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First-line treatment of unresectable or metastatic HER2 positive esophagogastric adenocarcinoma: liquid biomarker analysis of the phase 2 INTEGA trial

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ABSTRACT

Background The addition of nivolumab to trastuzumab and chemotherapy in first-line unresectable or metastatic HER2 positive esophagogastric adenocarcinoma (HER2+ EGA) results in long progression-free and overall survival as shown by the INTEGA (ipilimumab or FOLFOX in combination with nivolumab and trastuzumab in HER2 positive esophagogastric adenocarcinoma) trial. This trial suggested that the chemotherapy backbone is needed in an unselected HER2+ patient population. Yet, it remains an open question if there are specific patient subsets that may benefit from an enhanced immunotherapeutic but chemotherapy-free approach.

Methods We analyzed blood T cell repertoire metrics determined by next-generation sequencing, circulating tumor cell (CTC) counts detected by CellSearch and their expression of HER2 and PD-L1 as potential liquid biomarkers predicting outcomes on ipilimumab versus FOLFOX (folinic acid, FOL, fluorouracil, F, oxaliplatin, OX) chemotherapy added to a backbone of trastuzumab and nivolumab in patients with HER2+ EGA in the INTEGA trial population.

Results Patients with two out of three baseline-determined liquid biomarkers—high T cell repertoire richness, absence of CTCs or HER2-expression on CTCs—made up approximately 44% of HER2+ EGA cases and did not show compromise in efficacy if treated with a chemotherapy-free regimen. Long-term responders showing a progression-free survival of >12 months were enriched in this biomarker triad, especially if treated on the chemotherapy-free arm.

Conclusion Prospective validation of this liquid biomarker triad is needed to molecularly define HER2+ EGA patient subsets with different needs in the first-line systemic treatment setting.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The INTEGA trial showed benefit of adding immunoncology drugs (IO) to first-line treatment of patients with advanced HER2+ esophagogastric adenocarcinoma, while supporting the role of chemotherapy in unselected patients. It remained largely unclear if there were patient subsets that could safely omit chemotherapy.

WHAT THIS STUDY ADDS

⇒ This study provides biomarker analyses from the INTEGA trial suggesting that patients with favorable blood T cell metrics, absence of circulating tumor cells (CTCs) or HER2 expression on CTCs may be a subset that derives at least equal benefit from an IO-only approach combined with HER2 targeting, while patients without these markers need additional chemotherapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The study contributes to a molecular definition of patient subsets with different needs in the first-line systemic treatment setting. The identified set of liquid biomarkers should be prospectively validated.

BACKGROUND

Globally, 1.1 million new cases of gastric and 600,000 new cases of esophageal cancer occur each year.[1] With 770,000 and 540,000 deaths per year, these cancers remain among the leading causes of cancer-related deaths worldwide.[1] The majority of patients—approximately 75%—suffer from recurrent or metastatic disease at some point. These patients are in need of an effective palliative therapy to extend overall survival (OS) with good quality of life.

For advanced HER2+ esophagogastric adenocarcinoma (EGA), the combination of HER2 inhibition, PD-1 inhibition and chemotherapy has evolved as a new standard of care.[2–4] The AIO INTEGA phase II trial randomized patients to either receive trastuzumab/nivolumab combined with a standard

chemotherapy backbone (FOLFOX arm) or a chemotherapy free combination with ipilimumab (Ipi arm).[5 6] This study demonstrated favorable activity of the FOLFOX arm, while the Ipi arm showed inferior outcomes. Efficacy of the chemotherapy-free Ipi arm was comparable to the previous standard of care with chemotherapy and HER2-directed treatment without the addition of a PD-1 antibody.[5 7] Therefore, an upfront chemotherapy free strategy is currently not advisable in unselected patients with HER2+ EGA.

Despite this, some long-lasting responses in the Ipi arm of the INTEGA trial were observed pointing at potential patient subsets in which a chemo-free approach may be equally effective or even superior to the chemotherapy-containing regimen. Identifying patients that are highly susceptible to intensified immunotherapy regimens would be highly desirable to work on the 'tail of the survival curve'. Moreover, pre-existing conditions (such as severe neuropathy) may preclude the use of chemotherapy regimens in subsets of patients. These patients need counseling for viable alternatives that do not put them at risk for early progression.

This translational study in patients treated on the INTEGA trial defined patients that may benefit at least equally from intensified immunotherapy combined with targeted therapy as compared with a chemotherapy-containing regimen.

METHODS

INTEGA trial design and biosampling

Patient eligibility for the trial is described in detail in the original publication.[5] In brief, eligible patients had HER2+ EGA with inoperable, locally advanced or metastatic disease. Patients were randomized to trastuzumab and nivolumab with either FOLFOX (FOLFOX arm) or ipilimumab (Ipi arm). For biomarker studies, peripheral blood was acquired to isolate leucocyte DNA for T cell repertoire analyzes and circulating tumor cells (CTCs) prior to treatment initiation and before the second treatment cycle. For T cell repertoire analyzes, blood was collected in cell-free DNA BCT tubes (STRECK, USA) and processed according to the manufacturer's instructions. The leucocyte pellet was frozen in 1 mL heat-inactivated Fetal Bovine Serum (Life Technologies, USA) with 10% dimethyl sulfoxide (Sigma, Germany). Enumeration of CTCs and phenotyping of HER2 and PD-L1 were performed using the CellSearch system (Menarini Silicon Biosystems; Bologna, Italy). From each patient, two samples of 7.5 mL blood at baseline (BL) and after the first treatment cycle were collected into CellSave tubes (Menarini) and processed within 96 hours storage at room temperature. In brief, after automatic EpCAM (Epithelial cell adhesion molecule)-based immunomagnetic enrichment and immunofluorescence staining, cells were automatically scanned by the CellTracks Analyzer II (Menarini) and keratin-positive/CD45-negative nucleated cells with a diameter of at least 4 µm were referred to as CTCs. Immunofluorescent HER2 and PD-L1 stainings of CTCs were described previously.[8 9]

For survival analyses, patients were divided into subgroups based on different CTC levels. For HER2-status and PD-L1-status of CTCs, the highest measured score per cell per patient was used. Patients were divided into subsets with a HER2-score or PD-L1-score of ≥1 vs no expression. Cases with missing values were excluded from the corresponding analyses.

T cell repertoire analyzes by next-generation immunosequencing

Amplification of the T cell receptor beta chain (TRB) repertoire was performed from 250 ng genomic DNA of circulating leucocytes as previously described.[10–16] Sequencing and demultiplexing was performed on the Illumina MiSeq platform (San Diego, USA) with V.3 chemistry in a 601-cycle single indexed, paired-end run at an average coverage of 80,000 reads per sample. The *TRB* locus was assembled using the MiXCR analysis tool V3.0.12.[13 17] Sequences with less than two reads and out-frame rearrangements were dropped. All repertoires were normalized to 20,000 productive reads. Analyses and data plotting were performed using R (V.4.2.1)[18] with package tidyverse.[19] TRB metrics including clonality, richness and diversity were calculated as described before[20] with the package tcR.[21] Richness was defined by the number of unique CDR3 amino acid sequences per repertoire. For survival analyses, patients were divided into subgroups with higher or lower richness than the median richness of the corresponding cohort per timepoint (median richness at BL=1131, after first treatment cycle=1348). Kaplan-Meier plots were generated with packages survminer[22] and survival.[23] Correlation analyses were computed using R package corrplot.[24] Principal component analysis including axis contributions was calculated using package ade4.[25] A $p<0.05$ was considered statistically significant. The datasets generated for this study can be found in the European Nucleotide Archive (ENA) under accession number ID: PRJEB55475.

RESULTS

High peripheral blood T cell richness is associated with favorable outcomes on regimens containing immune checkpoint inhibitors for HER2+ EGA

Richness in peripheral blood T cell clonotypes had been previously found associated with favorable outcomes on treatment with immune checkpoint inhibitors.[10 26–29] We investigated the peripheral blood T cell receptor repertoire by sequencing the *TRB* locus from genomic DNA of circulating leucocytes in the INTEGA trial. TRB metrics were similar before (BL) and after the first treatment cycle (C1) with a trend toward higher richness after C1 (figure 1). To explore potential predictive effects of TRB richness, we compared progression-free survival (PFS) and OS in patients with higher or lower than median T cell repertoire richness. Patients with high richness showed

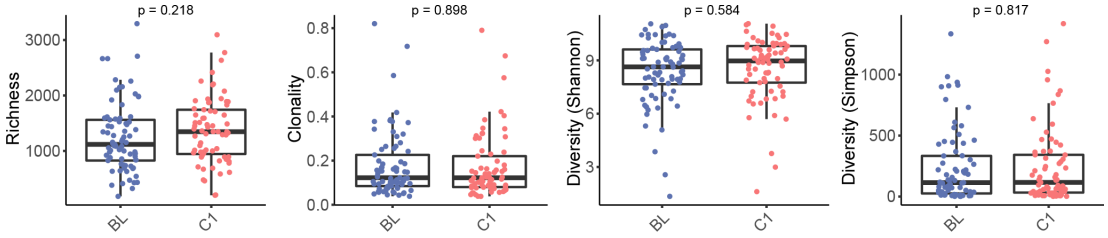

Figure 1 Metrics of the blood T cell receptor beta repertoire at baseline (BL) and after the first treatment cycle (C1). T cell repertoire metrics richness, clonality, Shannon diversity and Simpson diversity were calculated and plotted. Repertoires of all patients of the INTEGA trial with available samples were included in the analysis, both from the Ipi and FOLFOX arm. Statistical test: ANOVA. ANOVA, analysis of variance; FOLFOX, folinic acid (FOL), fluorouracil (F), oxaliplatin (OX).

significantly improved median PFS (9.5 vs 4.4 months for BL and C1 metrics) and a higher median OS (22.1 vs 12.6 months for BL and 31.2 vs 12.9 months for C1 metrics) (figure 2). The favorable effects of T cell repertoire richness were especially pronounced in patients that had high T cell repertoire richness both in the BL and C1 sample. Patients with high T cell richness at only one time point had somewhat shorter PFS and those with low T cell richness at both time points showed the most unfavorable outcomes (figure 2B,C).

The absence of CTCs and expression of HER2 on CTCs is associated with longer survival

CTCs represent a critical cellular compartment since they play a major role in tumor dissemination and metastasis. Their biological properties and biomarker profiles are therefore of great importance. CTCs were enumerated (table 1) and patients allocated to two groups with >0 or 0 CTC at BL and after the first treatment cycle. This analysis showed that the absence of CTCs after initiation of therapy was associated with numerically longer PFS (median 4.2 vs 2.4 months) and significantly longer OS (median 6.5 months vs NA) in the Ipi arm (figure 3). CTC quantification before treatment initiation was less informative for survival outcomes (online supplemental figure S1).

Due to the important biological role of CTCs, we reasoned that the analysis of biomarkers in this specific compartment might proof very useful to predict treatment outcomes. We, therefore, stained CTCs isolated at BL with antibodies against HER2 and PD-L1. The staining of CTCs showed partial overlap with the staining scores of tumor slides (retrieved from Stein *et al*[5] but added additional information in the majority of cases (figure 4A,B). Interestingly, HER2 expression of CTCs correlated strongly with improved outcomes in the Ipi arm (median PFS 8.4 vs 1.7 months, median OS 23.3 vs 7.9 months) but not in the FOLFOX arm (figure 4C). In contrast, PD-L1 expression did not show any association with treatment outcomes neither in the Ipi nor the FOLFOX arm consistent with the previously reported failure of PD-L1 tissue expression to predict treatment benefit in any of the two arms[5] (figure 4D).

Definition of marker panel for patients that may omit chemotherapy in first-line systemic treatment

Since high T cell richness, absence of CTCs or HER2 positivity of CTCs were associated with favorable outcomes on the Ipi arm, we wished to explore correlations between these biomarkers. Correlation analysis of all biomarkers—interestingly—showed no correlation between CTC counts at BL and C1 while T cell repertoire richness was correlated at BL and C1 (figure 5A). High CTC counts were weakly associated with high PD-L1 expression on CTC, but not with HER2 expression. This analysis indicated that all three parameters associated with favorable outcomes on the Ipi arm were independent parameters.

In a clinical perspective, the triad of high T cell repertoire richness, absent CTCs or HER2 expression of CTCs may enable clinicians to identify patients in whom replacement of chemotherapy by ipilimumab is safe. To explore the respective discriminative power of these markers, Kaplan-Maier analyzes examining different combinations of markers as well as principle component analyses were performed. We included only BL markers in this analysis since these were deemed most relevant for up-front patient identification. Patients who showed positivity for two out of three favorable liquid biomarkers comprised 44% of patients in the INTEGA cohort. When treated on the Ipi arm, these showed significantly higher median PFS (12.8 vs 3.2 months) and numerically higher OS (NA vs 8.2 months) than patients that were positive for only one or none of these biomarkers (figure 5B). The same analysis in the FOLFOX (folinic acid, FOL, fluorouracil, F, oxaliplatin, OX) arm showed no statistically significant effects on PFS (13.3 vs 9.6 months) or OS (22.1 vs 32.9 months). Patients with two out of three favorable liquid biomarkers showed no significant difference in PFS and OS on the two different treatment arms, although the median OS was not reached on the Ipi arm (figure 5C). Long-term responders that showed >12 months PFS on first-line systemic treatment were enriched in patients with the favorable triad of biomarkers, especially on the Ipi arm (table 2).

Together, this suggested that approximately 44% of patients with HER2+ EGA may derive at least equal benefit from a chemotherapy-containing and a chemotherapy-free intensified immunotherapy regimen.

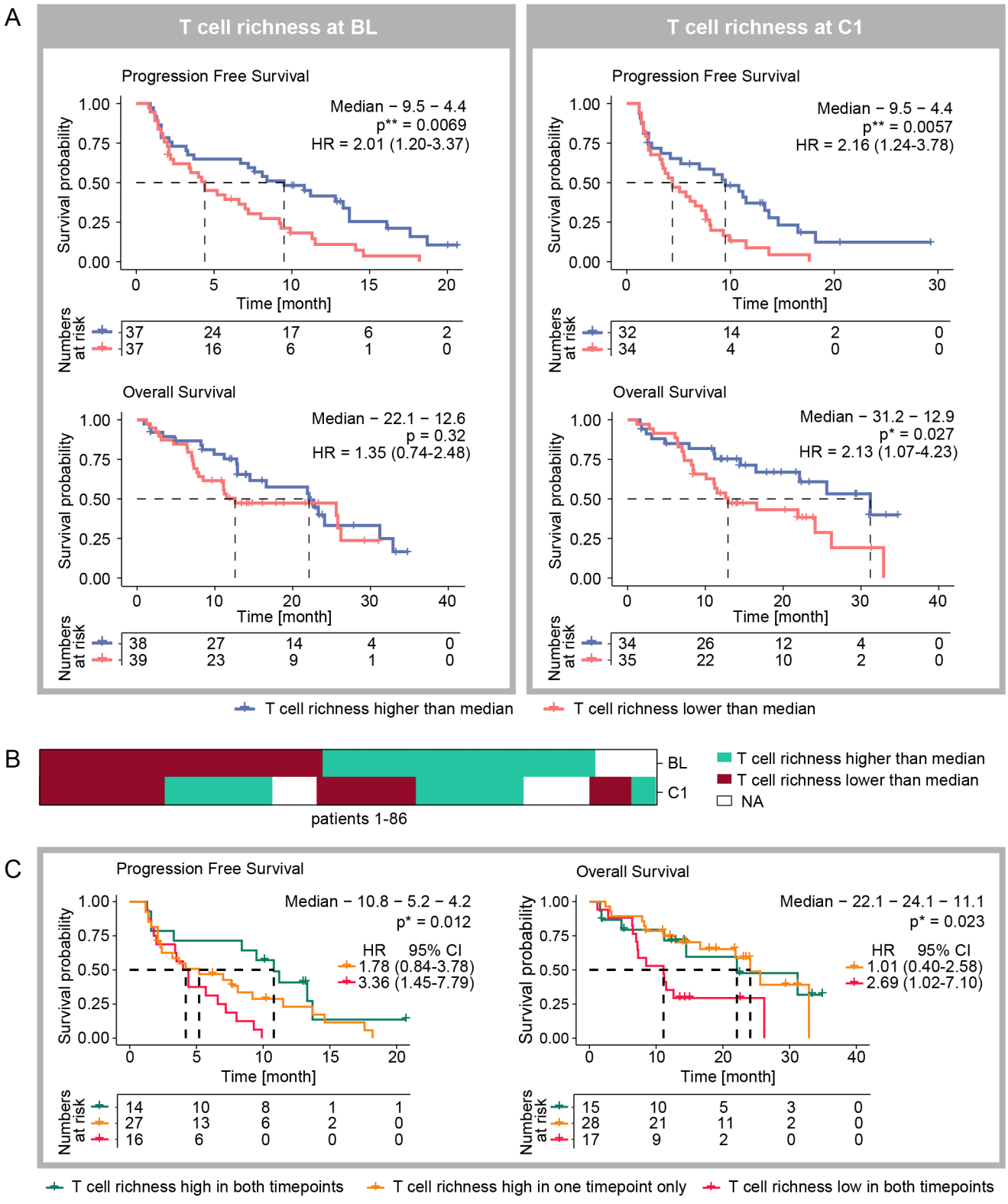

Figure 2 Survival probability in relation to blood T cell richness. Kaplan-Meier plots of progression-free survival (PFS) and overall survival (OS) of INTEGA patients. (A) Patients with high versus low T cell richness at baseline (BL) versus after the first treatment cycle (C1) are shown. (B) Distribution of T cell richness before and after treatment initiation referring to median in individual patients. (C) Kaplan-Meier plots of PFS and OS of patients in both treatment arms combined. Patients with T cell richness above median in both time points are grouped versus patients with T cell richness above median at either BL or C1 versus patients with T cell richness below median in both time points. The p values were calculated using log-rank test. Median survival is given in month. HR of Cox regression including 95% CIs in brackets; *, p<0.05, **, p<0.01.

Table 1 Number of patients with CTC counts at different cut-offs

Time point	CTC count measured	CTC count >0	CTC count ≥3	CTC count ≥5
Baseline	63	39	24	22
After first treatment cycle	33	14	7	7

CTC, circulating tumor cell.

DISCUSSION

Chemotherapy in combination with HER2 and PD-1-directed therapy is becoming the standard of care for locally advanced or unresectable HER2+ EGA. This new standard was again confirmed by the results of the INTEGA trial that showed high efficacy of the combination of chemotherapy, PD-1 and HER2 inhibition while pointing at overall inferior outcomes with a chemotherapy-free regimen in an unselected cohort of patients. These results are very consistent with previously published literature: In tumor entities that are generally considered chemotherapy-sensitive, this component of systemic treatment can only be omitted in biomarker-defined subgroups of patients (eg, PD-L1 expressing or microsatellite instability high tumors).[30 31] In HER2+ EGA, the clinical success of immunotherapy has unfortunately not yet generated deep insight that could help to define patient subsets that benefit most from immune checkpoint inhibitors plus targeted agents versus combinations including chemotherapy. Yet, there is an unmet clinical need of biomarker guidance in such treatment decisions to increase the rate of long-term responders and to spare chemotherapy in patients that do not need it in first-line treatment.

Replacing the chemotherapy backbone with ipilimumab failed to improve OS and underperformed in ORR and PFS compared with historical data in the INTEGA trial. Yet, consistent with comparable trials in other entities, we observed a number of long-term responders in the arm that included nivolumab and ipilimumab suggesting that a chemotherapy-free intensified immunotherapy approach may result in long-term disease control in some patients. For future studies and better treatment stratification, it is mandatory to molecularly define this subset of patients that displays exquisite sensitivity to immunotherapy.

To identify potential blood-based biomarkers, we performed TCR immune repertoire deep sequencing,

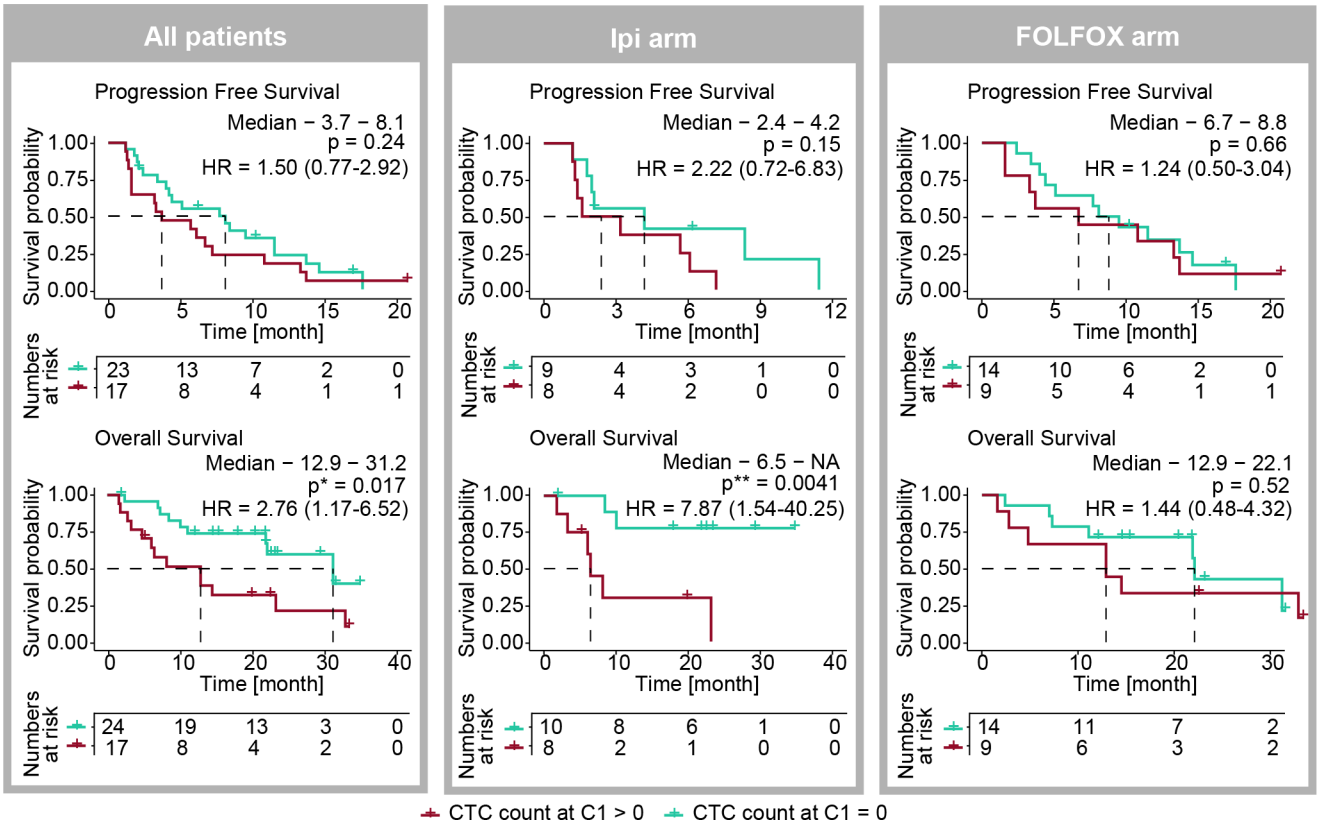

Figure 3 Survival probability in relation to the numbers of circulating tumor cells (CTCs) after the first treatment cycle (C1). Kaplan-Meier plots of progression-free survival and overall survival for all patients (left row), Ipi arm (middle row) and FOLFOX arm (right row). Patients with CTC counts >0 vs all others are shown. The p values were calculated using log-rank test. Median survival is given in month. HR of Cox regression including 95% CIs; FOLFOX, folinic acid (FOL), fluoruracil (F), oxaliplatin (OX).

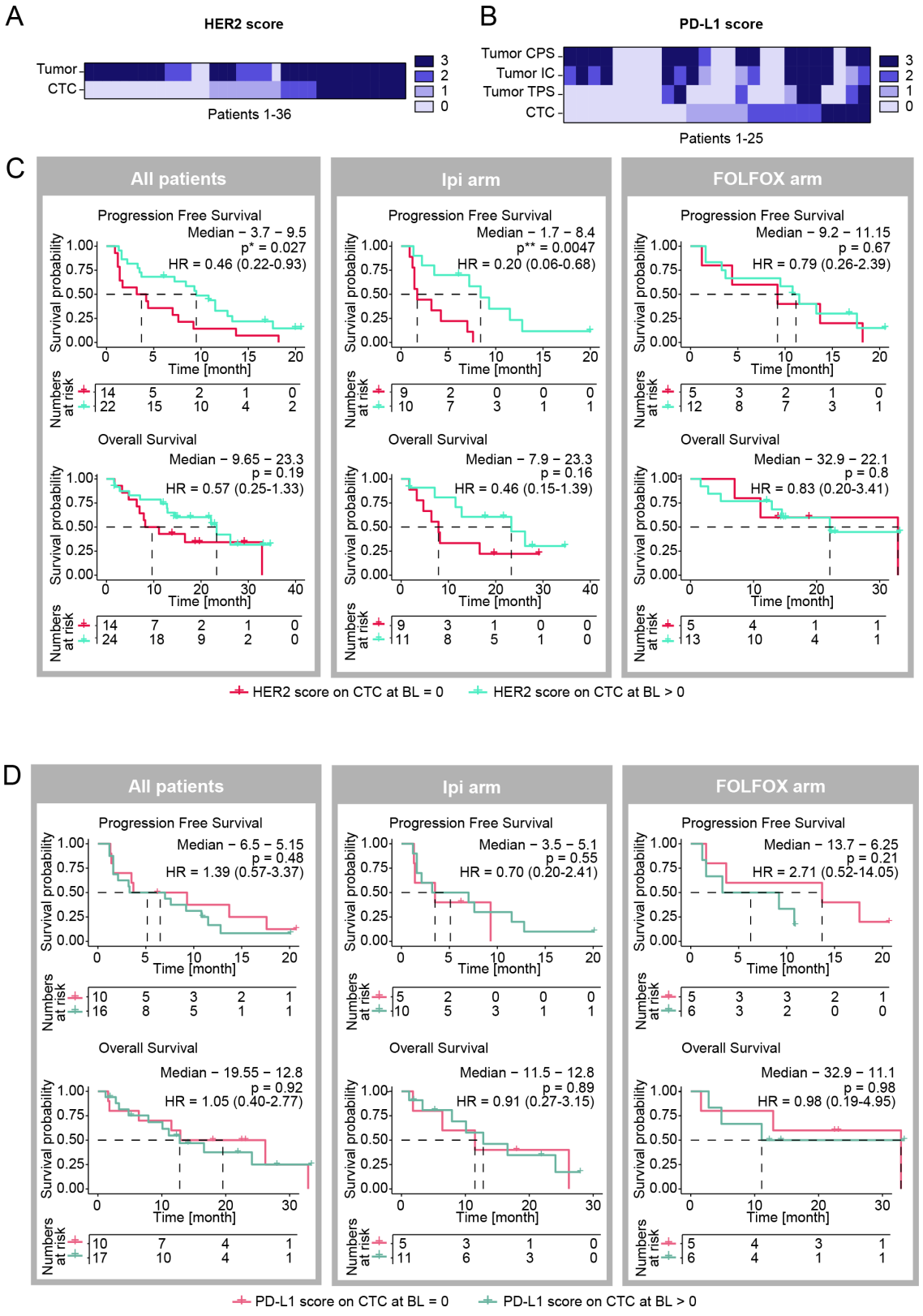

Figure 4 Survival probability based on HER2 and PD-L1 staining of circulating tumor cells (CTCs) at baseline (BL). Comparison of HER2 tissue staining (A) as well as Tumor Proportion Score (TPS), Combined Positive Score (CPS) and Immune Cell Score (IC) of PD-L1 tissue staining (B) compared with CTC staining scores. Tissue staining scores were obtained from ref[5] Kaplan-Meier plots of progression-free survival (PFS) and overall survival (OS) for all patients (left row), Ipi arm (middle row) and FOLFOX arm (right row). Patients with a HER2 score of 0 on CTC at baseline a HER2 score of >0 are shown (C) patients with a PD-L1 score of 0 vs those with a score of >0 are shown (D). The p values were calculated using log-rank test. Median survival is given in months. HR of Cox regression including 95% CIs in brackets; FOLFOX, folinic acid (FOL), fluorouracil (F), oxaliplatin (OX).

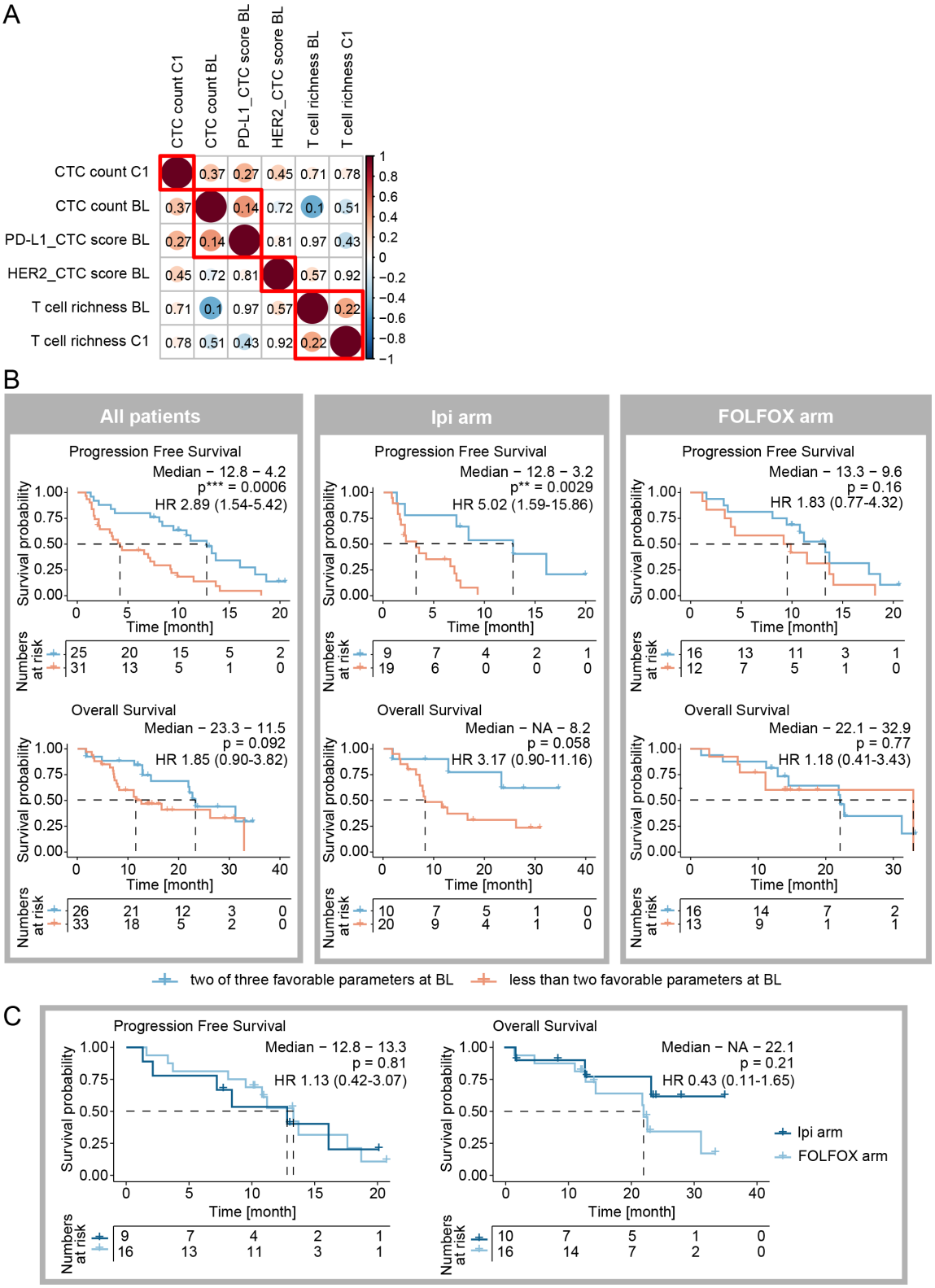

Figure 5 Survival analysis and correlation of multiple liquid biomarkers. (A) Correlation plot. Color and diameter of the circles in the matrix represent Pearson's correlation coefficients as indicated by the scale (red color=positive correlation, blue color=negative correlation). The numbers represent p values. Red rectangles show the results of hierarchical clustering. (B) Survival probability for patients with two of three favorable biomarkers at BL. Kaplan-Meier plots of progression-free survival (PFS) and overall survival (OS) of all patients (left row), Ipi arm (middle row) and FOLFOX arm (right row) are shown. The p values were calculated using log-rank test. Median survival is given in month. HR=HR ratio of Cox regression including 95% CIs in brackets. (C) Direct comparison of PFS and OS curves in patients with two of three favorable biomarkers treated on the Ipi versus the FOLFOX arm. **, p<0.01; ***, p<0.001; BL, baseline; C1, after first treatment cycle; CTC, circulating tumor cell; FOLFOX, folonic acid (FOL), fluorouracil (F), oxaliplatin (OX).

Table 2 Long-term progression-free survivors (>12 months) with favorable liquid biomarker profile (≥2 out of 3 favorable liquid biomarkers)

	Long-term progression-free survivors—% with favorable liquid biomarkers	Remaining patients—% with favorable liquid biomarkers
FOLFOX arm	6/9 (67%)	10/20 (50%)
Ipi arm	4/4 (100%)	6/26 (23%)

FOLFOX, folinic acid, FOL; fluorouracil, F; oxaliplatin, OX.

quantified CTCs and studied PD-1 and HER2 expression of CTCs in the INTEGA trial population. Overall, three markers appeared to be associated with favorable outcomes on the chemotherapy-free regimen: high richness of T cell repertoires, absence of CTCs or HER2 expression of CTCs. Our analysis showed that patients with two of the three favorable liquid biomarkers at BL evaluation—high T cell repertoire richness, no CTCs or HER2 expression on CTCs—did at least equally well on the Ipi arm compared with the FOLFOX arm with a median OS that was not reached on the Ipi arm compared with 22.1 months on the FOLFOX arm. In contrast, patients with only one or none of these markers exhibited drastically lower survival rates on the Ipi arm (median OS of only 8.2 months) and benefitted much more from the chemotherapy regimen (median OS not reached).

CTCs and also cell-free tumor DNA have been studied intensively across a wide variety of tumors including EGA.[32–36] It is generally accepted that their levels reflect tumor burden.[37–40] The association of high CTC counts with lack of benefit from the intensified immunotherapy protocol supports previously reported detrimental effects of tumor burden on the efficacy of immune checkpoint inhibitors.[41] Interestingly, PD-L1 expression on CTCs—despite showing a weak positive correlation with CTC counts—was not associated with specific outcomes neither in the Ipi nor the FOLFOX arm. This mirrored the failure of tissue PD-L1 scores to predict treatment outcomes in this setting, although it has to be noted that both treatment arms were experimental and included an immune checkpoint inhibitor.[5] The association of HER2 expression on CTCs with improved outcomes in the chemotherapy-free Ipi arm reflected the previously established predictive value of high HER2 expression in tumor tissue (HER2 3+ vs HER2 2+/ISH+).[5] However, HER2 expression on CTCs seemed to be a stronger discriminator than the HER2 tissue level, thus adding additional information compared with tissue staining. This might be explained by the fact that CTCs in blood do not only represent primary tumor tissue but also (micro)metastatic lesions and therefore provide more comprehensive information.[42 43] While the prognostic relevance of CTCs is well established and may even be more discriminative at later on-treatment sampling time points,[32 33] the idea of high T cell repertoire richness as prognostic marker in cancer treatment has only recently been emerging.[10 26–29 44 45] Especially in peripheral blood repertoires, T cell metrics are affected by thymic cell output and rich T cell repertoires likely reflect patients with higher immune fitness.[46] Our data support a novel emerging concept that the breadth of the T cell repertoire—regardless of functionality—is instrumental for treatment-enhanced tumor control.

While our study provides important insights, there are several limitations that should be considered when interpreting the results. One potential limitation of the study is the use of flat dosing versus weight-adjusted dosing of nivolumab in the two treatment arms. The dosing was chosen to ensure comparability across trials, but it may have had an impact. However, we believe that the impact of slightly different nivolumab dosing is probably negligible compared with the effects of adding chemotherapy or ipilimumab to the backbone, which are expected to be much more significant. Likely the most significant limitation is our small sample size, which could impact the statistical power of our analysis, particularly when comparing biomarker-defined subsets of patients. Our findings suggest that patients with favorable immune metrics and HER2 on CTCs may be effectively treated without chemotherapy, but these results need to be validated in a larger, prospective trial. Additionally, the complexity of biomarker testing presents a challenge for real-world application of our findings. While liquid biopsy and immune repertoire sequencing are becoming more common at specialized treatment centers, broader implementation of these technologies is necessary to ensure that predictive testing can guide treatment choices under real-world conditions. Overall, while our study provides important preliminary data, further research is needed to fully understand the potential implications of our findings for clinical practice.

In conclusion, our work establishes a new set of liquid biomarkers—T cell repertoire richness, CTC numbers and expression of HER2 on CTCs—which should be prospectively validated as molecular triad identifying responders to a chemotherapy-free first-line treatment regimen in HER2+EGA.

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Contributors Conception and design: MB, LP, AS and SR. Collection and assembly of data: AS, JT, SR, LP, MB, CS, and KP. Experimental work: LP, SR and S-SH. Data analysis and interpretation: LP and MB. Drafting of manuscript: MB, LP and BT. Guarantor: MB

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Competing interests AS received institutional research funding for this trial from and serves as an advisory board member for Bristol-Myers Squibb. All other authors report no potential conflict of interest related to this trial.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Independent Ethics Committee (IEC) for Germany §13, (2) and (3) GCP-V. Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available in a public, open access repository. The datasets generated for this study can be found in the European Nucleotide Archive (ENA) under accession number ID: PRJEB55475.

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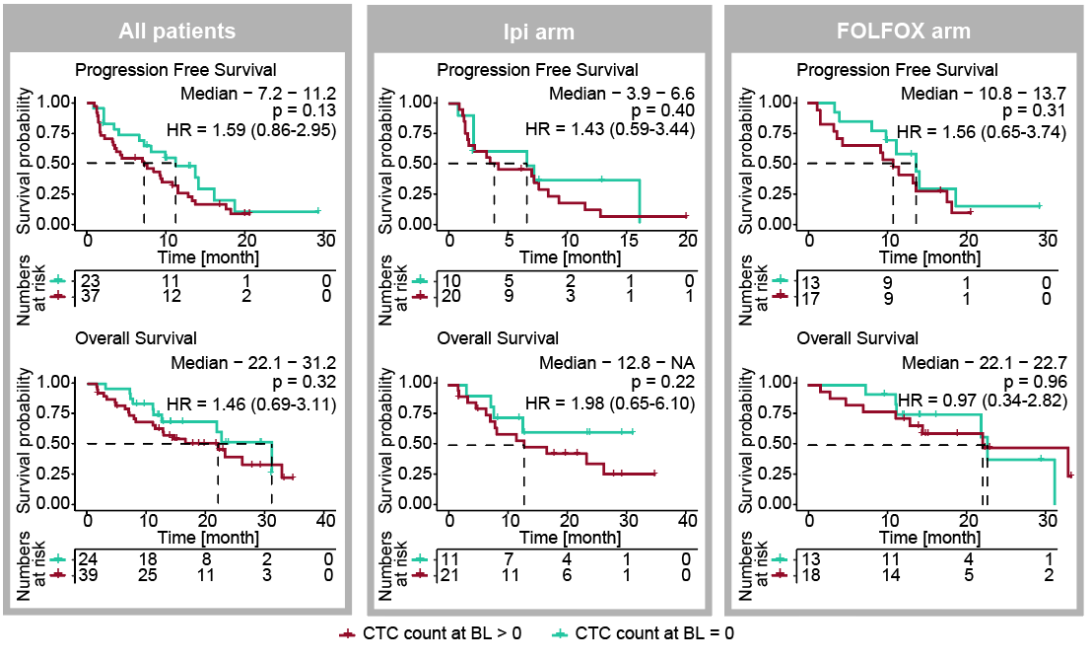
Supplemental Figure



Supplemental Figure S1: Survival probability in relation to the number of circulating tumor cells (CTC) at baseline. Kaplan-Meier plots of progression-free survival (PFS) and overall survival (OS) of all patients (left row), Ipi arm (middle row) and FOLFOX arm (right row). Patients with CTC counts > 0 are grouped versus patients with CTC counts = 0 before initiation of treatment. The p-values were calculated using log-rank test. Median survival is given in month. HR = hazard ratio of cox regression including 95% confidence intervals in brackets.