Supplementary Material: Supplementary Figures, Tables and Data

for Halama et al.

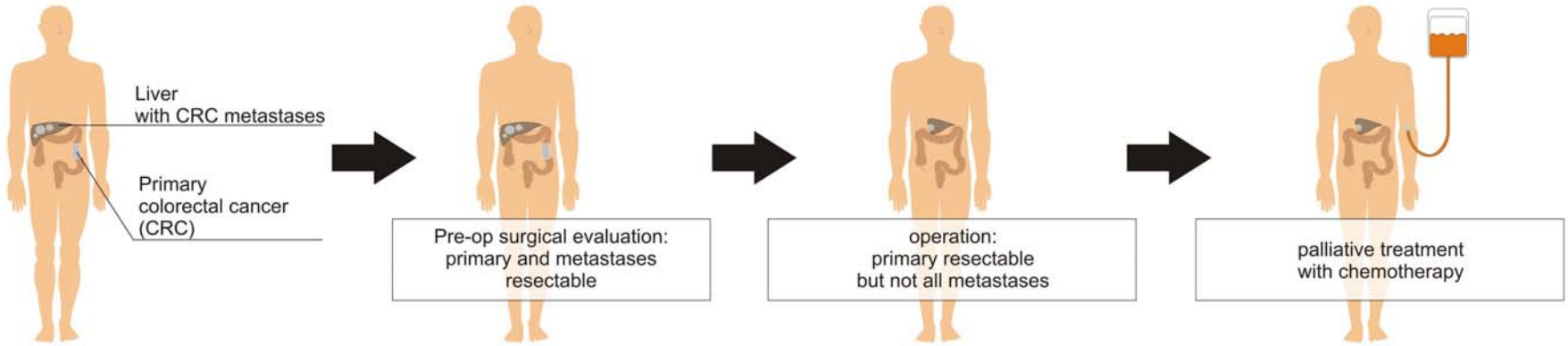

Figure S1: Patient selection for patients with diagnostic excision (or incomplete resection) of CRC liver metastases. The patient receives palliative treatment but part of the metastatic lesion can be analyzed, whereas measurable tumor burden in the liver can be observed for treatment response (according to RECIST criteria).

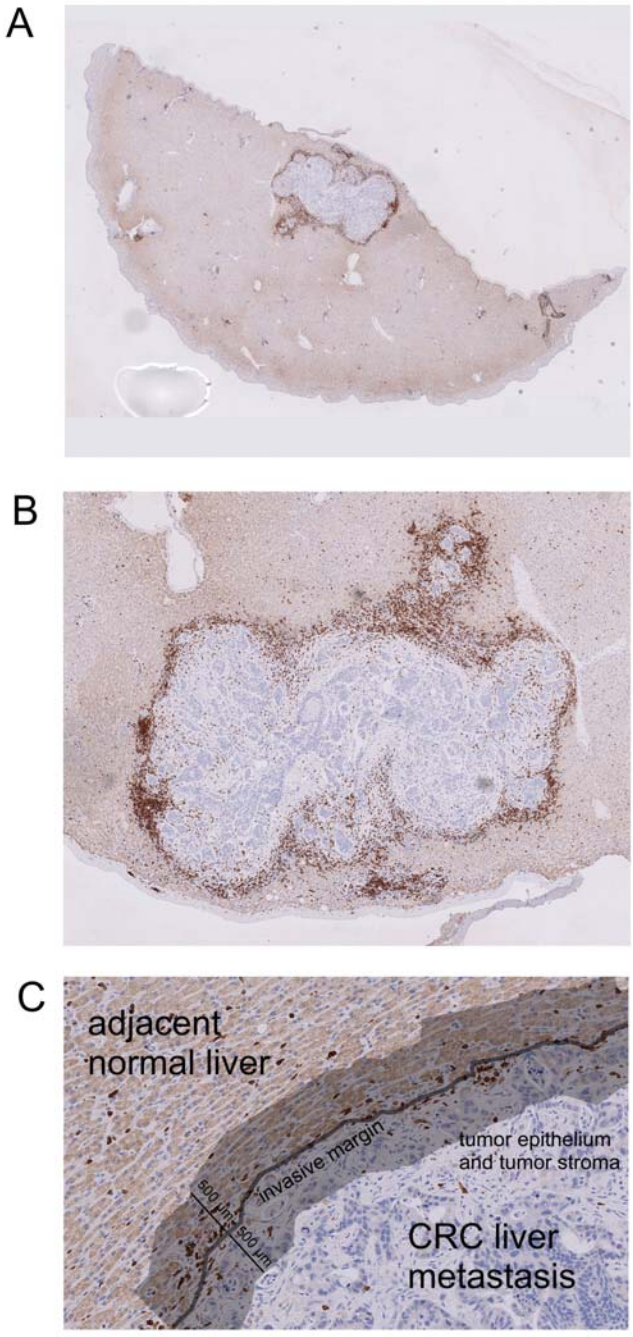

Figure S2: (A) Overview of a metastatic liver lesion (x1.5 magnification, CD3 staining) (B) Higher magnification of the liver metastasis, highlighting the invasive margin with the dense CD3 positive T cell infiltration (red spots) (C) Schematic representation of the definition of the invasive margin and the evaluated area of 500 µm in both directions.

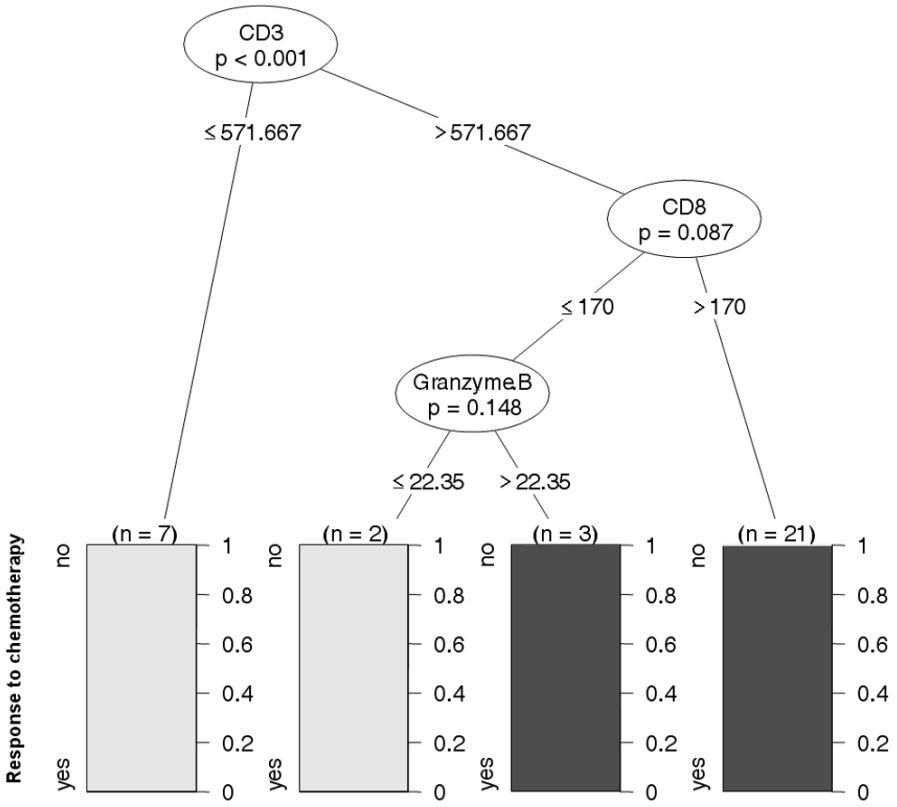

Figure S3: Conditional inference tree. The rule derived from the analysis of the generated cell densities by recursive partitioning is depicted as a tree with the decisive cell densities shown near the corresponding decision node. Light grey bars indicate the proportion of patients without response, dark grey bars indicate the proportion with response to chemotherapy.

Table S1: Patient characteristics

	Training set N (%)	Validation set N (%)	P
Gender			**0.83**
female	13 (39.4%)	24 (35.3%)	
male	20 (60.6%)	44 (64.7%)	
Age			**0.32**
Median	61.0	64.0	
Range	40.0 - 80.0	35.0 - 86.0	
T			**0.34**
1	0 (0.0%)	2 (2.9%)	
2	3 (9.1%)	11 (16.2%)	
3	24 (72.7%)	49 (72.1%)	
4	6 (18.2%)	6 (8.8%)	
N			**0.34**
0	8 (24.2%)	24 (35.3%)	
1	8 (24.2%)	18 (26.5%)	
2	17 (51.5%)	25 (36.8%)	
unknown	0 (0.0%)	1 (1.5%)	
Localization			**0.83**
Colon	23 (69.7%)	45 (66.2%)	
Rectum	10 (30.3%)	23 (33.8%)	
Chemotherapy			**0.47**
5-FU	0 (0.0%)	3 (4.4%)	
FOLFIRI	20 (60.6%)	36 (52.9%)	
FOLFOX	13 (39.4%)	29 (42.6%)	
Antibody			**0.006**
no	16 (48.5%)	14 (20.6%)	
yes	17 (51.5%)	54 (79.4%)	
Microsatellite status (metastasis)			**1.00**
Instable (MSI)	0 (0%)	0 (0%)	
Stable (MSS)	33 (100%)	68 (100%)	
CD3			**0.05**
Median	778.5	660.0	
Range	314.0 - 1899.0	398.0 - 2614.0	
CD3 > 600			**0.35**
no	7 (21.2%)	21 (30.9%)	
yes	26 (78.8%)	47 (69.1%)	
CD8			**0.005**

Median	395.0	273.5	
Range	32.0 - 1009.0	15.0 - 916.0	
CD8 > 200			**0.26**
no	8 (24.2%)	25 (36.8%)	
yes	25 (75.8%)	43 (63.2%)	
Granzyme B			**< 0.001**
Median	31.0	9.0	
Range	2.0 - 260.5	0.0 - 60.0	
Granzyme B > 25			**0.008**
no	14 (42.4%)	48 (70.6%)	
yes	19 (57.6%)	20 (29.4%)	

Antibody: EGFR antibody cetuximab and VEGF antibody bevacizumab.

FOXP3 positive regulatory T cell densities

FOXP3 positive regulatory T cells were found only in low numbers at the invasive margin. For 20 samples of the training set, FOXP3 staining was evaluated. Cell numbers ranged from 0 cells/mm² to 61 cells/mm², with a median of 3.5 cells/mm². Ratios of CD3 to FOXP3 positive cells in serial sections showed a range of 16.2 to infinity (FOXP3 positive cells being absent), with a median of 58.23. FOXP3 cell numbers were the lowest observed among the stained immune cell populations. Subsequent addition of FOXP3 cell numbers in the recursive partitioning approach did not reveal any relevance for these cell densities and the rules for prediction emerged unchanged as outlined in the main text.

Details of patients' characteristics in the training cohort

The analysis within the training cohort consisted of 33 well characterized patients. 13 (39%) were female patients and 20 (61%) were male. TNM classifications for the primary tumor were distributed as follows. The tumor in 10 patients (30.3%) was classified as T4, in 21 patients (63.6%) it was classified T3 and in two patients (6.1%) it was classified as T2. The lymph node status was classified N2 in 18 patients (54.6%), N1 in seven (21.2%) patients and N0 in eight (24.2%) patients. Six patients had metachronous metastases. Adjuvant therapy for three of these patients consisted of 5-FU (administered for six months), all three were diagnosed with metachrone liver metastases two years after diagnosis. One patient had an oxaliplatin-based adjuvant therapy (administered for six months) and liver metastases were identified one year after diagnosis. Two patients had no adjuvant treatment, one patient was found to have liver metastases six years after diagnosis, the other patient was identified to have liver metastases one year after diagnosis. The overall median age was 61 years with a range of 40 to 80 years. The subgroup with no marked response to chemotherapy (group

0-2) consisted of nine patients, five male patients and four female patients. Median age was 62 years in this subgroup. The subgroups with marked response to chemotherapy (groups 3 and 4) consisted of 24 patients, 15 male patients and 9 female patients. Median age was 61 years in this group. ECOG performance status was rated 1 or better for all patients at the beginning of the chemotherapy. Distribution of different treatment regimens and administered antibodies is similar between non-responders and responders.

The study population is heterogeneous with regard to the treatment regimens. Nevertheless, the observed strong relationship between the infiltrate density and PFS and treatment response was then validated in an independent data set. Therefore, there is a strong effect of the infiltrate density on treatment response, regardless of the treatment regimen. Given the heterogeneity of the treatment regimens, it strongly supports the prognostic value of infiltrate density because the marker is associated with a differential outcome regardless of the treatment given.

Sample characteristics

The average surface tissue area of invasive margin evaluated consisted of approx. 10 mm². 75% of the samples consisted of specimens of resected liver metastases with an evaluated invasive margin area of up to 49,8 mm². The remaining specimens were biopsies containing long stretches of invasive margin. Typical liver biopsies contain only <1 mm² of invasive margin, which makes evaluations extremely difficult due to heterogeneity (see Figure 4). Therefore our training set only contained samples from diagnostic excisions or partially resected liver metastases, containing ample invasive margin (>10mm²) for analysis. It was then to be expected that the precision of the predicted treatment outcome is lower for the biopsies in this analysis. 7 out of 10 (70%) of the samples in the group of incorrectly predicted treatment responses are biopsies, whereas in the correctly predicted group only 9/37 (24%) are biopsies.