associate with response to targeted therapy (14,15). To effectively identify candidate treatment targets, any assay must be able to discriminate between these two scenarios. Complicating assessment of cfDNA assays, direct comparisons to commonly utilized tissue-based approaches such as FISH and IHC are difficult, as differential shedding of tumor DNA into the circulation affects the pCN.

The goals of this study were: i.) to determine the sensitivity of *ERBB2* amplification detection in plasma using a CLIA-certified, CAP-accredited cfDNA assay relative to standard tissue testing; ii.) to assess *ERBB2* pCN as a predictor of HER2-targeted therapy benefit; and iii.) to establish a pCN threshold to enrich for patients potentially responsive to HER2-targeted.

Materials and Methods

HERACLES A *patient cohort*

HERACLES A patients had a histologically confirmed diagnosis of metastatic colorectal cancer wild-type for *KRAS* exon 2 (codons 12 and 13) and positive for HER2 as defined by 3+ staining in >50% of cells by IHC or 2+ staining and a HER2:CEP17 ratio >2 in >50% of cells by FISH (14). Tissue samples for HER2 testing were derived from primary tumors for 11 (39%) of 28 patients, from metastatic lesions for the remaining 17 (61%). The patient cohort is described in Table 1 and Figure 1. All had measurable disease according to Response Criteria Evaluation in Solid Tumors (RECIST) version 1.1. Patients received treatment and were assessed for objective response, PFS, best overall response and duration of response (11). As part of the trial, patients also underwent serial plasma collection for cfDNA analysis. Pre-treatment and at-progression plasma samples (N=48; 29 pre-treatment and 19 at progression) from 29 HER2-tissue positive patients were tested using the Guardant360® assay (Guardant Health, Inc. Redwood City, CA), and sensitivity was calculated. We also determined the observed *ERBB2* pCN cutoff that maximized the identification of the HERACLES A intent-to-treat population and best predicted response to trastuzumab plus lapatinib therapy.

Guardant360 database and historical mCRC cohort

ERBB2 amplification frequency, pCN distribution and RAS/RAF mutation co-occurrence were determined using the Guardant Health database (accessed April 11th, 2018). This cohort

comprised 4,294 plasma samples from individuals with stage III/IV colorectal cancer undergoing cfDNA analysis (Guardant360) as part of their routine care.

cfDNA analysis

cfDNA NGS analysis was performed at Guardant Health, Inc. (Guardant360; Redwood City, CA), a CLIA-certified, College of American Pathologists (CAP)-accredited, New York State Department of Health-approved laboratory (13,17). The Guardant360 assay detects single-nucleotide variants (SNV), indels, fusions and copy number alterations in 73 genes with a reportable range of ≥0.04%, ≥0.02%, ≥0.04%, and ≥2.12 copies, respectively, as well as microsatellite instability (Supp. Fig. 1A). For the HERACLES A trial, 10ml of whole blood was collected in EDTA tubes. Plasma was separated within 5 hours of collection using 2 different centrifugation steps. Plasma was stored at -80°C until cfDNA extraction. cfDNA was extracted from 1-2 ml of plasma (QIAmp Circulating Nucleic Acid Kit, Qiagen, Inc.), labeled with non-random oligonucleotide barcodes (IDT, Inc.) and used to prepare sequencing libraries, which were then enriched by hybrid capture (Agilent Technologies, Inc.), pooled, and sequenced by paired-end synthesis (NextSeq 500 and/or HiSeq 2500, Illumina, Inc.). Separate sequencing controls were utilized for SNVs and CNs/fusions/indels (CFI) (Supp. Fig. 1B).

Bioinformatic analysis and observed copy number determination

As previously described, base call files generated by Illumina's RTA software (v2.12) were de-multiplexed using bcl2fastq (v2.19) and processed with a custom pipeline for molecule barcode detection, sequencing adapter trimming, and base quality trimming (discarding bases below Q20 at the ends of the reads) (13). Processed reads were then aligned to hg19 using BWA-MEM (arXiv:1303.3997v2) and used to build double-stranded consensus representations of original unique cfDNA molecules using both inferred molecular barcodes and read start/stop positions. To detect copy number amplification, probe-level unique molecule coverage was

normalized for overall unique molecule throughput, probe efficiency, GC content, and signal saturation and robustly summarized at the gene level. pCN determinations were based on training set-established decision thresholds for both observed copy number deviation from per-sample diploid baseline and deviation from the baseline variation of probe-level normalized signal in the context of background variation within each sample's own diploid baseline. Per-sample relative tumor burden was determined by normalization to the mutational burden expected for tumor type and ctDNA fraction and reported as a z-score. Observed *ERBB2* pCN values representing the lower 50^{th}, 50^{th}-90^{th}, and the top 10th percentiles across all amplified samples in the Guardant Health database were calculated (13).

Correlation between ISH, tissue copy number and calculation of an adjusted plasma copy number

tCN in the HERACLES A trial was centrally determined using several methodologies including IHC, FISH and quantitative real-time PCR (qRT-PCR) (11). Observed pCN using cfDNA NGS was compared to FISH and qRT-PCR methods, and Spearman's correlations were calculated. To correct for variation in plasma tumor fraction between samples that can affect the tumor contribution to the circulating DNA pool and consequenctly pCN, we adjusted the observed pCN to the proportion of tumor DNA in each cfDNA sample. To do this, we used the maximum mutant allele fraction (MAF%/100) observed in each individual sample as a surrogate for plasma tumor fraction (T%), as this typically represents the earliest initiating mutation shared by all tumor clones. Genes with the highest MAF were *TP53* (n=15), *APC* (n=11), *PTEN* (n=1) and *RAF1* (n=1) (Supp table 2). We then calculated an adjusted pCN (ApCN); Adjusted pCN = [Observed pCN – 2*(1-T%)]/T% where T% = 2 X MAF^{max}/100. The methodology for calculating adjusted pCN was developed independently and outside of the context of current project and had been finalized prior to integration into the present study.

Clinical outcomes based on adjusted plasma copy number

We correlated ApCN with PFS and best objective response (BOR) on trastuzumab and lapatinib and calculated significance using the Mantel-Cox test. We used ROC curve and optimal cutoff analysis to determine ApCN cutoffs with the highest sensitivity and specificity to predict response.

All patients provided written informed consent. The study was done in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization and Good Clinical Practice guidelines and the US Common Rule. The institutional review boards of the participating centres approved the study procedures.

Results

ERBB2 pCN and RAF/RAF status in the HERACLES A cohort

Forty-eight banked plasma samples from 29 patients were available for analysis, including 29 pre-treatment and 19 at-progression plasma samples. One at-progression sample had no detectable ctDNA, leaving 47 (29 pre-treatment and 18 post-treatment) evaluable samples. *ERBB2* amplification was identified in 46 of 47 plasma samples (28/29 pre-treatment and 18/18 at-progression) for a sensitivity of 97.9% (95% CI = 87.2-99.8%). The mean pCN in pre-treatment samples was 23.1 copies (median = 9.28; range = 2.6-121.7 copies) and 16.76 (median = 8.48; range = 2.13-82.17) in at-progression samples.

To assess focal vs. non-focal amplification, we examined the copy number of genes neighboring *ERBB2* on chromosome 17 to differentiate aneuploidy or large (e.g. arm-level) events. Only a single sample in this study demonstrated a pattern suggestive of a large-scale chromosome 17 amplification event (Supp. Fig. 2A). This patient had stable disease and progressed after 6

months of therapy. The remainder of samples showed patterns most compatible with focal amplification (Supp. Fig. 2B).

Ten *KRAS, NRAS*, and *BRAF* mutations were identified in pre-treatment samples from 9 patients. There were 3 *RAS* codon 12/13 mutations, 5 non-codon 12/13 mutations (4 *KRAS* and 1 *NRAS*) and 3 *BRAF* mutations (V600E, G469A and G596R). *BRAF* G469A and *KRAS* Q61H co-occurred in one sample. Three patients with primary resistance to therapy (n=1) or radiographic progression (n=2) had clonal *RAS/RAF* driver mutations as defined by a *RAS/RAF*:maximum allelic fraction ratio of >0.3 (Fig. 2) (18).

ERBB2 amplification frequency in a historical ctDNA cohort

There were 4,294 unique mCRC patients in the Guardant Health database tested between February 5th, 2015 and April 11th, 2018. Centiles of *ERBB2* pCN were as follows: copy number 2.4, 50th percentile; copy number 4, 90th percentile (Supp. Fig. 3). Of the 4,294 patients, 247 (5.8%) had detectable *ERBB2* amplification, which is compatible with previous reports of prevalence (4,16,19) An *ERBB2* pCN cutoff of $\geq$ 2.4 copies in the historical cohort allowed for exclusion of 84% of all *KRAS, NRAS*, and *BRAF* driver mutations in the historical cohort (Supp. Fig. 4) and suggest that samples with pCN above this threshold represent those for which *ERBB2* amplification is the primary driver of malignancy. An observed pCN cutoff of 2.4 accurately identified 100% of the intent to treat HERACLES A population (Fig. 2).

Correlation between tissue copy number and observed and adjusted plasma copy number (ApCN)

To determine the correlation between tCN and pCN, we compared *ERBB2* observed pCN values with ISH and with tCN as measured by qRT-PCR. There was modest correlation between observed pCN and ISH (Spearman r = 0.49; Fig. 3A) and observed pCN and qRT-PCR

tCN (Spearman r=0.52; Fig. 3C). Compared with observed pCN, adjusted pCN (ApCN) showed stronger correlation with ISH and *ERBB2* pCN by qRT-PCR (Fig. 3B and 3D) with a Spearman R of 0.77 and 0.86, respectively.

Response to therapy based on ApCN and co-occurring alterations

Radiographic response was assessed in 26 patients with *ERBB2* amplification detected in plasma to determine if ApCN correlated with BOR (Fig. 4). Two patients had early clinical progression and were not imaged. We determined an *ERBB2* ApCN cutoff value of 25.82 for optimal segregation of responders versus non-responders using ROC analysis (Supp. Table 2). There were 6 patients with RECIST-defined progressive disease (PD) and 2 patients with clinical evidence of primary resistance to therapy for whom imaging was not available at the time of progression. Of these 8 cases, 6 had an ApCN below 25.82 and 2 had a pCN $\geq$ 25.82 (Supp. Table 2). In addition to a pCN below 25.83, 3 patients with progressive disease had clonal *KRAS* or *BRAF* mutations (KRAS G12V, G12D and *BRAF* V600E) identifed in plasma (Supp. Table 2). Twenty patients (20/28, 71%) had some degree of clinical benefit, including 13 with stable disease, 6 partial response and 1 complete response. Thirteen of these 20 patients had a ApCN $\geq$ 25.82. Of these 13 patients with a ApCN $\geq$25.82, 7 had their disease controlled by anti-HER2 treatment: 1 achieved a partial response and the remaining 6 had stable disease according to RECIST. We also assessed the correlation between ApCN and PFS. The median PFS in individuals with a ApCN < 25.82 was 14.8 weeks, as compared to 22.5 weeks in those with a ApCN $\geq$ 25.82 (Mantel Cox p = 0.0347, Fig. 5).

Discussion

Non-invasive methods of comprehensive genomic profiling are becoming standard clinical practice because they provide rapid and accurate identification of clonal driver alterations and selection of appropriate targeted therapy and allow for serial assessment of clonal tumor

dynamics (20). In mCRC, HER2-targeted therapy is emerging as an active therapy, but it has not entered widespread use, in part, due to difficulties in identifying HER2-positive mCRC patients who are likely to experience clinical benefit (18). In this context, not all cfDNA assays are well-validated, and many have limitations around the types of alterations they can detect with high sensitivity and specificity (21). Similarly, attempts in mCRC to isolate circulating tumor cells have resulted in limited and mixed success (22). Therefore, we utilized a cfDNA NGS assay that has been extensively validated for all four major types of genomic alterations and microsatellite instability (13). Copy number amplification is a challenging alteration type to detect in cfDNA due to the high ratio of diploid leukocyte DNA to tumor DNA in circulation, which dilutes tumor copy number signals. In our analysis, the cfDNA assay utilized detected *ERBB2* amplification in 28 of 29 pre-treatment and 18 of 18 evaluable at-progression plasma samples from the HERACLES A cohort.

In the present series we observed weak correlation between observed pCN and tCN using a variety of methods, including IHC, ISH and qRT-PCR. This is in part due to the fact that different methodologies are measuring different analytes (protein over-expression vs. number of gene copies) and are not all quantitative (IHC, ISH), which can make direct comparisons difficult. However, the major confounding factor when comparing blood and tissue methods of copy number assessment is the variation in the amount of tumor DNA shed into the bloodstream. Concordance between tissue and plasma is often high when two criteria are met: 1) the observed copy number in the tumor is high and 2) there is ample shedding of DNA into circulation, i.e. the tumor fraction in circulation is high. In addition, pCN represents a summary of all amplified lesions that may be shedding DNA into circulation. As a result, pCN may be impacted by the heterogeneity of actual copy number across tumor sites. These factors may limit the sensitivity of cfDNA assessment, and make comparison of copy number between tissue and plasma challenging. Furthermore, observed pCN is often misleadingly low in samples with

low tumor fraction, despite high tCN, which could prevent patients with true oncogenic driver amplifications from receiving appropriate targeted therapy. To overcome some of these technical barriers, we adjusted the observed pCN for the amount of tumor DNA shedding using the maximum mutant allele fraction as a surrogate for tumor content. The resulting adjusted plasma copy number (ApCN) correlated with tCN and response to therapy in our analysis.

Our results demonstrate a correlation between PFS/BOR and level of *ERBB2* amplification; however, not all patients with high *ERBB2* pCN responded (Fig. 4). One patient (121024) showing progressive disease had *ERBB2* ApCN of 27.38, but there were no co-occurring mutations present in the baseline sample to explain the lack of response. There were also 5 patients with stable disease, 1 with an unconfirmed partial response and 1 with a partial response who exhibited plasma copy numbers below the cutoff. Some of these patients showed evidence of acquired resistance mutations at the time of progression. Resistance mechanisms and ctDNA dynamics over the course of treatment in the HERACLES cohort have been extensively studied and are described in detail elsewhere (18). Interestingly, 3 patients with either progressive disease or primary clinical progression harbored clonal *KRAS* (n=2) *BRAF* (n=1) mutations, as assessed by cfDNA analysis. Although the HERACLES A trial excluded patients with *KRAS* mutations in their diagnostic tissue in most cases (the treatment-naïve primary tumor), these *RAS/RAF* mutations presumably developed during the prior course of anti-EGFR therapy and dominated the original *KRAS* WT clonal populations under selective pressure. Screening for *BRAF* mutations was not required for entry into HERACLES A but is now part of the NCCN guidelines, given the 4-5% mutation frequency in mCRC and associated lack of response to anti-EGFR therapy (3).

Several studies have discussed the utility of cfDNA in assessing copy number amplification. Liang et al. showed perfect concordance (n=7 of 7 patients) between tissue and plasma-

identified *ERBB2* CN in metastatic breast cancer patients (23). All 7 patients were given anti-HER2 therapy, and 6 of the 7 had a clinical response to therapy, underscoring the therapeutic relevance of *ERBB2* pCN assessment. Similarly, in two separate studies of untreated gastric/gastroesophogaeal cancer patients, high *ERBB2* pCN was a positive predictor of patient response and plasma was able to capture copy number changes present in both the primary tumor and the metastases (24). These papers highlight the fact that pCN measurements, unlike tissue measurements, are often a summary of all shedding lesions and can be influenced by tissue heterogeneity. The HERACLES A study further highlights the benefits of cfDNA copy number analysis and underscores the ability of cfDNA to capture tumor heterogeneity in mCRC patients. This latter capability may be similarly important in mCRC as, despite high concordance for somatic mutations between primary tumors and metastases, there is significant discordance (6%-15%) for tissue-assessed *ERBB2* copy number amplifications (25,26).

Limitations of this study include the small sample size and the lack of HER2-negative patients in the HERACLES A cohort. While the latter prohibits calculation of specificity, positive predictive value and negative predictive value in this cohort, the specificity and positive predictive value of the copy number assessment method utilized here have been described elsewhere (13). In that study, analytic specificity was $\geq$ 99.9% and PPV was 100% when compared to ddPCR of cell lines with known gene copy number status. Another possible limitation of the methods employed here is distinction between focal copy number amplification, which in the case of *ERBB2* in mCRC, is a druggable target, as compared to aneuploidy, which may not always result in protein over-expression and thus may not always respond to targeted agents. Despite the clear pattern of focal amplification in this study and others (19), these results cannot be considered representative of focal vs. non-focal amplification rates in other contexts as these vary widely by chromosome, cancer type, and treatment context. Another limitation of the study is the lack of detailed clinical information on the historical ctDNA cohort. The ctDNA test utilized here is

performed in a large reference laboratory where details such as previous therapy and current therapy at the time of the blood draw, histology, stage of disease at diagnosis, previous genomic testing results, etc. are not typically provided by the ordering clinician. Therefore, although the historical cohort represents later stage mCRC patients, the cohort is likely heterogeneous in terms of previous and current therapy status and direct comparisons between this cohort and the HERACLES A cohort must be done with caution. Lastly, the pCN adjustment method utilized here remains exploratory in nature and has not been validated in a separate cohort. Although the correlation of adjusted pCN with both FISH and tCN as determined by qRT-PCR and PFS/BOR suggest that this is a robust correction method, further validation of the model is necessary in additional larger cohorts. Furthermore, the current adjustment method does not consider copy number amplification or loss of heterozygosity of the gene comprising the mutation with the maximum mutant allele fraction from which tumor fraction is inferred. In particular, loss of heterozygosity in *TP53* or *APC*, which are often the mutations with the highest mutant allele fraction in mCRC samples, could result in an over-representation of mutant alleles in the cfDNA sample and therefore result in an overestimation of tumor fraction. Correction for this may provide more accurate estimates of tCN as reflected in the plasma and should be explored.

ERBB2 amplification is an emerging therapeutic target in the mCRC setting and may also be a negative predictor of response to anti-EGFR therapy (4,7). In this series, comprehensive cfDNA NGS accurately identified *ERBB2* amplification in 96.6% (28/29) of the intent-to-treat population, suggesting that ctDNA can be used as a surrogate for tissue especially in cases when archival tissue cannot be obtained in a timely manner and re-biopsy is not preferred. In an independent study (20), ERBB2 amplification was detected in 2 mCRC patients by both cfDNA profiling and chromogenic in situ hybridization, further attesting to the value of cfDNA analysis in capturing the tissue genomic make-up. Additional investigation is needed to determine if plasma CN can

replace tissue CN assessment. In addition, as previously described, the assay identified co-occurring mutations in *KRAS*, *BRAF* and *ERBB2* that were predictive of resistance to therapy (27). Furthermore, we describe an adjusted pCN threshold above which patients are more likely to respond to targeted therapy. These results support use of appropriately validated cfDNA tests as an alternative to tissue biopsy to identify individuals who may benefit from anti-HER2 therapy. Additional prospective studies in larger cohorts are needed, particularly in treatment-naïve mCRC patients where targeted therapy may be most efficacious.

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Table 1. Patient characteristics

Characteristic	N (28)
Gender	
Male	22
Female	6
Median age at enrollment	63 yrs. (range 41-86)
Primary tumor site	
Rectum	7
Proximal colon	5
Distal colon	16
HER2 IHC Score	
2+(FISH AMPLIFIED)	6
3+	22
No. of previous lines therapy	
<3	7
≥3	21

Figure legends

Figure 1. Consort diagram showing the HERACLES A cohort and the subset of patients included in the present study.

Figure 2. *ERBB2* pCN, RAS/RAF status and maximum mutant allele fraction (Max MAF) in baseline plasma samples from the HERACLES A cohort. An observed pCN cutoff of 2.4 (>50th %ile) allowed for identification of the HERACLES A intent-to-treat population. Both clonal (dark blue) and sub-clonal (light blue) RAS/RAF mutations were identified in pre-treatment plasma samples.

Figure 3.

Correlation between HER2/*ERBB2* status in tissue and *ERBB2* status in plasma. Observed pCN does not correlate strongly with HER2 ISH status (3A) or *ERBB2* copy number as measured by qRT-PCR (3C), but adjusted plasma copy number correlates well with ISH and *ERBB2* copy number as measured by qRT-PCR (3B and 3D).

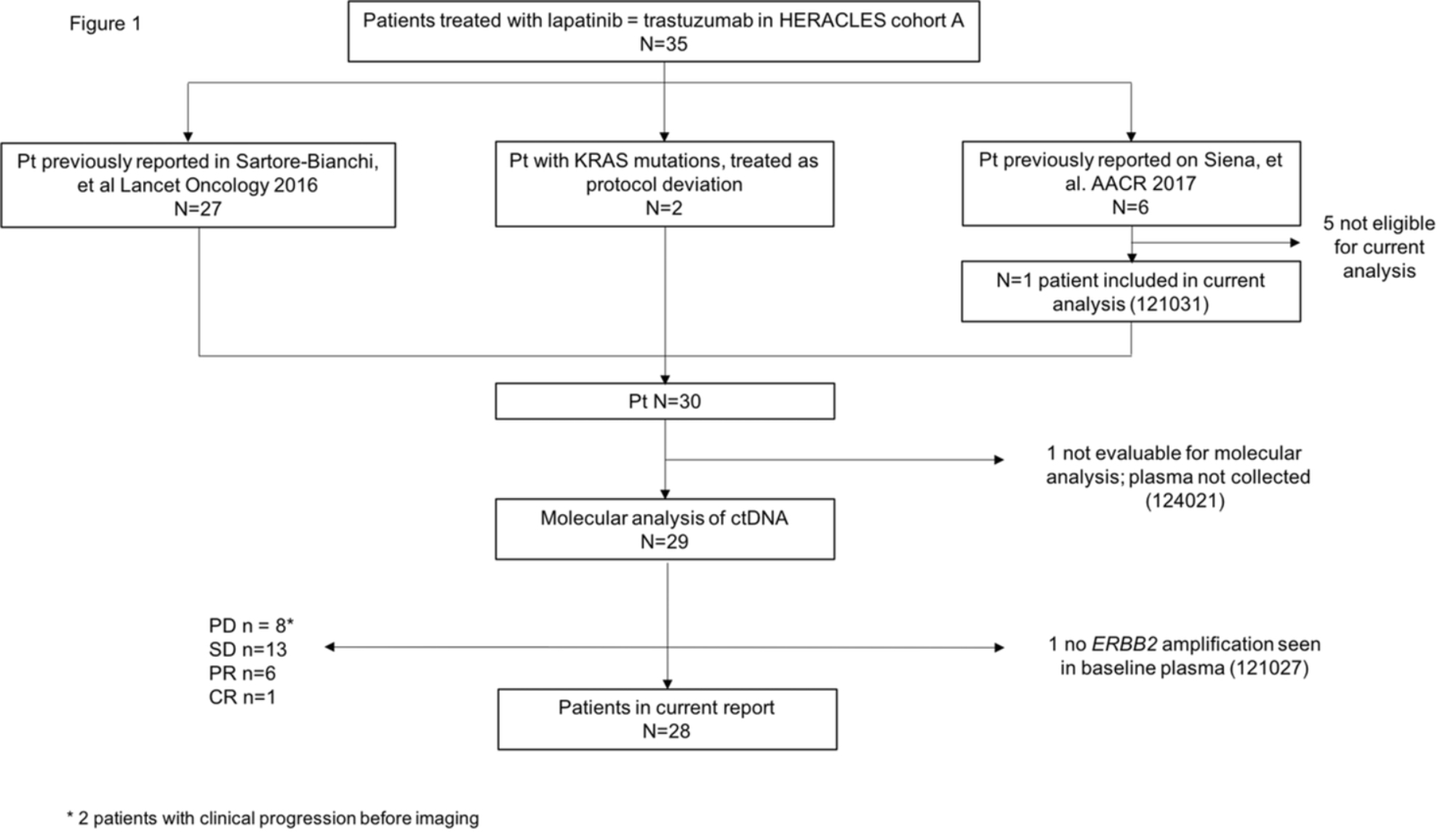
Figure 4.

Best objective response based on adjusted pCN in baseline HERACLES A samples. Bars show the change in target lesion size from baseline to first progression. Horizontal dotted lines correspond to a 20% increase in target lesion size from baseline (top line) and 30% decrease in target lesion size from baseline (bottom line). Red bars represent samples with an adjusted pCN (ApCN) $\geq$ 25.82 and blue bars an ApCN of < 25.82. * indicates patients with clonal *RAS/RAF* mutation in baseline plasma samples. Arrows indicate two patients with primary clinical progression who did not undergo radiographic imaging.

Figure 5.

Progression-free survival by adjusted pCN (ApCN). Red line shows patients with an ApCN $\geq$ 25.82 and the blue line patients with an ApCN of < 25.82.

Figure 1



* 2 patients with clinical progression before imaging

Figure 2

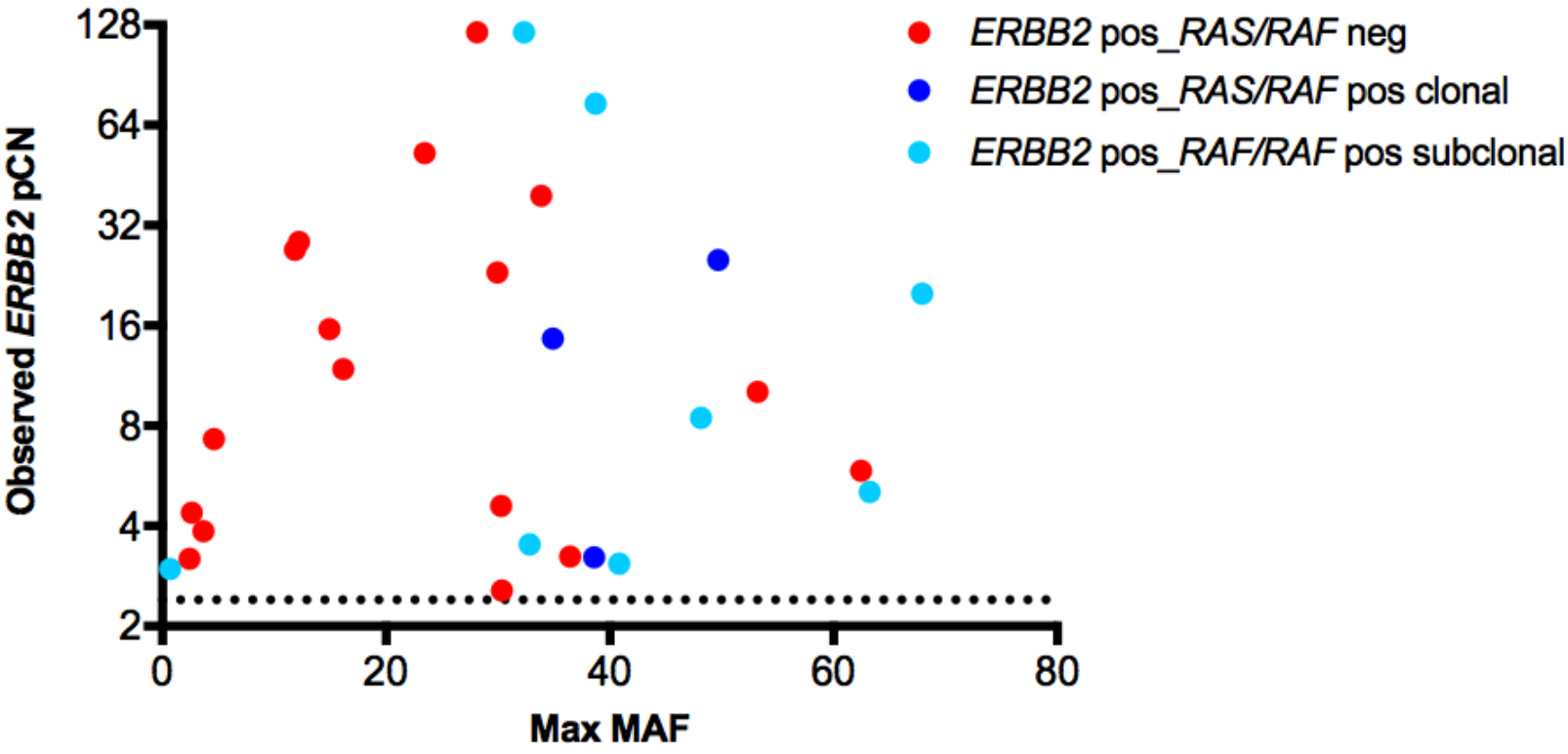

128
64
32
16
8
4
2
Observed ERBB2 pCN
0
20
40
60
80
Max MAF
ERBB2 pos_RAS/RAF neg
ERBB2 pos_RAS/RAF pos clonal
ERBB2 pos_RAF/RAF pos subclonal

Figure 3

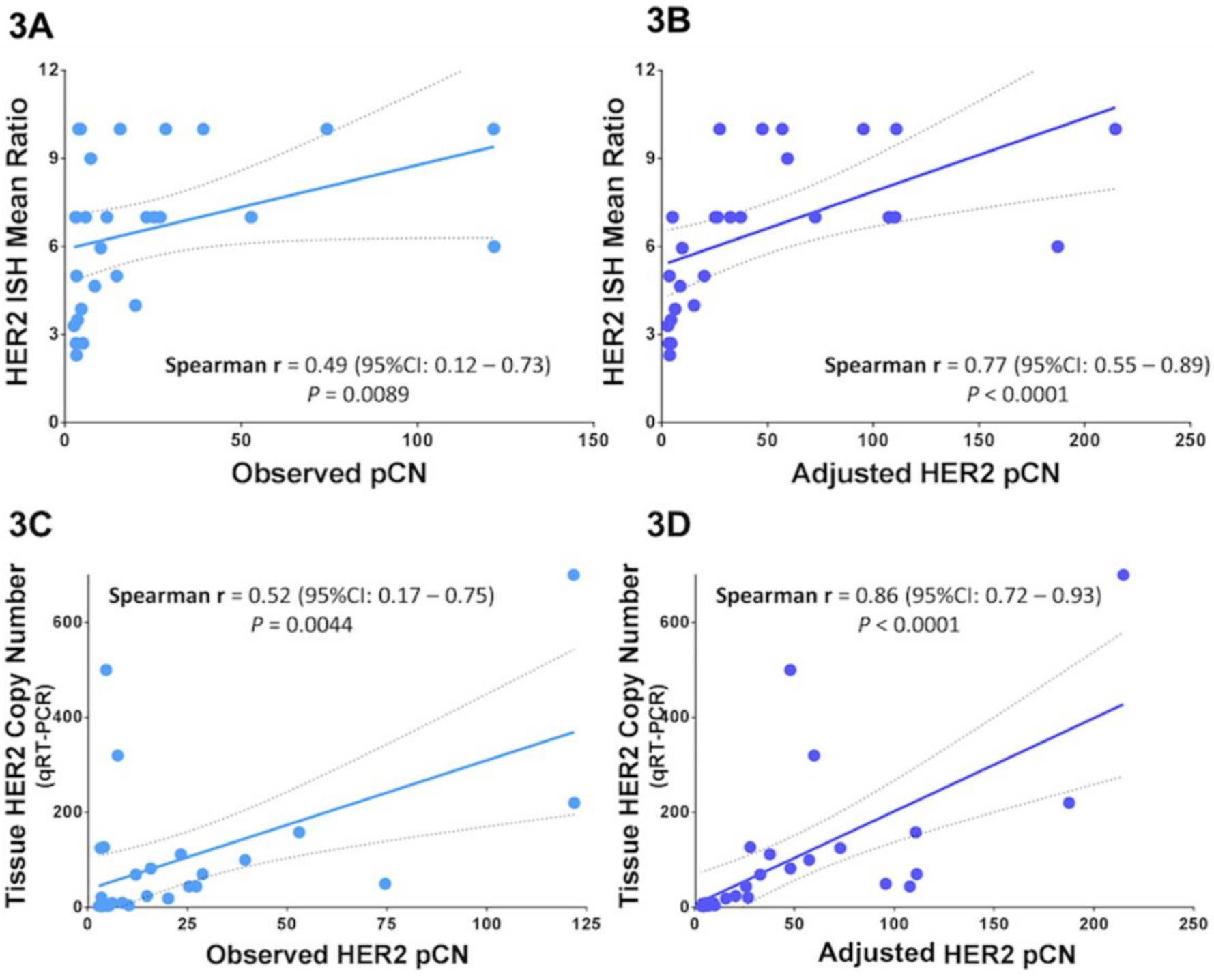

3A
HER2 ISH Mean Ratio
Observed pCN
Spearman r = 0.49 (95%CI: 0.12 – 0.73)
P = 0.0089
3B
HER2 ISH Mean Ratio
Adjusted HER2 pCN
Spearman r = 0.77 (95%CI: 0.55 – 0.89)
P < 0.0001
3C
Tissue HER2 Copy Number (qRT-PCR)
Observed HER2 pCN
Spearman r = 0.52 (95%CI: 0.17 – 0.75)
P = 0.0044
3D
Tissue HER2 Copy Number (qRT-PCR)
Adjusted HER2 pCN
Spearman r = 0.86 (95%CI: 0.72 – 0.93)
P < 0.0001

Figure 4

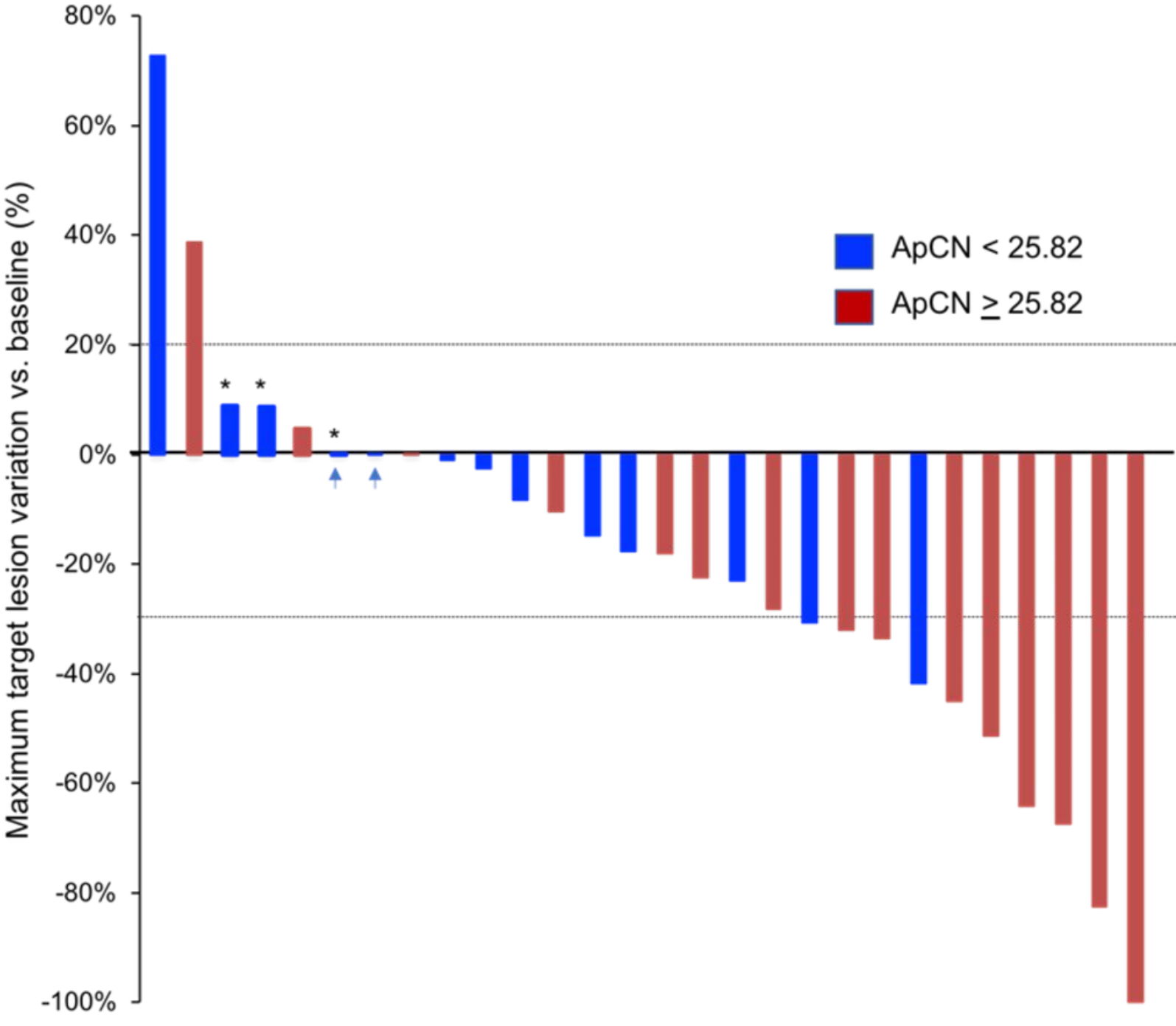
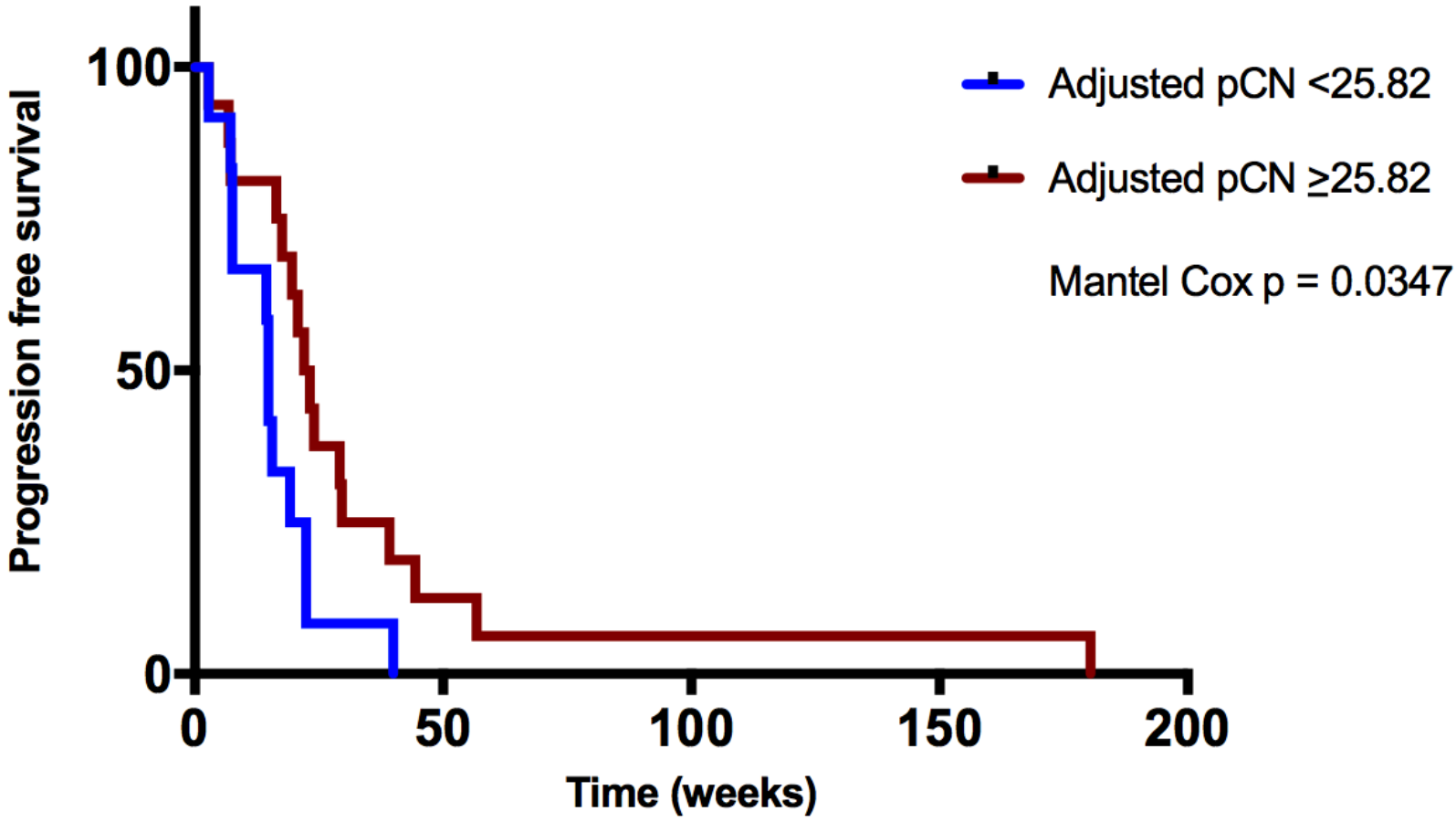

80%
60%
40%
20%
0%
-20%
-40%
-60%
-80%
-100%
Maximum target lesion variation vs. baseline (%)
ApCN < 25.82
ApCN ≥ 25.82
*
*
*

Figure 5


Progression free survival
100
50
0
0
50
100
150
200
Time (weeks)
Adjusted pCN <25.82
Adjusted pCN ≥25.82
Mantel Cox p = 0.0347