

## CANCER

# Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients

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There is a clear and unmet clinical need for biomarkers to predict responsiveness to chemotherapy for cancer. We developed an *in vitro* test based on patient-derived tumor organoids (PDOs) from metastatic lesions to identify nonresponders to standard-of-care chemotherapy in colorectal cancer (CRC). In a prospective clinical study, we show the feasibility of generating and testing PDOs for evaluation of sensitivity to chemotherapy. Our PDO test predicted response of the biopsied lesion in more than 80% of patients treated with irinotecan-based therapies without misclassifying patients who would have benefited from treatment. This correlation was specific to irinotecan-based chemotherapy, however, and the PDOs failed to predict outcome for treatment with 5-fluorouracil plus oxaliplatin. Our data suggest that PDOs could be used to prevent cancer patients from undergoing ineffective irinotecan-based chemotherapy.

## INTRODUCTION

Chemotherapy is still considered the backbone of anticancer therapy and has improved the life expectancy of countless patients (1, 2). Unfortunately, a large fraction of patients do not benefit from this treatment but still experience substantial side effects (3–6). Although genomics has greatly facilitated patient selection for targeted therapies, this has been unsuccessful for chemotherapy, in part due to its often incompletely understood and diverse mechanisms of action (7–9). A handful of clinical parameters can help provide prognosis, but most of the proposed biomarkers are not currently used to predict chemotherapy treatment outcome in the clinic (9–14). Previous attempts to use patient material to determine treatment responsiveness have had limited success due to the long turnaround times, poor scalability, or low success rates of establishing patient cell lines or xenografts (15, 16). Together, personalized cancer treatment for chemotherapy

is currently lacking, and new predictive assays to help match patients to treatments are highly needed.

Patient-derived tumor organoids (PDOs) are cultures of tumor cells that can be derived from individual patients with a high success rate and expanded indefinitely, and which recapitulate morphological and genetic features of the original tumor (17–19). Recent *post hoc* studies suggest that PDOs may mirror clinical responses of individual patients to therapy (20, 21). We therefore embarked on a multicenter, prospective, observational clinical study to determine the feasibility and potential value of PDOs as a predictive test for chemotherapy treatment regimens for colorectal cancer (CRC) patients. The Tumor Organoids: feasibility to predict sensitivity to treatment in cancer patients (TUMOROID) study is a multicenter study focused on regimens commonly used in CRC; patients received standard-of-care chemotherapy, including infusional 5-fluorouracil (5-FU) or capecitabine (oral prodrug of 5-FU), in combination with either oxaliplatin (referred to as FO) or irinotecan (FI), or irinotecan alone. Bevacizumab was allowed in all treatments, but patients who received additional cetuximab or panitumumab after the biopsy was taken were excluded. This criterion was chosen because the number of patients that received additional cetuximab or panitumumab was limited, and the selection based on wild-type *N/KRAS* would be a strong confounder. The primary objective was the development of an assay to accurately identify nonresponders to chemotherapy. Nonresponders were defined as patients with progressive disease (PD) after three cycles of chemotherapy according to the response evaluation criteria in solid tumors (RECIST) 1.1 (22).

## RESULTS

### Genetic and clinical characteristics of patients included in the TUMOROID study

We included 61 patients in the trial, from whom 67 biopsies were taken and cultured as previously described (19, 23). Of the total 67 included biopsies, tissue retrieval was unsuccessful for four biopsies

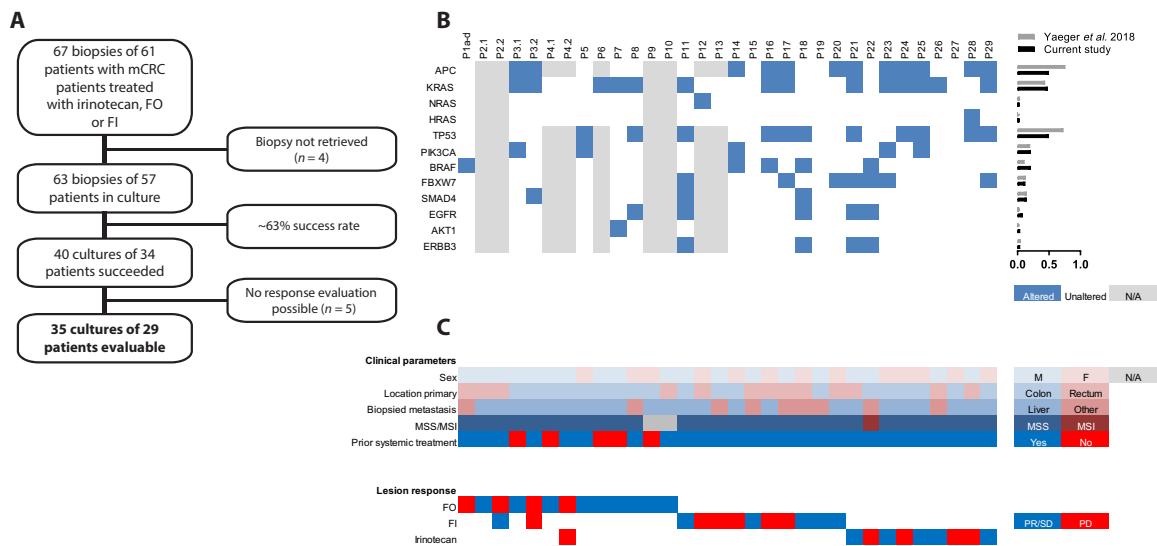
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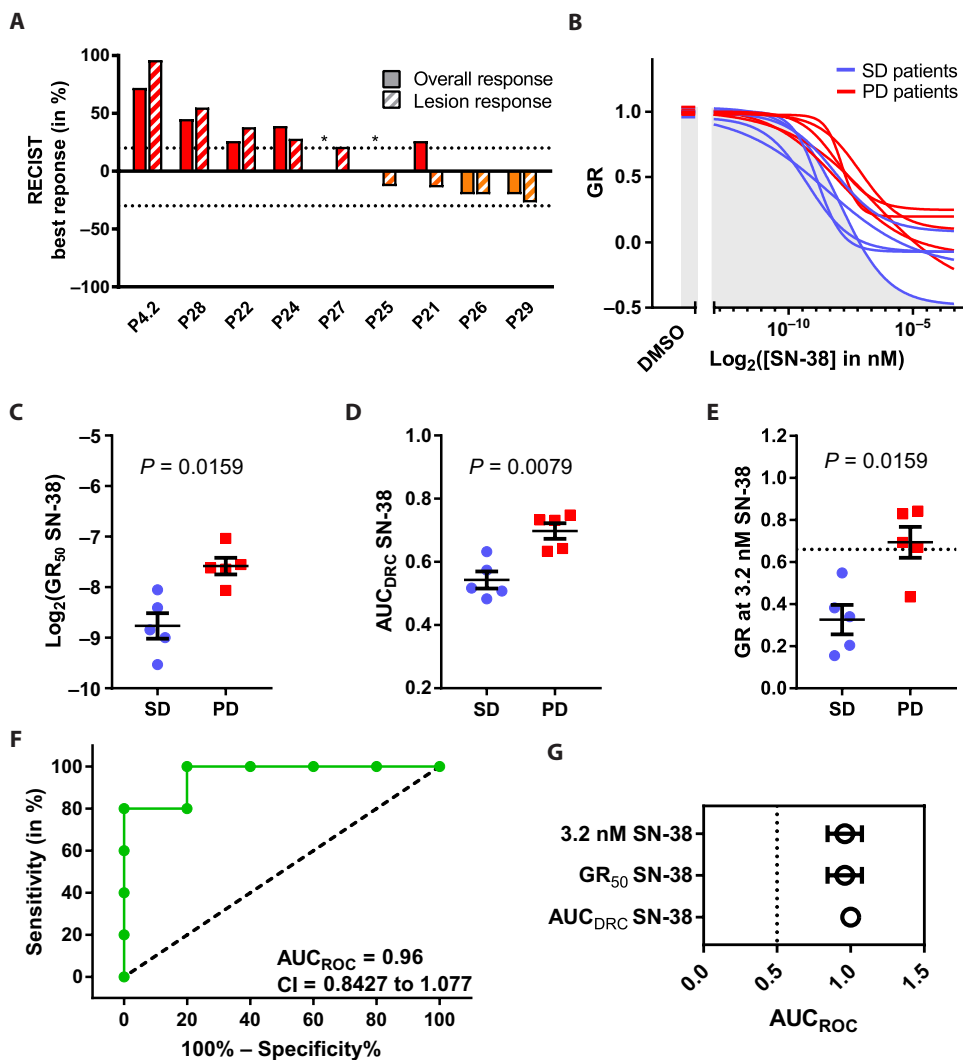
**Fig. 1. Generation of PDOs from patients with metastatic CRC enrolled in the TUMOROID study.** (A) Flow chart indicating the number of patients with metastatic CRC included, the number of evaluable patients, reasons for non-evaluability, and the success rate of establishing cultures from patients. (B) Overview of all patients, corresponding mutations in genes commonly mutated in CRC, and clinical parameters [sex, primary location, biopsied metastasis, microsatellite instability (MSI) status, and previous systemic treatment]. Gray boxes indicate that data were not available. On the right side are bar graphs representing the fraction of samples with a genetic aberration identified per gene, plotted and compared to those of Yaeger and colleagues (24). “Altered” was defined as a given variant being predicted pathogenic by COSMIC. (C) Clinical responses of patients, indicated in either blue (PR/SD) or red (PD), on the relevant treatment indicated on the left. The clinical and genetic data are described in more detail in tables S1 to S3.

(Fig. 1A). Culture of 23 of the remaining 63 biopsies failed due to no or too few tumor cells in the biopsy ( $n = 14$ ), quality control problem ( $n = 6$ ), or bacterial infection ( $n = 3$ ). Overall, we obtained a ~63% PDO culture success rate (40 of 63 cultures) across the whole study, which is in line with previous reports (18–20), and found that clinical parameters did not influence culture success (fig. S1A). For five patients, the culture succeeded, but the clinical response was not evaluable, and therefore, the PDOs could not be used for in vitro patient comparison of drug response. Of the resulting 35 PDOs, 16 were used to evaluate the PDO drug sensitivity for first-line FO, and 22 were used for second-line FI or irinotecan (12 and 10 PDOs, respectively) (Fig. 1A). In most cases, the PDOs were established before the start of treatment. In rare cases, PDOs were established after progression on treatment (P27 and P28) or could also be used for testing response to multiple treatment outcomes because the patients were biopsied immediately after progression on first-line treatment with FO and right before the start of second-line treatment with irinotecan or FI (P2.2, P3.2, and P4.2). Patient and tumor characteristics, clinical background, pathological parameters, genetic aberrations, and treatment history are presented in Fig. 1B and more elaborately in tables S1 to S3. We found that the frequency of known genetic drivers of CRC was similar between the first (FO)- and second (FI/D)-line treatment cohorts (Fig. 1B) and similar to a recent clinical sequencing study of metastatic CRC, suggesting that our study describes a representative population of patients (24). Clinical response data for each patient are depicted in Fig. 1C.

### PDOs predict response to irinotecan monotherapy

We first tested 10 PDOs from 10 patients treated with irinotecan (described in more detail in table S3). Five PDOs were derived from lesions that were classified as PD, and five were derived from lesions that were classified as stable disease (SD) (Fig. 2A; depicted in more detail in fig. S2A). This distribution of responses is in line with larger

studies using irinotecan (6). All PDOs were exposed to the active metabolite of irinotecan, SN-38, for 6 days, and each screen was repeated by a second person to determine interobserver reproducibility (average Pearson’s  $R = 0.947$ ; range, 0.796 to 0.996; fig. S2B). We calculated the growth rate inhibition metrics (GR) of each condition 6 days after drug exposure and fitted dose-response curves (DRCs) (Fig. 2B) (25). We quantified responses to SN-38 by calculating the  $GR_{50}$  and the area under the DRC ( $AUC_{DRC}$ ), both of which were significantly different between PDOs generated from PD versus SD lesions (Mann-Whitney test,  $P = 0.0159$  and  $P = 0.0079$ , respectively; Fig. 2, C and D). To reduce the number of organoids and data points required for testing, we refined the drug assay by determining the concentration in the DRC at which the window of effect (or “variance”) of chemosensitivity was the largest (fig. S3A). This strategy results in the elimination of drug concentrations for which the differential effect between PDOs was small. We found that 3.2 nM SN-38 yielded the largest window of effect, and PDOs from SD patients were more sensitive than PDOs from PD patients when exposed to this concentration (Mann-Whitney test,  $P = 0.0159$ ; Fig. 2E and fig. S3A). The receiver operating characteristic (ROC) curve generated from this window had an AUC of 0.96 [confidence interval (CI), 0.8427 to 1.1077; Fig. 2F], comparable to those generated based on  $GR_{50}$  and  $AUC_{DRC}$  (summarized in Fig. 2G). We next aimed to develop a GR score-based classifier that accurately identifies nonresponders to irinotecan, without misclassifying responders, and test its predictive performance using leave-one-out cross-validation (LOOCV; fig. S3B). LOOCV resulted in correct classification of 80% of patients (empirical  $P = 0.0061$ ; fig. S3B). These data demonstrate that PDOs have predictive value for irinotecan monotherapy, which can be captured by exposure to a single concentration of SN-38 and application of a cutoff of  $GR > 0.67$ . Such an assay only required ~5000 cells, which could be generated and screened within about 2 weeks.



**Fig. 2. PDO drug sensitivity predicts response to treatment with irinotecan.** (A) Waterfall plot of each patient's overall response and best response of the biopsied lesion in the irinotecan-treated PDO cohort. Red indicates progressive disease (PD), and orange indicates stable disease (SD). \*, new lesion(s). (B) Fitted dose-response curves (DRCs) of 10 PDOs exposed to SN-38 in vitro. Blue lines represent PDOs derived from SD patients, and red lines represent PDOs from PD patients. GR values represent in vitro sensitivity of PDOs to SN-38. The screen was plated in technical triplicate and performed twice, once each by independent researchers. Red indicates PD, and blue indicates SD. (C)  $\text{Log}_2(\text{GR}_{50} \text{ SN-38})$  was interpolated from the fitted DRCs shown in (B). Groups were compared using a two-tailed Mann-Whitney test. Dots/squares represent individual PDOs, horizontal bars represent the mean, and error bars indicate SEM. (D) The area under the DRC ( $\text{AUC}_{\text{DRC}}$ ) was calculated by integrating the DRC of each PDO in (B). Groups were compared using a two-tailed Mann-Whitney test. Dots/squares represent individual PDOs, horizontal bars represent the mean, and error bars indicate SEM. (E) The data point with the largest window of effect (captured by the variance) was calculated (3.2 nM SN-38; fig. S3A), and response was compared using a two-tailed Mann-Whitney test. (F) The data of (E) were plotted as an ROC curve. The dotted line represents an  $\text{AUC}_{\text{ROC}}$  of 0.5, which indicates no predictive value. CI, confidence interval. (G) Summary graph of the  $\text{AUC}_{\text{ROC}}$ s and 95% CIs on the basis of the in vitro parameters  $\text{GR}_{50}$  and  $\text{AUC}_{\text{DRC}}$ .

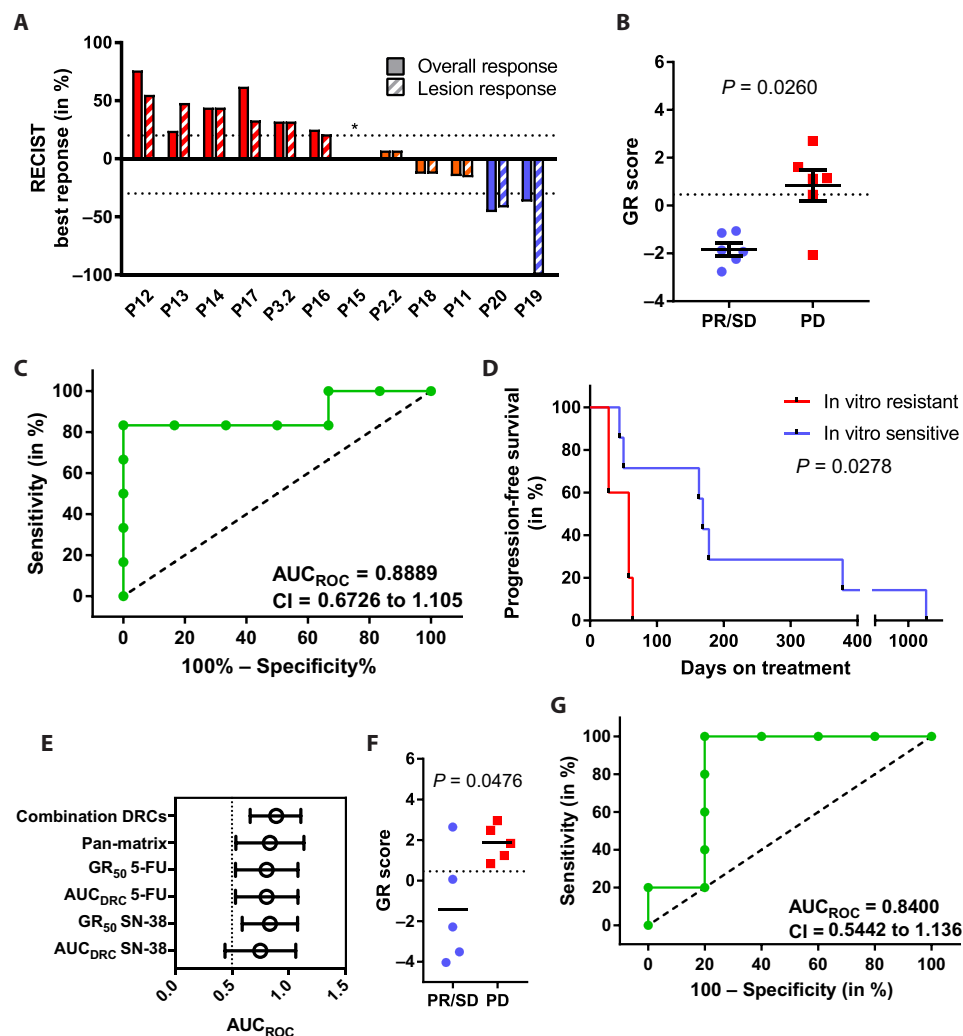
### PDOs predict response to 5-FU–irinotecan combination therapy

Next, we attempted to construct a PDO-based classifier that would predict response to combination therapy. We also tested PDOs from 12 patients treated with FI (Fig. 3A; more details in fig. S4A). Again, the distribution of clinical responses was representative of distributions found in larger studies (6). Because FI is a combination chemotherapy of two drugs, we designed a drug matrix with a broad

range of concentrations (fig. S4B). All 12 PDOs were screened in duplicate for response to FI (average Pearson's  $R = 0.922$ ; range, 0.819 to 0.991; fig. S4C), and responses were quantified by summing the GR values across the drug matrix to create a “pan-matrix GR score” and then compared to the RECIST best response of the lesion (fig. S4D). We observed a pattern that closely mirrored the clinical responses: Five PDOs of patients with PD were resistant to FI in vitro, whereas seven PDOs derived from patients with partial response (PR)/SD were sensitive to FI (P14 was derived from a patient with PD but clustered with PDOs sensitive to FI; fig. S4D). Responses to the individual drugs 5-FU and SN-38 were not significantly different between PR/SD and PD lesions (fig. S4E). Most PDOs were either sensitive or resistant to both SN-38 and 5-FU, although there were two exceptions: P19 was resistant to 5-FU but sensitive to SN-38, and P20 showed the opposite. These data suggest that, in most cases, 5-FU and irinotecan collectively contribute to the effect of FI and the correlation found in vitro (fig. S4F).

Analogous to the method described for the irinotecan monotherapy cohort, we refined the drug assay by determining the concentrations in the drug matrix at which the variance of in vitro chemosensitivity was the largest (figs. S4B and S5A). This analysis identified two complementary rows and columns in the drug matrix with large windows of effect: 200  $\mu\text{M}$  5-FU as fixed concentration (“anchor”) and a titration of SN-38, as well as 6.25 nM SN-38 as anchor and a titration of 5-FU (fig. S5A). For each PDO, we summed the 11 GR values within these two complementary DRCs to create a GR score and found these to differ between PR/SD and PD patients (Mann-Whitney test,  $P = 0.0260$ ; Fig. 3B and fig. S5A). Furthermore, an ROC based on this score produced an  $\text{AUC}$  of 0.89 (Fig. 3C), suggesting that PDOs may have predictive value for FI combination chemotherapy. In addition,

the 50% of PDOs that were most sensitive to FI in our assay had a significantly higher progression-free survival (PFS), indicating that in vitro sensitivity to FI is also associated with a longer response in the clinic (log-rank test,  $P = 0.0278$ ; Fig. 3D). When compared to other in vitro endpoints, the combined DRCs performed as well as or better than 5-FU and SN-38 as single agents (summarized in Fig. 3E). Last, for clinical implementation, the two complementary DRCs of the FI assay only required  $\sim 10,000$  cells,



**Fig. 3. PDO drug sensitivity predicts response to treatment with 5-FU/capecitabine and irinotecan.** (A) Waterfall plot of each patient's overall response and best response of the biopsied lesion in the FI-treated PDO cohort. Red indicates PD, orange indicates SD, and blue indicates partial response (PR). \*, new lesion(s). (B) GR scores of FI-treated PDOs derived from lesions with PR/SD and PD. Scores of PR/SD ( $n=6$ ) versus PD ( $n=6$ ) patients were compared using a two-tailed Mann-Whitney test. The screen was plated in technical triplicate and performed two times, once each by independent researchers. GR scores representing in vitro sensitivity of PDOs to FI were calculated by summing the 11 data points in the two complementary rows and columns in the drug matrix (fig. S5A). Dots/squares represent individual PDOs, horizontal bars represent the mean, and error bars indicate SEM. (C) ROC curve of the FI cohort illustrating the potential to predict response.  $AUC_{ROC}$  = area under the receiver operating characteristic curve. (D) Kaplan-Meier curve depicting the PFS of the 50% most sensitive versus 50% most resistant PDOs. Groups were compared using the Mantel-Cox log-rank test. (E) Summary of the  $AUC_{ROC}$  and 95% CI for lesion response, calculated based on the in vitro parameters  $GR_{50}$  and  $AUC_{DRC}$ . (F) GR scores of irinotecan-treated PDOs derived from lesions with PR/SD and PD. The test developed using the FI set [described in (B)] was applied to the 10 PDOs from the patients in the irinotecan monotherapy cohort: All PDOs were exposed to the 11 concentrations identified in (B), and GR scores representing in vitro sensitivity of PDOs to irinotecan were calculated analogous to the method described there. GR scores for PDO were compared to our threshold [ $GR > 0.46$ , represented by the dotted line in the figure, as identified previously in (B)] to determine whether PR/SD ( $n=5$ ) and PD ( $n=5$ ) patients were correctly classified by this method. Groups were compared using a two-tailed Fisher's exact test. (G) The data of (F) are plotted as an ROC curve.

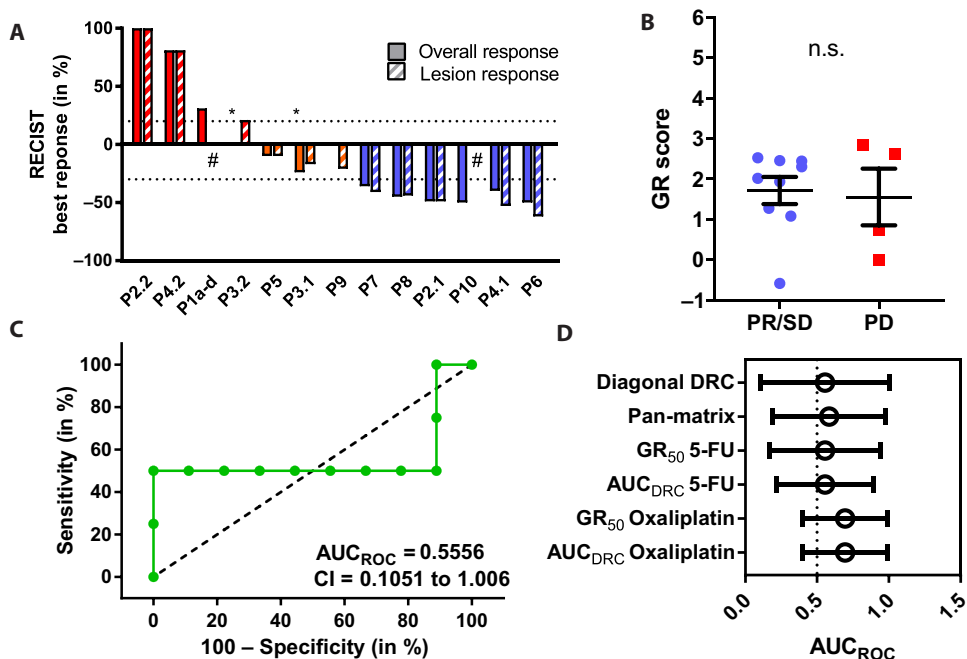
which can be readily generated and screened within 21 days. This turnaround time is a marked improvement over the previously reported 2 to 6 months for other cell culture models and more similar to sequencing of gene panels such as the Memorial Sloan Kettering IMPACT panel (21 days) or the turnaround times reported in precision medicine studies (I-PREDICT median time to start treatment, ~29 days) (15, 26, 27).

To assess the performance of the FI classifier, we first repeated the LOOCV for the FI dataset and found that 83.3% of patients were correctly classified (empirical  $P = 0.0017$ ; fig. S5B). We then tested the classifier for FI combination therapy on the cohort of patients described above, which received irinotecan monotherapy. All PDOs of the irinotecan cohort (Fig. 2A and fig. S2A) were exposed for 6 days to the complementary DRCs identified in the FI analysis above. When we applied the previously identified threshold of  $GR > 0.46$ , we correctly classified five of five resistant patients (90% correct; Fisher's exact test,  $P = 0.0476$ ) (Fig. 3F). Furthermore, the ROC curve generated from this GR score had an  $AUC_{ROC}$  of 0.84 (Fig. 3G). Together, these results demonstrate the predictive nature of our test in a second patient cohort.

### PDOs do not predict response to 5-FU-oxaliplatin combination therapy

We then performed similar experiments to test the predictive value of PDOs for FO chemotherapy using 16 PDOs derived from 10 patients (Fig. 4A and fig. S6A) (22). All samples were screened and analyzed as described above (inter-researcher reproducibility: average Pearson's  $R = 0.971$ ; range, 0.935 to 0.996; fig. S6B). In contrast to the irinotecan-based patient cohorts, we did not find a notable difference in PDOs generated from PD versus PR/SD lesions in response to any of the tested parameters (Fig. 4B and fig. S6, C and D). Furthermore, no correlation with clinical response was found for the response to either the combination treatment or the individual drugs (summarized in fig. S6E). Consequently, the ROC curves showed no predictive value (Fig. 4, C and D).

To analyze inpatient differences in drug responses before treatment (P1a-d) or over the course of treatment (P2 to P4), we calculated response to FO and individual drug responses of PDOs. Organoids were generated from multiple synchronous metastases of P1, as well as before and after treatment for P2 to P4. In line with a previous study, we found that responses to individual agents can differ substantially between lesions in a single patient (P1a-d; fig. S7, A and B) (21). However, all four lesions were comparably sensitive to the FO combination (fig. S7C), which contrasts with the heterogeneous responses to single agents (fig. S7, A and B, and summarized in fig. S7D). For three patients, we profiled drug



**Fig. 4. PDO drug sensitivity does not predict response to treatment with 5-FU/capecitabine and oxaliplatin.** (A) Waterfall plot of the best lesion and overall responses in the FO-treated PDO cohort. Red indicates PR, orange indicates SD, and blue indicates PD. \*, new lesion(s); #, lesion could not be measured. (B) GR scores of FO-treated PDOs derived from lesions with PR/SD and PD. Scores of PR/SD ( $n = 9$ ) versus PD ( $n = 4$ ) patients were compared using a two-tailed Mann-Whitney test. The screen was plated in technical triplicate and performed two times, once each by independent researchers. GR scores representing in vitro sensitivity of PDOs to FO were calculated by summing the six data points of equimolar concentrations of 5-FU and oxaliplatin to generate a GR score. Dots/squares represent scores for individual PDOs (except for P1a-d, which are represented as the average of these four samples), horizontal bars represent the mean, and error bars represent the SEM. n.s., not significant. (C) ROC curve of the FO cohort illustrating the potential to predict response. (D) Summary graph of the  $AUC_{ROC}$ s and 95% CIs calculated on the basis of in vitro parameters  $GR_{50}$  and  $AUC_{DRC}$ .

responses before treatment and upon clinical progression to FO (fig. S7E). P2.1 showed a considerable response to single-agent 5-FU and oxaliplatin, and to the FO combination, which was partly diminished in P2.2 and mirrored the clinical response of this patients (fig. S7, E to H). This contrasts with P3 and P4, for whom only minor changes between pre- and posttreatment cultures were found in response to single-agent 5-FU and oxaliplatin and to the FO combination (fig. S7, F to H). Moreover, baseline sensitivity to FO varied greatly between P2, P3, and P4 (fig. S7F). These observations suggest that the current PDO culture/screening system does not recapitulate the responses of patients to FO in the clinic as it does for second-line irinotecan-based therapy.

We performed several calculations to control for underlying factors that might contribute to the discrepancy found between first-line (FO;  $AUC_{ROC} = 0.5$ ) and second-line (FI/I;  $AUC_{ROC} > 0.8$ ) therapy response in vitro. We calculated the interaction (odds ratio) of clinical parameters or the presence of mutations in common CRC driver genes with clinical response (fig. S8, A and B) or with individual PDO responses to chemotherapy (fig. S8, C and D). We did not find a significant association with response or resistance, either clinical or in vitro, for any of these parameters.

## DISCUSSION

The discrepancy between the predictive value of PDOs for irinotecan-based treatment and FO suggests that the sensitivity and necessary

conditions of the test might differ across types of chemotherapy and underscores our limited knowledge with regard to the mechanism of action of chemotherapy, especially in combinations. Plausible explanations include the absence of stroma and an immune system in PDO cultures, which can dictate treatment outcome in various ways (8). Culture methods that at least partially retain the patient tumor microenvironment in vitro might provide a platform to overcome this hurdle and offer valuable insight into the multilevel interaction of 5-FU and oxaliplatin (23, 28). Another important factor in the clinical implementation of PDO-based tests is the culture success. Because culture is only successful for 63%, there is still a substantial fraction of patients for whom no PDO-informed decision can be made. Culture success might be further improved by obtaining multiple core biopsies, together with direct evaluation of the biopsies by a pathologist to identify samples with low cellularity, as suggested by Vlachogiannis *et al.* (20). Another technical limitation of functional tests is that they cannot be “rushed” as can be done for sequencing gene panels (26, 27). These two points highlight the need to further increase the efficacy of PDO culture.

In summary, to date, there are no predictive tests for responsiveness to standard-of-care chemotherapy in CRC. Our data support the use of PDOs as a predictive tool to prospectively identify patients with metastatic CRC who would not benefit from irinotecan-based palliative chemotherapy. We have demonstrated that it is clinically feasible to use PDOs to deliver a prediction on the outcome of irinotecan-based chemotherapy. Although encouraged by the data, we are mindful of several limitations to our study. We have performed analyses (LOOCV, data randomization, testing of the FI assay in an irinotecan monotherapy cohort) to assess the strength of the data, but the number of patients on which our initial test was designed is limited, and the performances of the classifiers should be tested in independent cohorts of patients. However, we have now provided a foundation for confirmatory trials to validate and refine PDO-based tests and identify alternative treatments for patients unlikely to respond to irinotecan-based therapies (20, 21). Our data suggest that it is clinically feasible to use PDOs to deliver a prediction for the outcome of irinotecan-based chemotherapy.

## MATERIALS AND METHODS

### Study design

The TUMOROID study is a Dutch multicenter observational cohort study (NL49002.031.14). The objective of the study was to evaluate the potential and feasibility of PDOs to distinguish patients with and without response to standard-of-care treatment, and the primary objective is a standardized PDO-based test with an  $AUC_{ROC}$

of >0.7 and a high negative predictive value (the ability to exclude nonresponders without withholding treatment to responders). The study was approved by the ethical review board of the Netherlands Cancer Institute. The protocol complies with the Declaration of Helsinki, Dutch law, and Good Clinical Practice. All patients provided written informed consent before any study-related procedures. Patients with metastatic CRC were accrued at the Netherlands Cancer Institute, Meander Medical Centre Amersfoort, and Elisabeth-TweeSteden Hospital Tilburg. Eligibility criteria included an Eastern Cooperative Oncology Group performance status of  $\leq 2$ , measurable disease, feasibility of tumor biopsy for histologic analysis, and age of 18 years or older. Patients underwent biopsies before start of treatment with clinically approved regimens of capecitabine/5-FU combined with oxaliplatin/irinotecan (described below). Patients were not randomized because the TUMOROID was an observational study. In select cases, posttreatment biopsies were obtained upon clinical progression. Patients underwent computed tomography scans at baseline and every 2 months to monitor response to treatment. Responses of the biopsied lesion were scored using RECIST 1.1 (22). All experiments were performed in parallel by two independent researchers. Researcher 2 was blinded to treatment outcome.

### Treatment

Patients were treated according to clinically approved regimens of irinotecan monotherapy or capecitabine/5-FU in combination with oxaliplatin or irinotecan. Bevacizumab was allowed in all regimens, but patients receiving additional panitumumab or cetuximab were excluded. Irinotecan monotherapy (350 mg/m<sup>2</sup>) was administered intravenously once every 3 weeks. Capecitabine plus oxaliplatin (CAPOX) was given in 3-week cycles; patients received capecitabine (1000 mg/m<sup>2</sup>) orally twice a day on days 1 to 14 and oxaliplatin (130 mg/m<sup>2</sup>) intravenously on day 1. 5-FU plus leucovorin plus oxaliplatin (FOLFOX) was given in 2-week cycles; patients received oxaliplatin (85 mg/m<sup>2</sup>), leucovorin (400 mg/m<sup>2</sup>), and 5-FU (400 mg/m<sup>2</sup> as a bolus, 600 mg/m<sup>2</sup> in 22 hours) intravenously on day 1. On day 2, the patients received leucovorin (200 mg/m<sup>2</sup>) and 5-FU (400 mg/m<sup>2</sup> as a bolus, 600 mg/m<sup>2</sup> in 22 hours) intravenously. Capecitabine plus irinotecan (CAPIRI) was given in 3-week cycles, where patients received capecitabine (1000 mg/m<sup>2</sup>) orally twice a day on days 1 to 14 and irinotecan (250 mg/m<sup>2</sup>) intravenously on day 1. 5-FU plus leucovorin plus irinotecan (FOLFIRI) was given in cycles of 2 weeks; patients received irinotecan (180 mg/m<sup>2</sup>), leucovorin (200 mg/m<sup>2</sup>), and 5-FU (400 mg/m<sup>2</sup> as a bolus, 600 mg/m<sup>2</sup> in 22 hours) intravenously on day 1. On day 2, the patients received leucovorin (200 mg/m<sup>2</sup>) and 5-FU (400 mg/m<sup>2</sup> as a bolus, 600 mg/m<sup>2</sup> in 22 hours) intravenously.

### Patient material processing and organoid culture

One or two 18-gauge tumor biopsies were used for organoid culture and DNA sequencing. Biopsies were collected in Advanced Dulbecco's Modified Eagle's Medium with Nutrient Mixture Ham's F-12 (Ad-DF) (#12634, Invitrogen), supplemented with 1% penicillin-streptomycin (#15140-122, Invitrogen), 1% HEPES (#15630-056, Invitrogen), and 1% GlutaMAX (#35050, Invitrogen) (hereafter referred to as Ad-DF+++). Biopsies were stored for a maximum of 24 hours at 4°C before being dissociated with sharp needles. Cells were counted, washed with Ad-DF+++ and cultured as previously described in CRC growth medium (17, 23). We could generally expand these biopsies to an average of  $\sim 2.0 \times 10^5$  cells at the first split (around day 10).

PDO cultures were checked for mycoplasma contamination every month using the MycoAlert Mycoplasma Detection Kit (Lonza). As part of quality control, PDOs were authenticated using a TaqMan-based single-nucleotide polymorphism (SNP) array targeting 26 SNPs [Hartwig Medical Foundation (HMF)]. Identity scores of tumor DNA versus DNA obtained from healthy blood were computed as described elsewhere (29). PDOs with identity scores of <0.9 were discarded.

### DNA sequencing

Part of the biopsied material of each patient was used for routine clinical sequencing of a panel of cancer genes (Illumina TruSeq; *ABL1*; *AKT1*; *ALK*; *APC*; *ATM*; *BRAF*; *CDH1*; *CDKN2A*; *CSF1R*; *CTNNA1*; *EGFR*; *ERBB2*; *ERBB4*; *FBXW7*; *FGFR1*; *FGFR2*; *FGFR3*; *FLT3*; *GNA11*; *GNAQ*; *GNAS*; *HNF1A*; *HRAS*; *ADH1*; *JAK2*; *JAK3*; *KDR*; *KIT*; *KRAS*; *MET*; *MLH1*; *MPL*; *NOTCH1*; *NPM1*; *NRAS*; *PDGFRA*; *PIK3CA*; *PTEN*; *PTPN11*; *RB1*; *RET*; *SMAD4*; *SMARCB1*; *SMO*; *SRC*; *STK11*; *TP53*; *VHL*) or whole-genome sequencing (WGS) by HMF. Both libraries were prepared according to the manufacturer's instructions (targeted sequencing: FC-130-1008; WGS: TruSeq Nano LT; FC-121-4001-3) and sequenced on the Illumina MiSeq (panel) or HiSeqX paired-end 2 × 150-base pair (WGS) platform. Analysis of the targeted panel was performed with Somatic Variant Caller v1.3 (Illumina). Analysis of the WGS data by the HMF was performed using their custom pipeline, which can be found online at BioRxiv/Github (30).

### Drug screening

All drug screens were performed two times, once each by independent researchers. PDOs were mechanically and enzymatically dissociated into single cells by incubating in TrypLE (#12604-013, Gibco) for 5 to 10 min, filtered, and replated to allow for formation of organoids over the course of 4 days. After 4 days, PDOs were collected, incubated with dispase II (2 mg/ml; #D4693, Sigma) for 15 min to remove Geltrex, and counted using a hemocytometer and trypan blue. PDOs were resuspended in 1:2 Ad-DF+++Geltrex at a concentration of 20 organoids/μl. Suspension (5 μl/well) was dispensed in clear-bottomed, white-walled 96-well plates (#3707, Corning) using an automated repeat pipet and overlaid with 200 μl of CRC growth medium. We generated six-step, fourfold drug matrices of 5-FU + oxaliplatin or 5-FU + SN-38 and 10-step, twofold single-drug DRCs in technical triplicate, covering physiological concentrations of 5-FU (5-FU C<sub>max</sub> in patients = 1.7 to 2.4 μM; 5-FU range in vitro = 0.319 to 200 μM), SN-38 (C<sub>max</sub> in patients ≈ 26 nM; SN-38 range in vitro = 0.195 to 100 nM), and oxaliplatin (oxaliplatin C<sub>max</sub> in patients = 3.8 to 10.1 μM; oxaliplatin range in vitro = 0.319 to 200 μM) in patients using a Tecan D300e digital dispenser (31–33). Readouts were obtained at day 0 ("baseline") and at day 6 in the positive control (10 μM phenylarsine oxide), negative control, and the drug-treated wells. Experiments that had poor or low cell growth (defined as less than twofold growth over 6 days) were excluded. Quantification of cell viability was done by replacing the CRC growth medium with 50 μl of CellTiter-Glo 3D (#G9681, Promega) mixed with 50 μl of Ad-DF+++ according to the manufacturer's instructions on an Infinite 200 Pro plate reader (Tecan Life Sciences).

### DRC fitting and correlation analysis

For P1a-d, the results reported are the average of the four samples. In cases where lesion response could not be accurately measured (P1 and P10), overall response was used. GRs were calculated on the

basis of median luminescence values of day 0, untreated day 6, and drug-treated wells at day 6, using the method described in more detail elsewhere (24). For the FI cohort, the GR value of each data point in the whole drug combination matrix or two DRCs (200  $\mu$ M 5-FU anchor plus a titration of SN-38 combined with 6.25 nM SN-38 anchor and a titration of 5-FU) was summed to create a GR score for each PDO line. Z-scores used in the Kaplan-Meier curve were calculated as  $(\mu - X)/\sigma$  ( $\mu$ , mean;  $X$ , score;  $\sigma$ , SD). Scores for response to combined FO in vitro were calculated on the basis of the 1:1 ratio (the “diagonal” in the combination matrix) of FO, again summing the GR value of each point to create an overall score. Curve fitting and estimation of GR<sub>50s</sub> was done using the GRmetrics package v.1.8.0 in R (34). AUC<sub>DRCs</sub> were inferred by integrating fitted curves. To analyze the reproducibility between drug screen 1 and drug screen 2, we calculated Pearson’s *R* and corresponding *P* value using either the GR value of the 11 data points in two complementary rows and columns identified as having the largest variance within our samples (FI cohort) or the GR value of the 36 data points in the full combination matrix (FO cohort) or all 10 data points in the DRCs (irinotecan monotherapy cohort). Correlation analyses, with associated *P* values, were performed using the COR function in R.

### LOOCV and data randomization

To identify data points that had the largest variance within our samples in the irinotecan set, we calculated the variance for each of the 10 data points in the DRCs, across all 10 PDOs, in a leave-one-out setting. To set the threshold for the irinotecan classifier, we selected the GR score within our set of 10 samples that correctly classified all sensitive patients while correctly classifying the maximum number of resistant patients (GR > 0.76 classified as resistant; Fig. 2E).

To test the predictive performance of this threshold, we applied LOOCV and data randomization. Here, we used nine samples as a training set to identify the data point with the highest variance, as described above. Next, we randomly reassigned our 10 GR scores among five sensitive and five resistant “patients.” Using the GR scores of the nine training samples, we set a threshold and used this cutoff to classify the 10th (validation) sample as either sensitive or resistant (fig. S3, A and B). In this manner, tested samples were never used to determine the threshold. As with our empirically determined threshold, we set the threshold by selecting the highest GR score that correctly classified all sensitive patients within the test set of nine PDOs while correctly classifying the maximum number of resistant patients within the test set of nine PDOs. Within each loop, the data randomization step was performed 120,000 times. This entire procedure was repeated 10 times in total, each time leaving out a different PDO. The *P* value for our empirically determined threshold was calculated by dividing the number of cases where a randomly generated classifier threshold performed as well as, or better than, our empirically determined classifier threshold by the number of iterations executed ( $P = R/I$ ; *P* is the *P* value; *R* is the number of times random data outperform our classifier; *I* is the number of iterations executed).

The analysis of the FI set was performed in a similar manner: We identified two complementary DRCs that had the largest variance within our samples in the FI set by calculating the variance for each of the 36 data points in the full drug matrix, across all 12 PDOs, in a leave-one-out setting. To set the threshold for the FI classifier, we selected the GR score within our set of 12 samples that correctly classified all sensitive patients while correctly classifying the maxi-

imum number of resistant patients (GR > 0.46 classified as resistant; Fig. 3B).

To test the predictive performance of this threshold, we applied LOOCV and data randomization. Here, we used 11 samples as a training set to identify the two complementary DRCs with the highest variance, as described above. Then, for each of our 12 PDOs, the GR score was determined by summing the GR value of the 11 data points within these two complementary DRCs. Next, we randomly reassigned our 12 GR scores among six sensitive and six resistant patients. Using the GR scores of the 11 training samples, we set a threshold and used this cutoff to classify the 12th (validation) sample as either sensitive or resistant (fig. S5, A and B). In this manner, tested samples were never used to determine the threshold. As with our empirically determined threshold, we set the threshold by selecting the highest GR score that correctly classified all sensitive patients within the test set of 11 PDOs while correctly classifying the maximum number of resistant patients within the test set of 11 PDOs. Within each loop, the data randomization step was performed 120,000 times. This entire procedure was repeated 12 times in total, each time leaving out a different PDO. The *P* value for our empirically determined threshold was calculated by dividing the number of cases where a randomly generated classifier threshold performed as well as, or better than, our empirically determined classifier threshold by the number of iterations executed.

### Statistical analysis

Groups were compared using a Mann-Whitney test in case of continuous variables and Fisher’s exact test in case of categorical values. The Mann-Whitney test was applied because data were not normally distributed, and Fisher’s exact test was applied because of the relatively small sample size. Difference in PFS was calculated using the log-rank test. Concordance between replicates was calculated using Pearson’s *R*. All statistical tests were performed two-tailed in GraphPad Prism V7.03, with the exception of the correlation analysis, which was done in R. *P* values were corrected for multiple testing using Bonferroni correction when required, as mentioned in the figure legends. *P* values of <0.05 were considered significant.

### SUPPLEMENTARY MATERIALS

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Fig. S1. Overview of clinical and genetic parameters in PDO cohorts.

Fig. S2. Overview of the irinotecan PDO-patient cohort.

Fig. S3. Development and cross-validation of an irinotecan classifier.

Fig. S4. Overview of the 5-FU-irinotecan PDO-patient cohort.

Fig. S5. Development and cross-validation of a 5-FU-irinotecan classifier.

Fig. S6. Overview of the 5-FU-oxaliplatin PDO-patient cohort.

Fig. S7. 5-FU-oxaliplatin drug responses of synchronous and paired metastases.

Fig. S8. The effects of clinical and genetic parameters on patients and PDO responses.

Table S1. Characteristics and clinical history of all patients included in the TUMOROID study.

Table S2. Genetic and pathological characteristics of patients in the TUMOROID study.

Table S3. Overview of the three cohorts and previous treatments and procedures.

[View/request a protocol for this paper from Bio-protocol.](#)

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expertise. D.v.d.V. provided assistance in coordinating the clinical trial. M.K. performed experiments. M.C., H.B., M.v.L., H.J.B., and L.V.B. included patients. L.W. provided bioinformatics expertise. E.C. coordinated WGS. H.C. contributed to the design and conceptual basis of the study. E.E.V. contributed to the (experimental) design and conceptual basis of the study, and supervised the overall execution of the study. **Competing interests:** E.E.V. is the medical director of the Netherlands Cancer Institute. E.E.V. and S.N.O. are inventors on patent application EP18207636.4 submitted by Stichting Het Nederlands Kanker Instituut—Antoni van Leeuwenhoek Ziekenhuis that covers a method of predicting responsiveness of a gastrointestinal cancer patient to a chemotherapy treatment. H.C. is an inventor on several patents regarding organoid culture and a cofounder and scientific advisory board member of Surrozen. H.C. is a scientific advisory board (SAB) member of the biotech firms Kallyope (New York), Merus (Utrecht), and Decibel (Boston). H.C. is a non-executive board member of Roche (Basel) and SAB member of the Roche subsidiary Genentech (San Francisco) since 2019. H.C. is a scientific advisor for Life Sciences Partners, a biotech venture capital firm located in Amsterdam. All other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. R-spondin1-producing cells are available from C. Kuo under a material transfer agreement with Stanford University. Wnt3a- and Noggin-producing cells are available from H.C. under a material transfer agreement with the Hubrecht Institute. Distribution of PDOs and deposition of DNA sequencing data in publicly available databases are regulated by the informed consent that participants to this study signed. PDOs, clinical outcome, DNA sequencing data, and safety data on a per-patient level can be obtained through the Institutional Review Board of the Netherlands Cancer Institute (IRB@nki.nl). All other codes and materials used in this study are freely or commercially available.

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## Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients

Salo N. Ooft, Fleur Weeber, Krijn K. Dijkstra, Chelsea M. McLean, Sovann Kaing, Erik van Werkhoven, Luuk Schipper, Louisa Hoes, Daniel J. Vis, Joris van de Haar, Warner Prevoo, Petur Snaebjornsson, Daphne van der Velden, Michelle Klein, Myriam Chalabi, Henk Boot, Monique van Leerdam, Haiko J. Bloemendal, Laurens V. Beerepoot, Lodewyk Wessels, Edwin Cuppen, Hans Clevers and Emile E. Voest

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### Dishing out treatment recommendations

The number of treatment options for cancer patients keeps expanding, but it remains difficult to predict which tumors will be sensitive to which treatments. As a result, most patients receive treatment according to standardized protocols. With this approach, some patients respond to treatment, but others only experience side effects. To help address this situation, Ooft *et al.* developed a method of testing drugs in patient-derived organoids, biopsy-derived cells from individual patients grown in a dish. In a clinical study, the responses of organoids to irinotecan correlated with patients' responses to the drug, suggesting that organoids could help avoid giving irinotecan to patients who would not benefit.

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